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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF METFORMIN, EMPAGLIFLOZIN AND LINAGLIPTIN IN ITS BULK AND COMBINED TABLET DOSEAGE FORM

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ABSTRACT

A simple, precise, accurate, and stability-indicating reverse phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated for the simultaneous estimation of Metformin (MET), Empagliflozin (EMP), and Linagliptin (LIN) in bulk drugs and combined tablet dosage forms. Chromatographic separation was achieved using a C18 column with a mobile phase consisting of phosphate buffer and methanol in an optimized ratio, delivered at a suitable flow rate, and detection was carried out using a UV/PDA detector. The method provided well-resolved peaks with satisfactory retention times, good peak symmetry, and acceptable system suitability parameters. The proposed method was validated according to ICH guidelines for parameters including accuracy, precision, linearity, range, specificity, robustness, limit of detection (LOD), and limit of quantification (LOQ). Linearity was observed over the selected concentration ranges for all three drugs with correlation coefficients greater than 0.99. Accuracy studies showed percentage recoveries within acceptable limits, indicating the reliability of the method. Precision studies demonstrated low %RSD values, confirming repeatability and intermediate precision. The method was found to be robust against small deliberate variations in chromatographic conditions. Forced degradation studies under acidic, alkaline, oxidative, thermal, and photolytic conditions confirmed that the method is stability-indicating, as degradation products did not interfere with the analyte peaks. The developed RP-HPLC method is suitable for routine quality control analysis and stability testing of MET, EMP, and LIN in pharmaceutical formulations.

KEYWORDS: RP-HPLC, Metformin, Empagliflozin, Linagliptin, Method Validation, Stability-Indicating Method, ICH Guidelines, Pharmaceutical Analysis

INTRODUCTION

1.1 Importance of Analytical Methods

Quality is important in every product or service, but it is vital in medicine as it involves life. Unlike other consumer goods, there can be, and there is no second quality. Therefore, analytical methods, which are a measure of quality of the drugs, play a very comprehensive role in drug development and follow-up activities, to ensure that a drug product meets the established standard, is stable and will continue to meet the purported quality throughout its shelf life.

These methods should be selective and sensitive to monitor the known and unknown impurities, have to be written in a format such that they can be produced over a period of time and from laboratory to laboratory, i.e. these methods should be validated.

1.2 Introduction to Analytical Methods

Analytical methods are required to characterize drug substance and drug product composition during all phases of pharmaceutical development. Early phase methods must support changes in synthetic routes and dosage form and elucidate the structures and levels of impurities. In later phases, goals change to the development of rapid and robust methods for release and stability evaluation.

Analysis includes a wide range of simple and instrumental analytical methods, but the most widely used analytical methods for quality assurance are spectroscopy and chromatography-based. Most quantitative analysis requires measuring specified components in the presence of a sample matrix and /or related substances; isolation or separation of the components is required before quantitative analysis. In such cases, chromatographic techniques are used for quantitative analysis. In cases where matrix interference is not observed, quantitative measurements are made using spectroscopic or titration methods directly.[3]

For the present studies, analytical methods based on Reversed Phase High Performance Liquid Chromatography (RP-HPLC), Reversed Phase and Normal Phase High Performance Thin Layer Chromatography HPTLC and Infra-Red Spectrophotometry have been developed.

Chromatography Techniques

In chromatographic methods, separation is based on variation in the distribution of different Compounds between two dissimilar phases - a stationary phase and a mobile phase. Further differentiation can be made between chromatographic procedures in which the individual components are monitored online (HPLC) and procedures in which the components are measured in situ on the chromatographic stationary phase (TLC).[4]

High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) is an advanced form of liquid chromatography used in separating the complex mixture of molecules encountered in chemical and biological systems, to better understand the role of individual molecules. In liquid chromatography, a mixture of molecules dissolved in a solution (mobile phase) is separated into its constituent parts by passing through a column of tightly packed solid particles (stationary phase). The separation occurs because each component in the mixture interacts differently with the stationary phase. Molecules that interact strongly with the stationary phase will move slowly through the column, while the molecules that interact less strongly will move rapidly through the column. This differential rate of migration facilitates the separation of the molecules.

HPLC utilizes different types of stationary phases, a pump that moves the mobile phase(s) and analyte through the column, and a detector to signal the characteristic retention time for the analyte. Analyte retention time varies depending on the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used, and the flow rate of the mobile phase.[5] A block diagram of the HPLC System is given below.

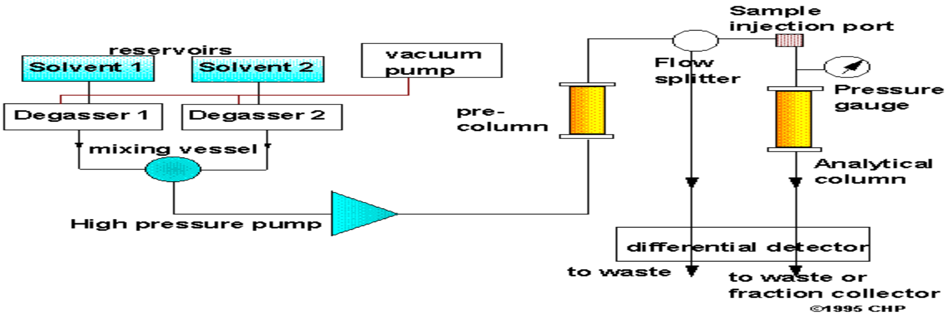


Figure 1: Block diagram of HPLC System.

In HPLC, a pump provides the higher pressure required to move the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes. This allows for better separation of shorter columns compared to ordinary column chromatography.

The sample to be analysed is introduced into the mobile phase stream in small volumes. The solution moved through the column is slowed by specific chemical or physical interactions with the stationary phase present within the column. The velocity of the solution depends on the nature of the sample and on the composition of the stationary (column) phase. The time at which a specific sample elutes (comes out of the end of the column) is called the retention time; the retention time under specified conditions is considered an identifying characteristic of a given sample.

There are two types of stationary phases, mainly reverse phase and normal phase. The nature of the stationary phase will determine whether a column can be used for normal phase or reverse phase chromatography.

a. Normal phase HPLC

Normal phase chromatography utilizes a polar stationary phase and a non-polar mobile phase. Generally, more polar compounds elute later than non-polar compounds.

b. Reversed-phase HPLC

About 75% of current HPLC analysis is performed using the reverse phase. In reversed-phase chromatography, the Stationary phase is mainly silica chemically bonded through a siloxane (Si-o-Si-C) linkage to a low-polarity functional group. These phases are prepared by treating the surface silanol groups of silica with an organochorosilane reagent[7]. The polarity of the column can be changed by varying the alkyl chain length in R.

Thus, reversed-phase HPLC has a non-polar stationary phase and an aqueous, moderately polar mobile phase. With these stationary phases, retention time is longer for molecules of less polarity, while polar molecules elute more readily.

Retention time can be increased by adding more water to the mobile phase, thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase. Similarly, an investigator can decrease retention time by adding more organic solvent to the eluent.

HPLC Detectors

Based on the method or principle used in detection, the detectors available are RI detectors, UV detectors, Fluorescent detectors, electrochemical detectors and Photo diode-array detectors (PDA). For the present study, UV and PDA detectors are mainly used. The refractive index detector is known as a universal detector, but it is not a very sensitive detector.

UV Detectors

In these systems, detection depends on the absorption of UV ray energy by the sample. The equipment comprises accessories such as a UV source, grating (for light diffraction), a sample passing through tubing exposed to rays, a photo cell, a charge conductor, etc.

When the UV rays emitted by the lamp pass through the gratings, the rays split into different wavelengths. One specific wavelength of rays is passed through the sample. Some amount of light is absorbed by the sample, and the unabsorbed rays that fall on the photo cell.

These rays on collision on photo cell, produce electrons whose current is recorded. This is indicative of the nature and quantity of the sample. This UV wavelength range of absorption is specific for sample. These are the HPLC detectors used in general, unless there is a requirement for analysis of special compounds. They are capable to detect very wide range of compounds. The sensitivity ranges from a microgram quantity of estimation.

PDA detectors

These are detectors which follow principle similar to UV detectors but the only advantages are higher sensitivity and measure the entire absorption range i.e.it gives scan of entire spectrum.

HPLC method development[9]

The wide variety of equipment, columns, eluent and operational parameters involved makes high performance liquid chromatography (HPLC) method development seem complex. The process is influenced by the nature of the analytes and generally follows the following steps:

Step 1 –Initial studies
Step 2 -Selection of initial conditions
Step 3 -Selectivity optimization
Step 4 -System optimization

Step-1 Initial studies

When developing an HPLC method, the first step is always to consult the literature to know the physico chemical properties of analyte. The properties like solubility, polarity, molecular weight and partition coefficient are key parameters in selecting the right column and mobile phase for a successful chromatographic separation.

Sample preparation.

The sample matrix determines whether the sample requires dissolution, filtration, and extraction, pre-concentration or clean up. Chemical derivatization may be required to assist detection sensitivity or selectivity.

Types of chromatography

Reverse phase is the choice for the majority of samples, but if acidic or basic analytes are present then reverse phase ion suppression (for weak acids or bases) or reverse phase ion pairing (for strong acids or bases) should be used. The stationary phase should be C18 bonded. For low/medium polarity analytes, normal phase HPLC is a potential candidate, particularly if the separation of isomers is required.

Gradient HPLC

This is only a requirement for complex samples with a large number of components (.20–30) because the maximum number of peaks that can be resolved with a given resolution is much higher than in isocratic HPLC.

Gradient HPLC will also give greater sensitivity, particularly for analytes with longer retention times, because of the more constant peak width (for a given peak area, peak height is inversely proportional to peak width).

Detectors UV detectors, single channel, multi channel or Photodiode array are the most common detectors used in HPLC.
Fluorescence or electrochemical detectors are used for trace analysis. For preparative HPLC, refractive index is preferred because it can handle high concentrations without overloading the detector.

The analytical sensitivity is maximum at λmax, but the wavelengths showing high absorbance can be used as analytical wavelengths. The UV wavelengths below 200 nm are avoided because mobile phase interferences and detector noise increase in this region. Higher wavelengths give greater selectivity.

Step 2 -Selection of chromatographic conditions
This step determines the optimum conditions to adequately retain all analytes; that is, ensures no analyte has a capacity factor of less than 0.5 (poor retention could result in peak overlapping) and no analyte has a capacity factor greater than 10–15 (excessive retention leads to long analysis time and broad peaks with poor detectability). Selection of the following is then required.

Mobile phase solvent strength
The solvent strength is a measure of its ability to sweep analytes from the column. It is generally controlled by the concentration of the solvent with the highest strength; for example, in reverse phase HPLC with aqueous mobile phases, the strong solvent would be the organic modifier; in normal phase HPLC, it would be the most polar one. The aim is to find the correct concentration of the strong solvent.

Gradient HPLC
With samples containing a large number of analytes (20–30) or with a wide range of analyte retentivities, gradient elution becomes necessary to avoid excessive retention.

Step 3 -Selectivity optimization
The aim of this step is to achieve adequate selectivity (peak spacing). The mobile phase and stationary phase compositions need to be taken into account. To select these, the nature of the analytes must be considered.

Once the analyte types are identified, the relevant optimization parameters may be selected the optimization of mobile phase parameters is always considered first as this is much easier and convenient than stationary phase optimization.

Step 4 -system parameter optimization
This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved. The parameters involved include column dimensions, column-packing particle size and flow rate. These parameters may be changed without affecting capacity factors or selectivity.

Step 5 -method validation
Proper validation of analytical methods is important for pharmaceutical analysis when ensurance of the continuing efficacy and safety of each batch manufactured relies solely on the determination of quality. The ability to control this quality is dependent upon the ability of the analytical methods, as applied under well-defined conditions and at an established level of sensitivity, to give a reliable demonstration of all deviation from target criteria.

1.3 Validation of analytical methods

The objective of any analytical measurement is to obtain consistent, reliable and accurate data. Validated analytical methods play a major role in achieving this goal. The results from method validation can be used to judge the quality, reliability and consistency of analytical results, which is an integral part of any good analytical practice. Validation of analytical methods is also required by most regulations and quality standards that impact laboratories.[13]

“Validation is the process of collecting documented evidence that the method performs according to its intended purpose”. This is based on analytical experiments performed according to the validation protocols that comply with the international guidelines i.e. ICH guidelines on method validation. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) is a unique project that brings together the regulatory authorities of Europe, Japan and the United States and experts from the pharmaceutical industry in the three regions to discuss scientific and technical aspects of product registration.

Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the International Conference on Harmonization (ICH) guidelines (Q2A and Q2B)[14,15] The US Food and Drug Administration (FDA)[16,17] and US Pharmacopoeia (USP)both refer to ICH guidelines, i.e. these methods should be validated.

All analytical procedures require some form of validation, regardless of whether the method is used for stability, in–process analysis, release, or acceptance testing. The extent of guidelines for validation requirements provided by different organizations varies widely, but the objective of validation is always to achieve valid analytical test results. This is important to ensure the quality and safety of products that are measured using the analytical method.

Method validation has received considerable attention in literature from various industrial committees and regulatory agencies. There is a wide variety of information and guidance available, as mentioned below;

Table 1: Literature from industrial committees and regulatory agencies.

Committees and regulatory agencies	**Guidelines available**
ICH	a) Q_2R_1 Guidelines are guidelines for new method development and its validation. b) Q_1R_1 Guidelines are for development and validation of stability indicating analytical methods includes methodology
The United States Food and Drug Administration	Two industry guidelines: a) for the validation of analytical methods 2 b) For the validation of bioanalytical methods 3.
IUPAC	"Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis".
EURACHEM	detailed guide for method validation primarily developed for ISO/IEC accredited laboratories but because of its completeness it is also a good source for (bio) pharmaceutical laboratories
AOAC	Technical document for the verification of analytical methods for the ISO 17025 accreditation.
Huber	Has published a technical document for the verification of analytical methods for the ISO 17025 accreditation.

Validation Parameters

As per ICH guidelines following types of analytical procedures need to be validated:

- Identification test
- Quantitation tests for impurity content
- Limit test for the control of impurities
- Quantitative tests of the active ingredient or other main components of the drug

These various parameters for validation of any analytical method are:

1. Accuracy

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

Accuracy can also be described as the extent to which test results generated by the method and the true value agree.

The ICH document on validation methodology recommends accuracy to be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range (for example, three concentrations with three replicates each). Accuracy should be reported as per cent recovery by the assay of a known added amount of standard in the sample or as the difference between the mean and the accepted true value, together with the confidence intervals.

Thus, the accuracy of the method was studied by recovery experiments using the standard addition method at three different levels (80%, 100% and 120. Known amounts of Standard solutions containing the analyte were added to pre-quantified sample solutions to get 80,100 and 120 %. These samples were analyzed by injecting the sample solution, and the % recovery was calculated. In the present study %recovery was calculated by the following formula.

$$\frac{\text{Area of spiked sample area of unspiked sample}}{\text{Area of standard}} \text{X} \frac{\text{Concentration of standard}}{\text{Concentration of added drug}} \text{X100}$$

Acceptance limit for % recovery is 98-100%

2. Precision

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

Precision is a measure of the reproducibility of the whole analytical method (including sampling, sample preparation and analysis) under normal operating circumstances. Precision is determined by using the method to assay a sample for a sufficient number of times to obtain statistically valid results (i.e. between 6 - 1 0). Therefore, for the present work sample was assayed six times, and the %RSD for the obtained assay values was obtained. Precision is then expressed as the relative standard deviation

$$\%RSD = \frac{STD\ DEV}{MEAN} * 100$$

Assay values were calculated with the following formula

$$\%\text{Assay} = \frac{\text{Area of sample}}{\text{Area of standard}} \text{X} \frac{\text{Concentration of standard}}{\text{Concentration of sample}} \text{X} \frac{\text{Purity of standard}}{100} \text{X100}$$

Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

Reproducibility

Reproducibility refers to the precision between laboratories (collaborative studies, usually applied to the standardisation of methodology).

3. Specificity

ICH defines specificity as “the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, this might include impurities, degradants, matrix, etc.” USP<1225> refers to the same definition and also notes that other reputable authorities, such as IUPAC and AOAC, use the term “selectivity” with the same meaning. This reserves the use of “specific” for those procedures that produce a response for a single analyte only. ISO/IEC most likely has the same understanding because it requires a method to be “selective” rather than specific. Our goal is to distinguish and quantify the response of the target compounds from the responses of all other compounds. Selectivity is the ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix.

Specificity for an assay ensures that the signal measured comes from the substance of interest, and that there is no interference from excipients and/or degradation products and/or impurities. Determination of this can be carried out by assessing the peak identity and purity.

4. Detection Limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact value. The limit of detection (LOD) was calculated by using the formula

$$\text{LOD} = 3.3 \times \sigma/S$$

Where,
σ = Standard deviation of response
S = Slope of the regression equation.

5. Quantitation Limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

$$\text{LOQ} = 10 \times \sigma/S$$

Where,
σ = Standard deviation of response,
S = Slope of the regression equation.

6. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample. This is the method's ability (within a given range) to obtain results that are either directly, or after mathematical transformation, proportional to the concentration of the analyte within a given range.

Linearity is determined by calculating the regression line using a mathematical treatment of the results (i.e. least mean squares) vs. analyte concentration... A calibration curve was constructed by plotting peak area vs concentrations of analyte.

7. Range

The range of an analytical procedure is the interval between the upper and lower concentration (amount) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

The range is normally expressed in the same units as the test results. For assay tests, ICH requires the minimum specified range to be 80 to 120 per cent of the test concentration.

8. Robustness

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

Thus Ruggedness is the degree of reproducibility of results obtained by the analysis of the same Sample under a variety of normal test conditions, i.e. different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different daysetc. (i.e. From laboratory to laboratory, from analyst to analyst).

1.4 System Suitability Tests (SST)

System suitability testing is an integral part of HPLC procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. Once a method or system has been validated, the task becomes one of routinely checking the suitability of the system to perform within the validated limits.

System suitability test parameters are:

- Plate number or number of theoretical plates (n)
- Capacity factor (capacity ratio) k
- The selectivity or Separation Factor (relative retention) α
- Peak Resolution R
- Peak asymmetry factor (As).

These are measured on a peak or peaks of known retention time and peak width.

1. Plate number or number of theoretical plates (n)

This is a measure of the sharpness of the peaks and therefore the efficiency of the column. This can be calculated in various ways, for example, the USP uses the peak width at the base and the BP at half the height.

Therefore, the higher the plate number, the more efficient the column. The plate number depends on column length -i.e. the longer the column, the larger the plate number

2. Capacity factor (capacity ratio) k

The capacity factor is the ratio of the reduced retention volume to the dead volume. Capacity factor, k‟, is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. The capacity factor is a measure of how well the sample molecule is retained by a column during an isocratic separation. The ideal value of k‟ ranges from 2 to 10.

3. The selectivity or Separation Factor (relative retention) α

The selectivity (or separation factor), α, is a measure of the relative retention of two components in a mixture. Selectivity is the ratio of the capacity factors of both peaks and the ratio of their adjusted retention times. Selectivity represents the separation power of a particular adsorbent to the mixture of these particular components.

This parameter is independent of the column efficiency; it only depends on the nature of the components, eluent type, and eluent composition, and adsorbent surface chemistry. In general, if the selectivity of two components is equal to 1, then there is no way to separate them by improving the column efficiency.

4. Peak Resolution R

Resolution (Rs)

Resolution is the parameter describing the separation power of the complete chromatographic system relative to the particular components of the mixture.

The resolution, Rs, of two neighboring peaks is defined as the ratio of the distance between two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of Rs is 1.5. It is calculated by using the formula

This is not only a measure of the separation between two peaks, but also the efficiency of the column. It is expressed as the ratio of the distance between the two peak maxima. (At) to the mean value of the peak width at base (Wb)

5. Peak asymmetry factor (Tf)

Peak asymmetry factor, Tf, can be used as a criterion of column performance. The peak half-width, b, of a peak at 10% of the peak height, divided by the corresponding front half-width, gives the asymmetry factor.

1.5 Stability-indicating assay method

Patients taking a pharmaceutical product for a particular malady expect the product to be safe and efficacious. Pharmaceutical regulatory agencies worldwide demand that the product retain its identity, quality, purity, and potency for the time the product is commercially available. Consequently, the agencies expect to see stability data supporting the proposed expiration date of the product in the marketing submission. Therefore, it is necessary to conduct stability studies to predict, evaluate, and ensure drug product safety.

Stability studies of drug substances via acid hydrolysis, base hydrolysis, oxidation and thermal and photolytic stress testing are a part of the development strategy under the ICH requirements.

These studies provide information on a drug’s inherent stability and help to validate analytical methods to be used for evaluating stability. Stability assays are currently being developed by using the stress testing approach of the ICH guidelines, Q1 A[R2]. The approach has been further extended to a stress test of drug combinations. These tests allow accurate and precise quantification of drugs, their degradation products, and their interaction products.

Stability-indicating methods according to the 1987 guideline were defined as the ‘Quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured.’

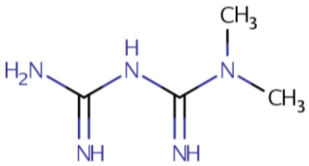
The revised drug stability guideline Q1AR from the International Conference on Harmonization (ICH) requires that stability samples be tested using validated stability-indicating assay methods. The guideline suggests that stress testing be performed to establish the intrinsic stability of the drug substance and to validate the stability-indicating nature of the analytical method. It also recommends that stress testing include the evaluation of the effect of temperature, humidity (when appropriate), oxidation, and photolysis on the drug substance, plus its susceptibility to hydrolysis across a wide range of pH values when in solution or suspension. Although applicable to new drugs, the ICH guidelines have recently been sought to be extended to generic drugs.

DRUG PROFILE

2.1. METFORMIN

Description: Metformin is a biguanide antihyperglycemic agent used for treating non-insulin-dependent diabetes mellitus (NIDDM). It improves glycemic control by decreasing hepatic glucose production, decreasing glucose absorption and increasing insulin-mediated glucose uptake. Metformin is the only oral antihyperglycemic agent that is not associated with weight gain. Metformin may induce weight loss and is the drug of choice for obese NIDDM patients. When used alone, Metformin does not cause hypoglycemia; however, it may potentiate the hypoglycemic effects of sulfonylureas and insulin. Its main side effects are dyspepsia, nausea and diarrhea. Dose titration and/or use of smaller divided doses may decrease side effects. Metformin should be avoided in those with severely compromised renal function (creatinine clearance < 30 ml/min), acute/decompensated heart failure, and severe liver disease and for 48 hours after the use of iodinated contrast dyes due to the risk of lactic acidosis. Lower doses should be used in the elderly and those with decreased renal function. Metformin decreases fasting plasma glucose, postprandial blood glucose and glycosylated haemoglobin (HbA1c) levels, which are reflective of the last 8-10 weeks of glucose control. Metformin may also have a positive effect on lipid levels. In 2012, a combination tablet of linagliptin plus Metformin hydrochloride was marketed under the name Jentadueto for use in patients when treatment with both linagliptin and Metformin is appropriate.

Structure



IUPAC Name: 1-carbamimidamido-N,N-dimethylmethanimidamide
Molecular Formula: $C_4H_{11}N_5$
Molecular mass:129.1636 g/mol
Category: Antidiabetic Agent
Appearance: White Crystalline powder
Melting Point: 223-226 °C
Solubility: It is soluble in Water and Ethanol

Mechanism of action: Metformin's mechanisms of action differ from other classes of oral antihyperglycemic agents. Metformin decreases blood glucose levels by decreasing hepatic glucose production, decreasing intestinal absorption of glucose, and improving insulin sensitivity by increasing peripheral glucose uptake and utilization. These effects are mediated by the initial activation by Metformin of AMP-activated protein kinase (AMPK), a liver enzyme that plays an important role in insulin signalling, whole body energy balance, and the metabolism of glucose and fats. Activation of AMPK is required for Metformin's inhibitory effect on the production of glucose by liver cells. Increased peripheral utilization of glucose may be due to improved insulin binding to insulin receptors. Metformin administration also increases AMPK activity in skeletal muscle. AMPK is known to cause GLUT4 deployment to the plasma membrane, resulting in insulin-independent glucose uptake. The rare side effect, lactic acidosis, is thought to be caused by decreased liver uptake of serum lactate, one of the substrates of gluconeogenesis. In those with healthy renal function, the slight excess is simply cleared. However, those with severe renal impairment may accumulate clinically significant serum lactic acid levels. Other conditions that may precipitate lactic acidosis include severe hepatic disease and acute/decompensated heart failure.

Pharmacokinetic data Absorption: Absorbed over 6 hours, bioavailability is 50 to 60% under fasting conditions. Some evidence indicates that the level of absorption is not dose-related, suggesting that absorption occurs through a saturable process. Limited data from animal and human cell cultures indicate that absorption occurs through a passive, non-saturable process, possibly involving a paracellular route. Peak action occurs 3 hours after oral administration.

Volume of distribution: 63-273L
Protein binding: negligibly bound to plasma proteins.
Metabolism: not metabolized.

Route of elimination: Intravenous single-dose studies in normal subjects demonstrate that Metformin is excreted unchanged in the urine and does not undergo hepatic metabolism (no metabolites have been identified in humans) or biliary excretion. Approximately 90% of the drug is eliminated in 24 hours in those with healthy renal function. Renal clearance of Metformin is approximately 3.5 times that of creatinine clearance, indicating the tubular secretion is the primary mode of Metformin elimination.

Half life: 6.2 hours.

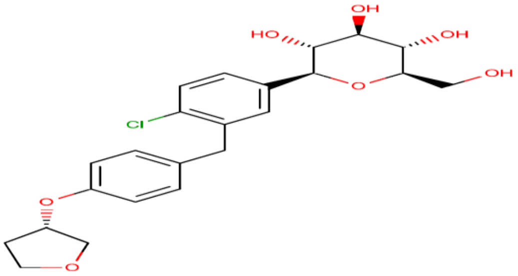
Pharmacodynamics: Metformin reduces liver (hepatic) production of glucose, decreases the intestinal absorption of glucose, and enhances insulin sensitivity by increasing both peripheral glucose uptake and utilization.

2.2 EMPAGLIFLOZIN

DESCRIPTION

Empagliflozin is a sodium glucose co-transporter-2 (sglt-2) inhibitor indicated as an adjunct to diet and exercise to improve glycemic control in adult patients with type 2 diabetes. Sglt2 co-transporters are responsible for reabsorption of glucose from the glomerular filtrate in the kidney. The glucouretic effect resulting from sglt2 inhibition reduces renal absorption and lowers the renal threshold for glucose, therefore resulting in increased glucose excretion. Additionally, it contributes to reduced hyperglycemia and also assists weight loss and blood pressure reduction.

Structure



IUPAC name: (1S)-1, 5-anhydro-1-(4-chloro-3-{4-[(3S)-tetrahydrofuran-3-yloxy] benzyl} phenyl)-D-glucitol
Molecular Formula: $C_{23}H_{27}ClO_7$
Molecular weight: 450.91 g/mol
Category: Antidiabetic Agent
Appearance: white to yellowish and non-hygroscopic crystalline solid
Melting point: 127-130 °C

Solubility: Empagliflozin is a white to yellowish, non-hygroscopic powder. It is very slightly soluble in water, sparingly soluble in methanol, slightly soluble in ethanol and acetonitrile; soluble in 50% acetonitrile/water; and practically insoluble in toluene.

Mechanism of action: Empagliflozin is a sodium glucose co-transporter-2 (SGLT-2) inhibitor. SGLT2 co-transporters are responsible for the reabsorption of glucose from the glomerular filtrate in the kidney. The glucouretic effect of SGLT2 inhibition reduces renal glucose reabsorption and lowers the renal threshold for glucose, leading to increased glucose excretion. Additionally, it helps reduce hyperglycemia, supports weight loss, and lowers blood pressure.

Pharmacokinetic Data Absorption: Following oral administration, peak plasma concentrations were reached at 1.5 hours post-dose and then declined in a biphasic manner with a rapid distribution phase and a relatively slow terminal phase. Administration follows a high-fat and high-calorie meal results in a slightly lower exposure with AUC decreasing by approximately 16% and Cmax decreasing by approximately 37% compared to fasted condition.

Volume of distribution: 73.8L
Protein binding: Plasma protein binding was found to be 86.2%

Metabolism: In vitro studies suggest that Empagliflozin is primarily metabolized by glucuronidation by 5'-diphospho-glucuronosyltransferases UG2B7, UGT1A3, UGT1A8, and UGT1A9. The most abundant metabolites are three glucuronide metabolites: 2-O-, 3-O-, and 6-O-glucuronide. Empagliflozin does not inhibit, Terminal inactivate, or induce CYP450 isoforms. It is a substrate for p-glycoprotein (p-gp), however in vitro studies suggest that it is unlikely to cause interactions with drugs that are p-gp substrates.

Route of elimination: After oral administration, Empagliflozin was 41.2% eliminated in feces and 54.4% eliminated in urine.

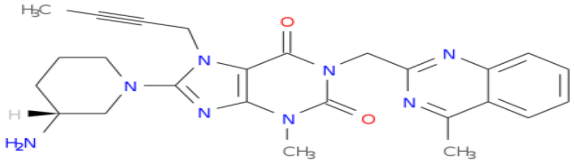
Half-life: elimination half-life was found to be 12.4 h based on population pharmacokinetic analysis.

Pharmacodynamics: Empagliflozin lowers blood glucose levels by preventing glucose reabsorption in the kidneys, thereby increasing the amount of glucose excreted in the urine.18 It has a relatively long duration of action requiring only once-daily dosing.

2.3 LINAGLIPTIN

Description: Linagliptin is a DPP-4 inhibitor used for the treatment of type II diabetes. Linagliptin differs from other DPP-4 inhibitors in that it has a non-linear pharmacokinetic profile, is not primarily eliminated by the renal system, and obeys concentration dependant protein binding

Structure



IUPAC Name: 8-[(3R)-3-aminopiperidin-1-yl]-7-(but-2-yn-1-yl)-3-methyl-1-[(4-methylquinazolin-2-yl) methyl]-2, 3, 6, 7-tetrahydro-1H-purine-2, 6-dione
Mol Formula: $C_{25}H_{28}N_8O_2$
Molecular Weight: 472.542 g/mol
Category: Antidiabetic agent
Appearance: White to yellowish and slightly hygroscopic solid substance
Melting point: 190-196^{0}C
Solubility: soluble in methanol

Mechanism of action: Linagliptin is a competitive, reversible DPP-4 inhibitor. Inhibition of this enzyme slows the breakdown of GLP-1 and glucose-dependent insulinotropic polypeptide (GIP)3,5. GLP-1 and GIP stimulate the release of insulin from beta cells in the pancreas while inhibiting release of glucagon from pancreatic beta cells. These effects together reduce the breakdown of glycogen in the liver and increase insulin release in response to glucose.

Pharmacokinetics Absorption: Oral bioavailability of linagliptin is 30%3.

Volume of distribution: A single intravenous dose of 5mg results in a volume of distribution of 1110L.

However an intravenous infusion of 0.5-10mg results in a volume of distribution of 380-1540L.

Metabolism: minimal

Protein Binding: 70-80% protein bound, the extent to which is concentration dependent. Because of the propensity of linagliptin to bind to plasma protein, it has a long terminal half-life and a non-linear pharmacokinetic profile. In contrast, other DPP-4 inhibitors have a linear pharmacokinetic profile which makes linagliptin unique.

Route of elimination: 84.7% of linagliptin is eliminated in the feces and 5.4% is eliminated in the urine.

Half Life: Terminal half-life = 131 hours. Because of this long half-life, inhibition of DPP-4 activity is sustained, which indicates that once-daily dosing is appropriate. Effective half-life for the accumulation of the drug is 12 hours when multiple oral doses of 5 mg are given.

Pharmacodynamics: A 5mg oral dose of linagliptin results in >80% inhibition of dipeptidyl peptidase 4 (DPP-4) for ≥24 hours. Inhibition of DPP-4 increases the concentration of glucagon-like peptide 1 (GLP-1), leading to decreased glycosylated hemoglobin and fasting plasma glucose.

RATIONALE OF STUDY

Reversed-phase HPLC has a non-polar stationary phase and an aqueous, moderately polar mobile phase. With these stationary phases, retention time is longer for molecules of less polarity, while polar molecules elute more readily, as most of the drugs are polar in nature. Based on these parameters, the method has been selected.

TRIJARDY XR is a combination of Empagliflozin, a sodium-glucose cotransporter-2 (Sglt2) inhibitor, Linagliptin, a Dipeptidyl peptidase-4 (DPP-4) inhibitor, and Metformin hydrochloride (HCl), a biguanide, indicated as an adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes mellitus.

According to the literature survey, there was no report published on the concurrent quantification of Metformin hydrochloride (MET), Empagliflozin (EMP) and Linagliptin (LIN) in a fixed dose using the HPLC technique.

MATERIALS AND METHODS

3.1 INSTRUMENTS USED

Table 2: Instruments used.

SL.No	Instrument	Model
1	HPLC	WATERS, software: Empower, 2695 separation module, UV detector.
2	UV/VIS spectrophotometer	LABINDIA UV 3000$^+$
3	pH meter	Adwa – AD 1020
4	Weighing machine	Afcoset ER-200A

3.2 CHEMICALS USED

Table 3: CHEMICALS USED

S. No	Chemical	Brand
1	Metformin	In-house
2	Empagliflozin	In-house
3	Linagliptin	In-house
4	KH_2PO_4	FINER chemical Ltd
5	Water and Methanol for HPLC	LICHROSOLV (MERCK)
6	Acetonitrile for HPLC	MOLYCHEM
7	Ortho-phosphoric acid	MERCK

HPLC METHOD DEVELOPMENT

Mobile Phase Optimization

Initially, the mobile phase tried was methanol: Ammonium acetate buffer and Methanol: phosphate buffer with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to Phosphate Buffer, in proportion 60:40 v/v, respectively.

PREPARATION OF BUFFER AND MOBILE PHASE

Preparation of 0.1M KH_2PO_4 buffer

Accurately taken 2.68 gm in a 100 ml volumetric flask, dissolved and diluted to 100 ml with HPLC water and the volume was adjusted. The pH of the solution was adjusted by using ortho-phosphoric acid.

Preparation of mobile phase

Accurately measured 400 ml (40%) of the above buffer and 600 ml of Methanol were mixed and degassed in an ultrasonic water bath for 10 minutes, and then filtered through a 0.45 µ filter under vacuum filtration.

Diluent Preparation

The Mobile phase was used as the diluent.

METFORMIN, EMPAGLIFLOZIN & LINAGLIPTIN STANDARD

Standard Solution Preparation

Accurately weigh and transfer 1000 mg of Metformin and 2.5 mg of Linagliptin, and 5 mg of Empagliflozin working standards into a 25 ml clean dry volumetric flask, add about 15 mL of Diluent and sonicate to dissolve it completely and make the volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (1000 ppm MET, 3 ppm of Lina and 6 ppm of EMPA).

Sample Solution Preparation

Accurately weigh 10 tablets of Each Drug and crush in mortar and pestle and transfer equivalent Tablet powder of Metformin, Empagliflozin and Linagliptin individually to 1000 mg of Metformin and 5 mg Empagliflozin and 2.5 mg of Linagliptin samples into a 50mL clean dry volumetric flask add about 15 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (1200 ppm MET, 3 ppm of LINA and 6 ppm of EMPA).

Procedure

Inject 10 µL of the standard sample into the chromatographic system and measure the areas for Metformin, Linagliptin and Empagliflozin peaks and calculate the %Assay by using the formulae.

Calculation: (For Metformin)

$$\textbf{Assay \%} = \frac{\textbf{AT}}{\textbf{AS}} \times \frac{\textbf{WS}}{\textbf{DS}} \times \frac{\textbf{DT}}{\textbf{WT}} \times \frac{\textbf{P}}{\textbf{100}} \times \frac{\textbf{Avg. Wt.}}{\textbf{Label Claim}} \quad \textbf{X 100}$$

Where:

AT = average area counts of sample preparation.
As = average area counts of standard preparation.
WS = Weight of working standard taken in mg.
P = Percentage purity of working standard
LC = Label Claim of Metformin mg/ml.

Calculation: (For Empagliflozin)

$$\textbf{Assay \%} = \frac{\textbf{AT}}{\textbf{AS}} \times \frac{\textbf{WS}}{\textbf{DS}} \times \frac{\textbf{DT}}{\textbf{WT}} \times \frac{\textbf{P}}{\textbf{100}} \times \frac{\textbf{Avg. Wt.}}{\textbf{Label Claim}} \textbf{X 100}$$

Where

AT = average area counts of sample preparation.
As = average area counts of standard preparation.
WS = Weight of working standard taken in mg.
P = Percentage purity of working standard
LC = Label Claim of Empagliflozin

SYSTEM SUITABILITY

Tailing factor for the peaks due to Metformin and Empagliflozin in the standard solution
Should not be more than 2.0

Theoretical plates for the Metformin and Empagliflozin peaks in the standard solution should not be less than 2000.

SAMPLE AND STANDARD DETAILS

Table 4: Sample And Standard Details

S. No.	Samples
1	Metformin, Empagliflozin &Linagliptin Tablets 1000mg, 5 mg&2.5 mg
2	Metformin, Empagliflozin &Linagliptin

METHOD VALIDATION SUMMARY

PRECISION

Preparation of stock solution

Accurately weigh and transfer 1000 mg of Metformin and 2.5 mg of Linagliptin, and 5 mg of Empagliflozin working standards into a 25 ml clean, dry volumetric flask. Add about 15 mL of Diluent and sonicate to dissolve it completely, and make the volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (1200 ppm MET, 3 ppm of Lina and 6 ppm of EMPA).

Procedure

The standard solution was injected six times, and the area for all six injections was measured in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Acceptance Criteria

The % RSD for the area of six standard injections results should not be more than 2%**.**

INTERMEDIATE PRECISION/RUGGEDNESS

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days.

Preparation of stock solution

Accurately weigh and transfer 1000 mg of Metformin and 2.5 mg of Linagliptin and 5 mg of Empagliflozin working standards into a 25 ml clean dry volumetric flask add about 15 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (1200 ppm MET, 3 ppm of Lina and 6 ppm of EMPA).

Procedure

The standard solutions prepared in the precision were injected on the other day, for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Acceptance Criteria

The % RSD for the area of six standard injections results should not be more than 2%.

ACCURACY

Preparation of Standard stock solution

Accurately weigh and transfer 1000 mg of Metformin and 2.5 mg of Linagliptin and 5 mg of Empagliflozin working standards into a 25 ml clean, dry volumetric flask. Add about 15 mL of Diluent and sonicate to dissolve it completely and make the volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (1200 ppm MET, 3 ppm of Lina and 6 ppm of EMPA).

Preparation Sample solutions

For preparation of 50% solution (With respect to target Assay concentration)

Accurately weigh and transfer 500 mg of Metformin and 1.25 mg of Linagliptin and 2.5 mg of Empagliflozin working standards into a 25 ml clean dry volumetric flask add about 15 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (600 ppm MET, 1.5 ppm of Lina and 3 ppm of EMPA).

For preparation of 100% solution (With respect to target Assay concentration)

Accurately weigh and transfer 1000 mg of Metformin and 2.5 mg of Linagliptin and 5 mg of Empagliflozin working standards into a 25 ml clean dry volumetric flask add about 15 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (1200 ppm MET, 3 ppm of Lina and 6 ppm of EMPA).

For preparation of 150% solution (With respect to the target Assay concentration)

Accurately weigh and transfer 1500 mg of Metformin and 5 mg of Linagliptin, and 10 mg of Empagliflozin working standards into a 25 ml clean, dry volumetric flask. Add about 15 mL of Diluent and sonicate to dissolve it completely, and make the volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (1800 ppm MET, 5 ppm of Lina and 10 ppm of EMPA).

Procedure

Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150%solutions.

Calculate the Amount found and Amount added for Metformin& Empagliflozin and calculate the individual recovery and mean recovery values.

Acceptance Criteria

- The % Recovery for each level should be between 98.0 to 102.0%.

LINEARITY

Preparation of stock solution

Accurately weigh and transfer 1000 mg of Metformin and 2.5 mg of Linagliptin, and 5 mg of Empagliflozin working standards into a 25 ml clean, dry volumetric flask. Add about 15 mL of Diluent and sonicate to dissolve it completely, and make the volume up to the mark with the same solvent. (Stock solution).

Preparation of Level – I (600 ppm of Metformin, 1 of Linagliptin& 2 ppm of Empagliflozin)

0.1 ml of the above stock solutions has taken in different 10ml of volumetric flasks, diluted up to the mark with diluent.

Preparation of Level – II (900 ppm of Metformin, 2 ppm of Linagliptin&4 ppm of Empagliflozin)

0.2 ml of the above stock solutions has taken in different 10ml of volumetric flasks, diluted up to the mark with diluent.

Preparation of Level – III (1200 ppm of Metformin, 3 ppm of Linagliptin&6 ppm of Empagliflozin)

0.3 ml of the above stock solutions has taken in different 10ml of volumetric flasks, diluted up to the mark with diluent.

Preparation of Level – IV (1500 ppm of Metformin, 4 ppm of Linagliptin&8 ppm of Empagliflozin)

0.4 ml of the above stock solutions has taken in different 10ml of volumetric flasks, diluted up to the mark with diluent.

Preparation of Level – V (1800 ppm of Metformin, 5 ppm of Linagliptin&10 ppm of Empagliflozin)

0.5 ml of the above stock solutions has taken in different 10ml of volumetric flasks, diluted up to the mark with diluent.

Procedure

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on the X-axis, concentration and on the Y-axis Peak area) and calculate the correlation coefficient.

Acceptance Criteria

- The correlation coefficient should be not less than 0.99.

ROBUSTNESS

As part of the Robustness, deliberate changes in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

A) The flow rate was varied from 0.9 ml/min to 1.1ml/min.

Standard solution 1200ppm of Metformin, 3 ppm of Linagliptin, and 6 ppm ofEmpagliflozin wasprepared and analysed using the varied flow rates along with the method flow rate.

On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence, it indicates that the method is robust even with a change in the flow rate ±10%.

B) The Organic composition in the Mobile phase was varied from 50% to 50%

Standard solution 1200 µg/ml of Metformin,3µg/ml of Linagliptin&6 µg/ml of Empagliflozin **were** prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method. On evaluation of the above results, it can be concluded that the variation in 10%.

Organic composition in the mobile phase affected the method significantly.

Hence it Indicates that the method is robust even with a change in the Mobile phase ±10

DEGRADATION STUDIES

The International Conference on Harmonization (ICH) guideline entitled Stability Testing of New Drug Substances and Products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. This work aimed to perform the stress degradation studies on the Metformin, Linagliptin and Empagliflozin using the proposed method.

Preparation of stock

Accurately weigh and transfer 1000 mg of Metformin and 2.5 mg of Linagliptin, and 5 mg of Empagliflozin working standards into a 25 ml clean, dry volumetric flask. Add about 15 mL of Diluent and sonicate to dissolve it completely, and make the volume up to the mark with the same solvent. (Stock solution).

Hydrolytic degradation under acidic condition

Pipette 0.3 ml of the above solution into a 10ml volumetric flask, and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Hydrolytic degradation under alkaline condition

Pipette 0.3 ml of the above solution into a 10ml volumetric flask into a 10ml volumetric flask and add 3 ml of 0.1N NaOH was added in 10 ml of volumetric flask. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1N HCl and made up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Thermally induced degradation

Metformin, Linagliptin and Empagliflozin sample was taken in a Petri dish and kept in a hot air oven at 110^0 C for 24 hours. Then the sample was taken, diluted with diluents and injected into the HPLC and analyzed.

Oxidative degradation

Pipette 0.3ml above stock solution 2 into a 10ml volumetric flask solution into a 10ml volumetric flask 1 ml of 3% w/v of hydrogen peroxide to a 10ml volumetric flask, and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials.

Photo Stability studies

Metformin, Linagliptin and Empagliflozin sample solutions was taken into a beaker and exposed to UV light for 1 day. Then the solutions were injected into the chromatographic system.

RESULTS

Trail 1

Buffer: 0.1% OPA
Mobile Phase: Buffer: Methanol (30:70)
Column: Thermo Hypersil C_{18} 250×4.6mm, 5um
Flow Rate: 1.0 ml/min
Temperature: Ambient
Volume: 10ul
Detector: 224 nm
Diluents: Water: Methanol 50:50

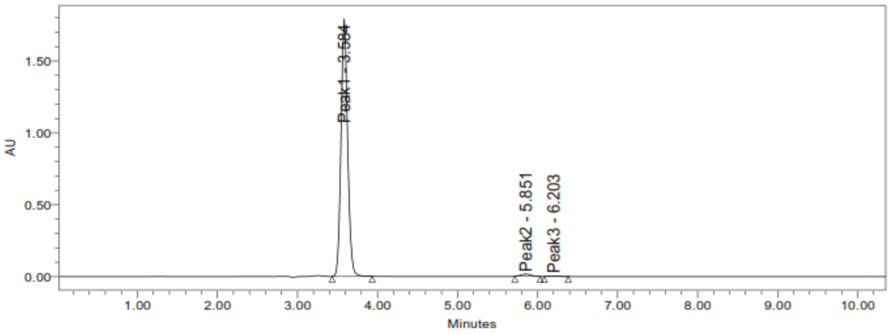


Table 5: data obtained in trial 1.

SL. NO	PEAK NAME	RT	AREA	% AREA	USP PLATE COUNT	USP PLATE RESOLUTION	USP TAILING
1	Metformin	3.584	10191283	98.41	8761		1.10
2	Empagliflozin	5.851	141084	1.36	8965	11.29	1.12
3	Linagliptin	6.203	23796	0.23	12400	1.49	1.17

Observation: Resolution between Linagliptin and Empagliflozin was not obtained.

Trail 2

Buffer: Phosphate Buffer
Mobile Phase: Buffer: Acetonitrile (40:60)
Column: Phenominex C_{18} 250×4.6mm, 5um
Flow Rate: 1.0ml/min
Temperature: Ambient
Volume: 10ul
Detector: 224 nm
Diluents: Water: Methanol 50:50

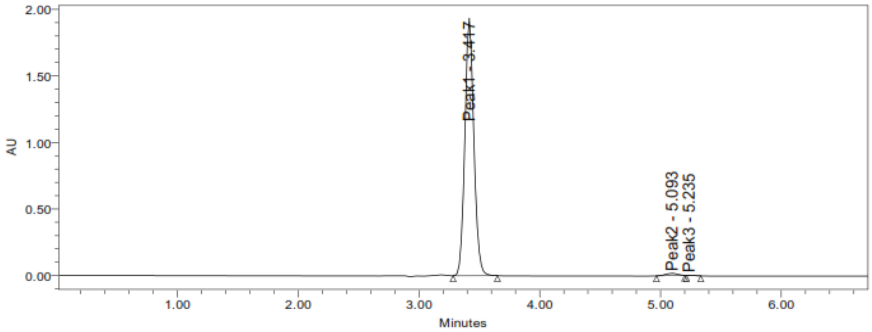


Table 6: data obtained in trial 2.

SL.NO	PEAK NAME	RT	AREA	% AREA	USP PLATE COUNT	USP PLATE RESOLUTION	USP TAILING
1	Metformin	3.417	9994768	98.82	9410		1.09
2	Empagliflozin	5.093	115442	1.14	10980	9.85	0.93
3	Linagliptin	5.235	3620	0.04	30527	0.90	3.14

Observation: Resolution between Linagliptin and Empagliflozin was not obtained.

Trail 3
Buffer: Trifluroacetic acid
Mobile Phase: Buffer: Methanol (35:65)
Column: X Bridge C_{18} 250×4.6mm, 5um
Flow Rate: 1.0ml/min
Temperature: Ambient
Volume: 10ul
Detector: 224 nm
Diluents: Water: Methanol 50:50

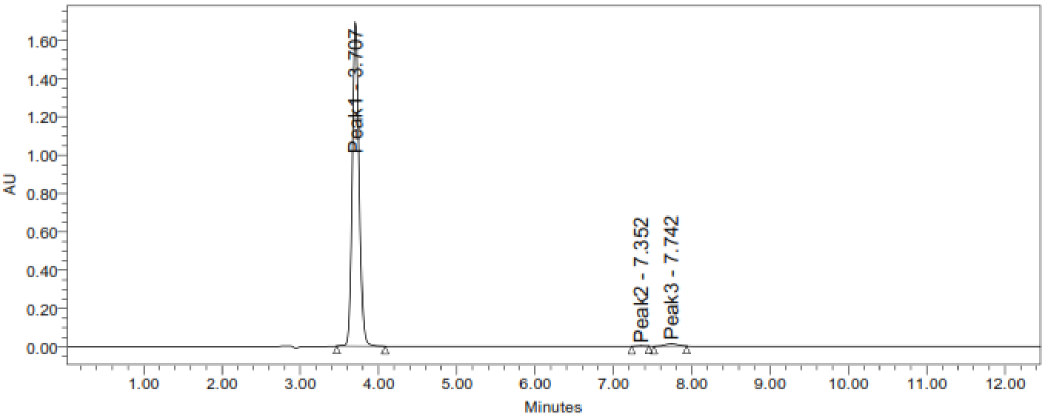


Table 7: data obtained in trial 3

SL.NO	PEAK NAME	RT	AREA	% AREA	USP PLATE COUNT	USP PLATE RESOLUTION	USP TAILING
1	Metformin	3.707	10332177	98.49	8741		1.11
2	Empagliflozin	7.352	14306	0.14	19249	19.44	0.92
3	Linagliptin	7.742	144124	1.37	9286	1.46	0.96

Observation: Resolution between Linagliptin and Empagliflozin was not obtained.

Trail 4
Buffer: Trifluroacetic acid
Mobile Phase: Buffer: Methanol: ACN (40:55:5)
Column: X Bridge C_{18} 250×4.6mm, 5um
Flow Rate: 1.0ml/min
Temperature: Ambient
Volume: 10ul
Detector: 224 nm
Diluents: Water: Methanol 50:50

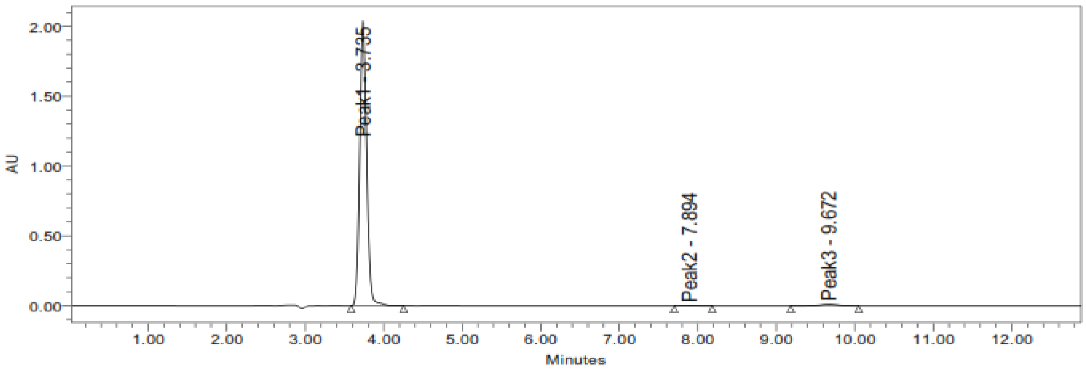


Table 8: data obtained in trial 4.

SL.NO	PEAK NAME	RT	AREA	% AREA	USP PLATE COUNT	USP PLATE RESOLUTION	USP TAILING
1	Metformin	3.735	12441097	98.44	8648		1.10
2	Empagliflozin	7.894	24990	0.20	12195	18.31	1.01
3	Linagliptin	9.672	172289	1.36	8771	5.01	0.98

Observation: Resolution Obtained but get more retention time

Trail 5
Buffer: 0.1 % Trifluroacetic acid
Mobile Phase: Buffer: Methanol: ACN (40:50:10)
Column: X Bridge C_{18} 250×4.6mm, 5um
Flow Rate: 1.0ml/min
Temperature: Ambient
Volume: 10ul
Detector: 224 nm
Diluents: Water: Methanol 50:50

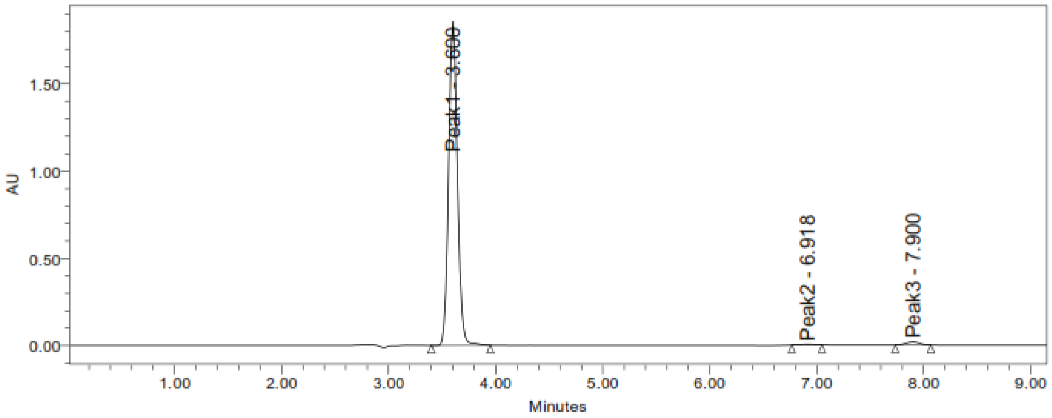


Table 9: data obtained in trial 5.

SL.NO	PEAK NAME	RT	AREA	% AREA	USP PLATE COUNT	USP PLATE RESOLUTION	USP TAILING
1	Metformin	3.600	10314593	98.14	9190		1.09
2	Empagliflozin	6.918	35909	0.34	13221	16.95	0.93
3	Linagliptin	7.900	159903	1.52	17513	4.04	1.00

Observation: Resolution Obtained but get more retention time.

Trail 6 (OPTIMIZED CHROMATOGRAPHIC CONDITIONS)
Instrument used: Waters HPLC with auto sampler and PDA detector.
Buffer: 0.1M KH_2PO_4
Mobile Phase: Buffer: Methanol (40:60)

Column: Thermo HYpesil C_{18} 150×4.6mm, 5um
Flow Rate: 1.0ml/min
Temperature: Ambient
Volume: 10ul
Detector 224 nm

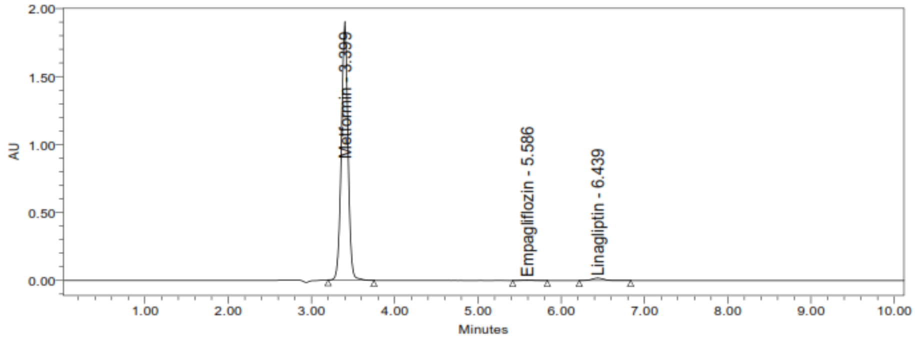
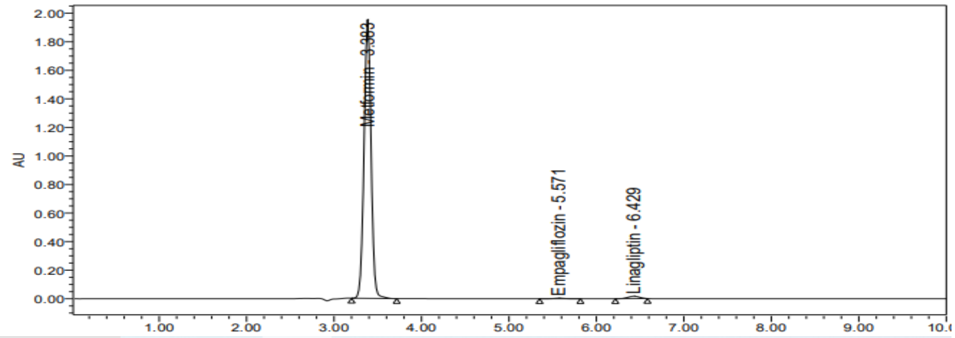


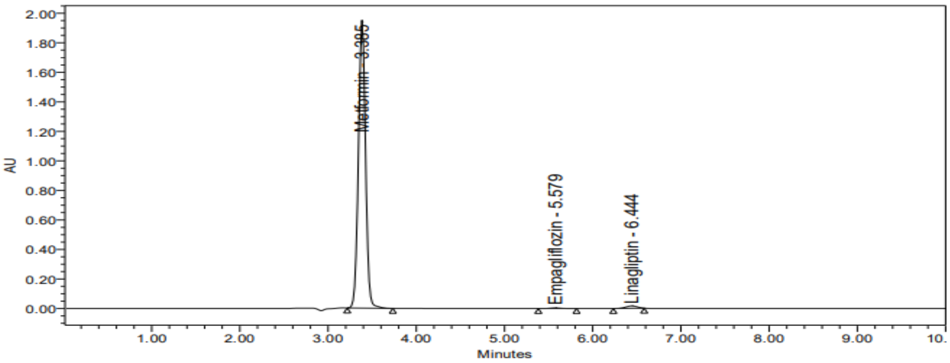
Table 10: data obtained in trial 6.

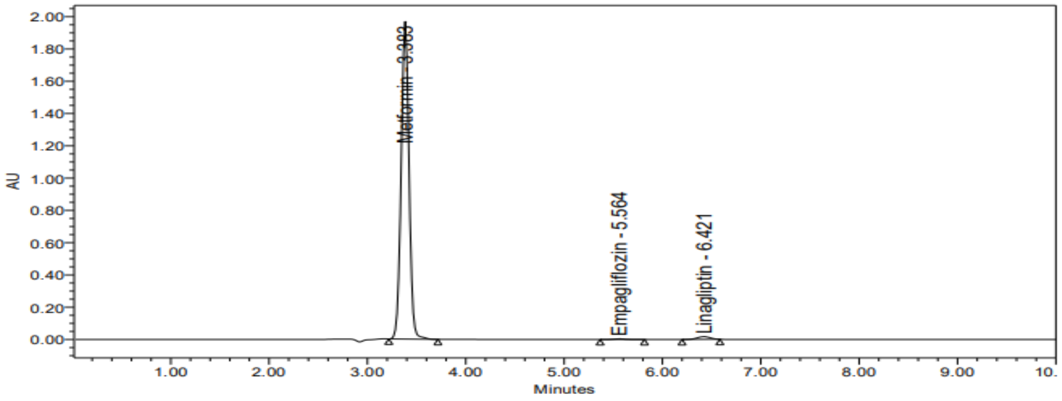
SL.NO	PEAK NAME	RT	AREA	% AREA	USP PLATE COUNT	USP PLATE RESOLUTION	USP TAILING
1	Metformin	3.399	10299304	98.05	8655		1.00
2	Empagliflozin	5.586	30802	0.29	11451	12.24	1.07
3	Linagliptin	6.439	174057	1.66	9657	3.57	1.02

Observation: This trail exhibited proper peak shape, plate count, good resolution, and retention time. So, this method is optimized.

CHROMATOGRAMS OF SAMPLE



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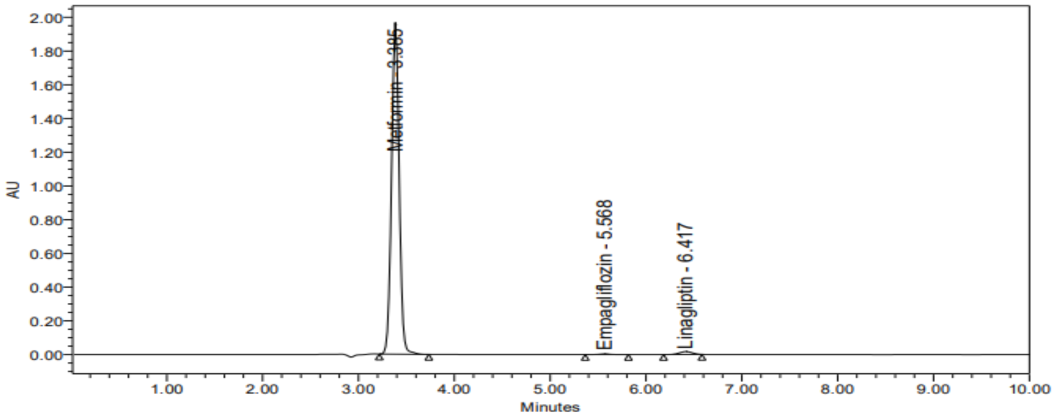
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Empagliflozin - 5.568
Linagliptin - 6.417
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2.00
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5.00
6.00
7.00
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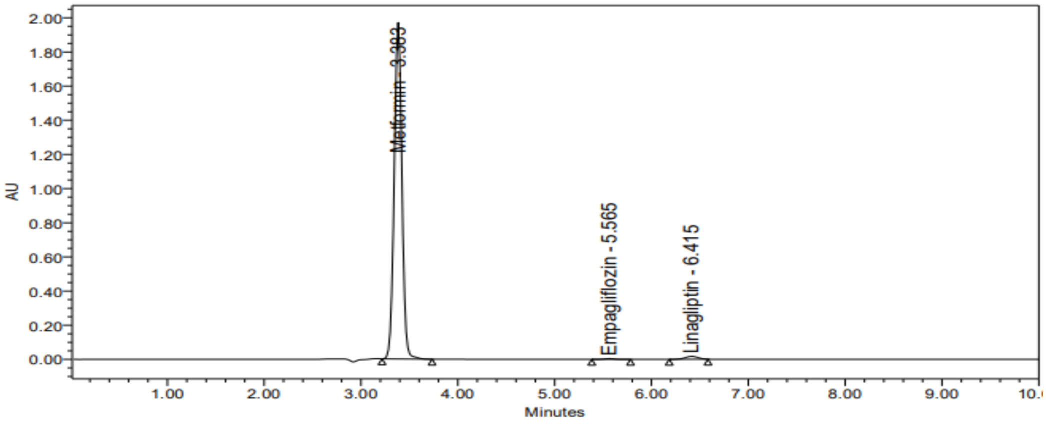
10.00
Minutes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1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
AU
Metformin - 3.383
Empagliflozin - 5.565
Linagliptin - 6.415
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2.00
3.00
4.00
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8.00
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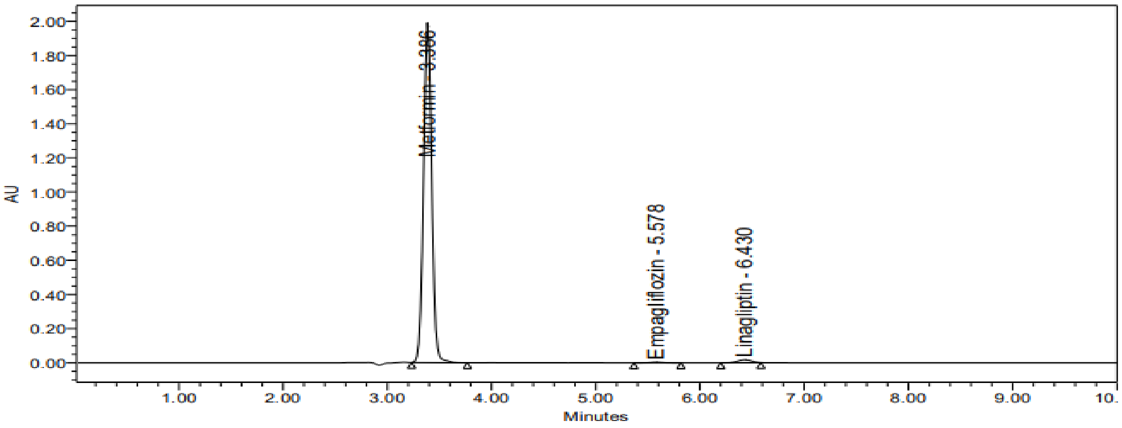
10.
Minutes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1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
AU
Metformin - 3.386
Empagliflozin - 5.578
Linagliptin - 6.430
1.00
2.00
3.00
4.00
5.00
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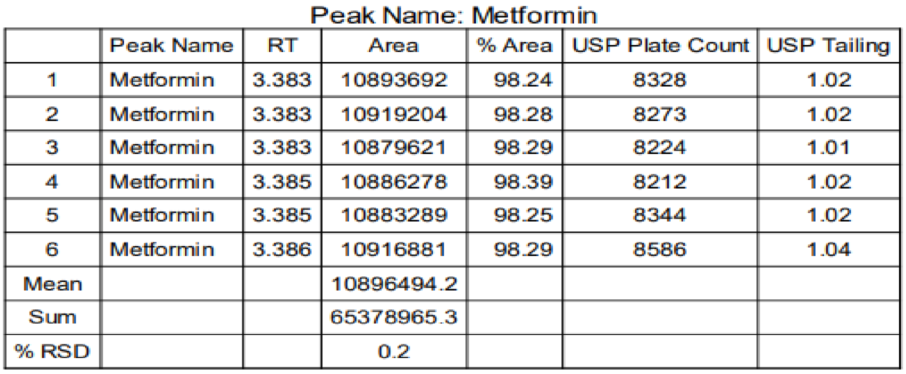
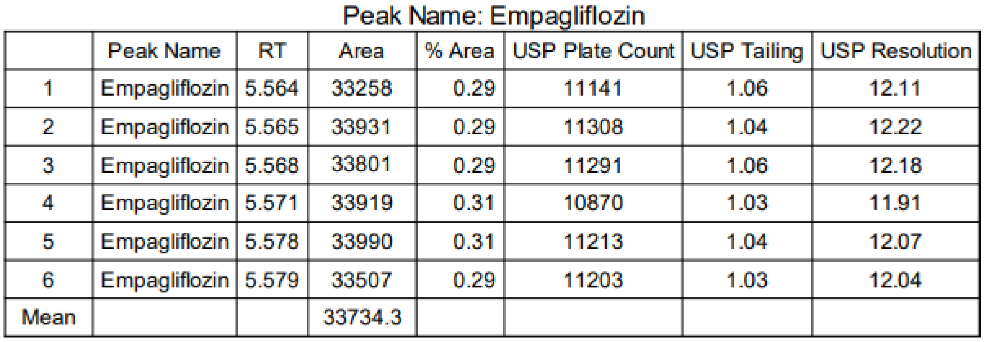
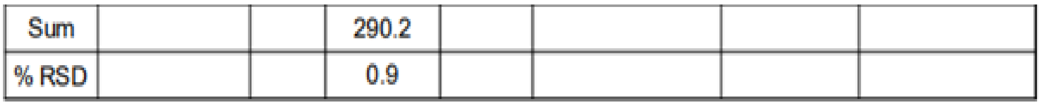
Minutes



Peak Name: Metformin

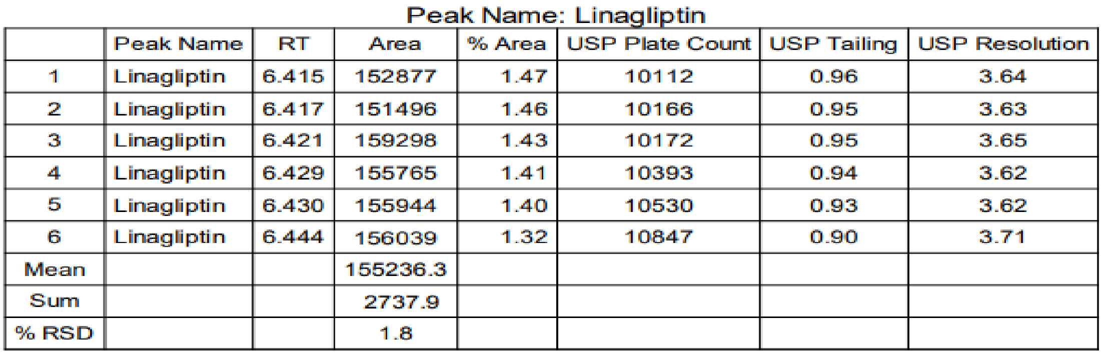
	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing
1	Metformin	3.383	10893692	98.24	8328	1.02
2	Metformin	3.383	10919204	98.28	8273	1.02
3	Metformin	3.383	10879621	98.29	8224	1.01
4	Metformin	3.385	10886278	98.39	8212	1.02
5	Metformin	3.385	10883289	98.25	8344	1.02
6	Metformin	3.386	10916881	98.29	8586	1.04
Mean			10896494.2			
Sum			65378965.3			
% RSD			0.2			





Peak Name: Empagliflozin

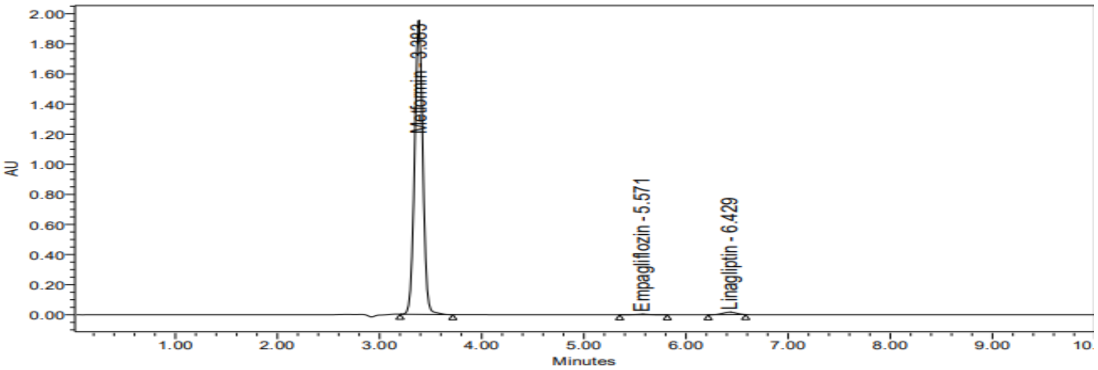
	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing	USP Resolution
1	Empagliflozin	5.564	33258	0.29	11141	1.06	12.11
2	Empagliflozin	5.565	33931	0.29	11308	1.04	12.22
3	Empagliflozin	5.568	33801	0.29	11291	1.06	12.18
4	Empagliflozin	5.571	33919	0.31	10870	1.03	11.91
5	Empagliflozin	5.578	33990	0.31	11213	1.04	12.07
6	Empagliflozin	5.579	33507	0.29	11203	1.03	12.04
Mean			33734.3				
Sum			290.2				
% RSD			0.9				

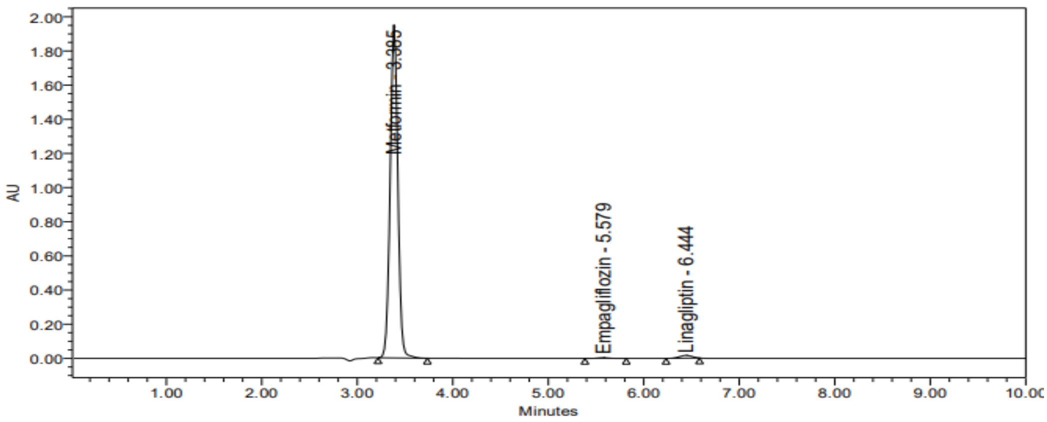


Peak Name: Linagliptin

	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing	USP Resolution
1	Linagliptin	6.415	152877	1.47	10112	0.96	3.64
2	Linagliptin	6.417	151496	1.46	10166	0.95	3.63
3	Linagliptin	6.421	159298	1.43	10172	0.95	3.65
4	Linagliptin	6.429	155765	1.41	10393	0.94	3.62
5	Linagliptin	6.430	155944	1.40	10530	0.93	3.62
6	Linagliptin	6.444	156039	1.32	10847	0.90	3.71
Mean			155236.3				
Sum			2737.9				
% RSD			1.8				

CHROMATOGRAMS OF STANDARD

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0.00
0.20
0.40
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0.80
1.00
1.20
1.40
1.60
1.80
2.00
Metformin - 3.385
Empagliflozin - 5.579
Linagliptin - 6.444
1.00
2.00
3.00
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7.00
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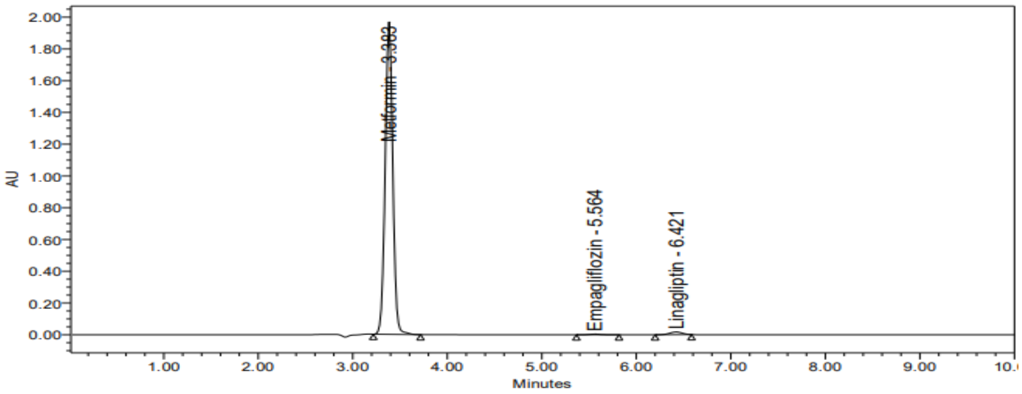
Minutes


AU
0.00
0.20
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1.20
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1.60
1.80
2.00
Metformin - 3.383
Empagliflozin - 5.564
Linagliptin - 6.421
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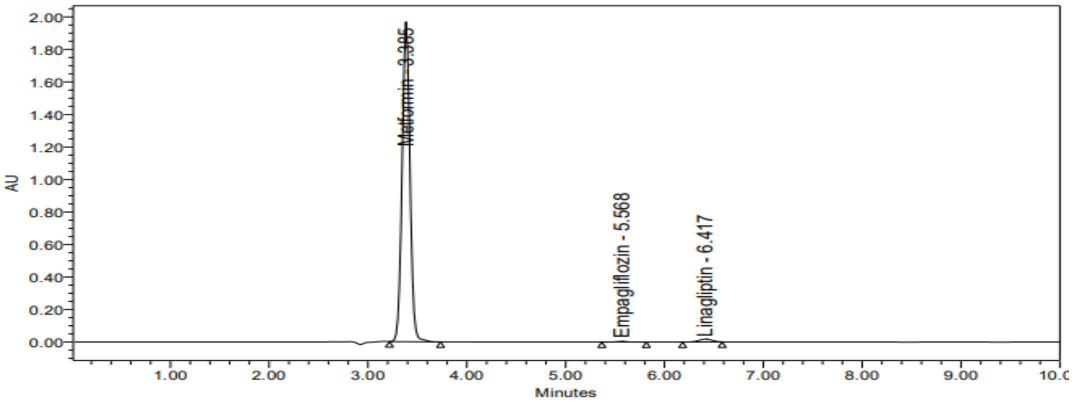
Minutes


AU
0.00
0.20
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0.80
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2.00
Metformin - 3.385
Empagliflozin - 5.568
Linagliptin - 6.417
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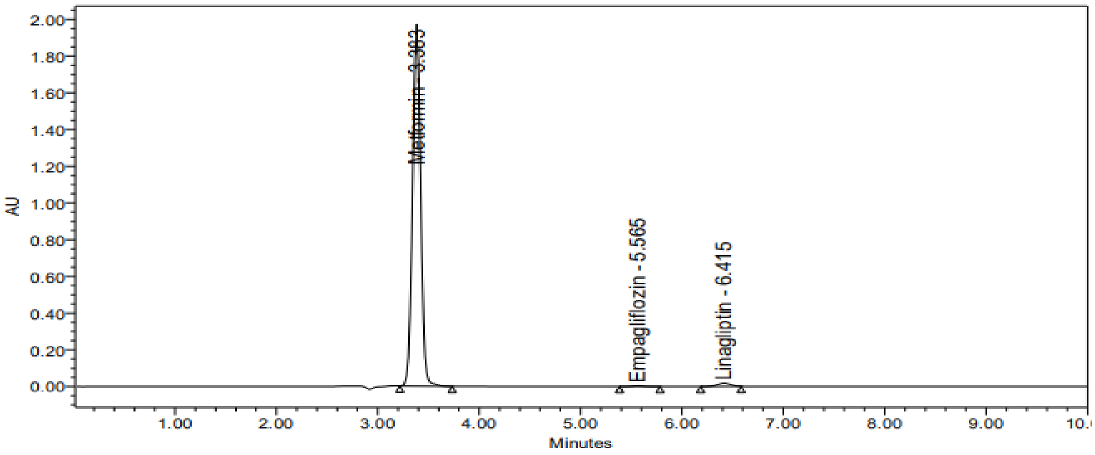
Minutes


AU
0.00
0.20
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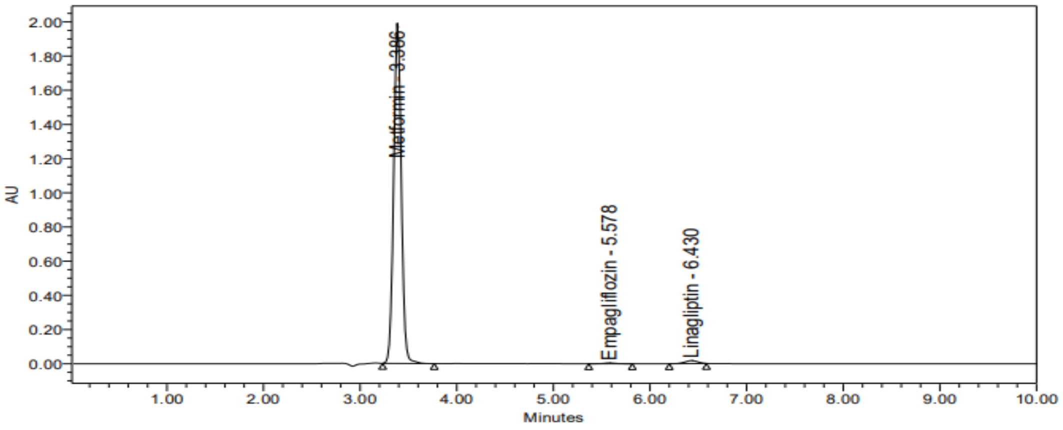
1.80
2.00
Metformin - 3.383
Empagliflozin - 5.565
Linagliptin - 6.415
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.
Minutes



Peak Name: Metformin

	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing
1	Metformin	3.383	10893692	98.24	8328	1.02
2	Metformin	3.383	10919204	98.28	8273	1.02
3	Metformin	3.383	10879621	98.29	8224	1.01
4	Metformin	3.385	10886278	98.39	8212	1.02
5	Metformin	3.385	10883289	98.25	8344	1.02
6	Metformin	3.386	10916881	98.29	8586	1.04
Mean			10896494.2			
Sum			65378965.3			
% RSD			0.2			

Peak Name: Empagliflozin

	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing	USP Resolution
1	Empagliflozin	5.564	32258	0.29	11141	1.06	12.11
2	Empagliflozin	5.565	32931	0.29	11308	1.04	12.22
3	Empagliflozin	5.568	32801	0.29	11291	1.06	12.18
4	Empagliflozin	5.571	32919	0.31	10870	1.03	11.91
5	Empagliflozin	5.578	33990	0.31	11213	1.04	12.07
6	Empagliflozin	5.579	32507	0.29	11203	1.03	12.04
Mean			32901.3				
Sum			594.7				
% RSD			1.8				

Peak Name: Linagliptin

	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing	USP Resolution
1	Linagliptin	6.415	152877	1.47	10112	0.96	3.64
2	Linagliptin	6.417	151496	1.46	10166	0.95	3.63
3	Linagliptin	6.421	159298	1.43	10172	0.95	3.65
4	Linagliptin	6.429	155765	1.41	10393	0.94	3.62
5	Linagliptin	6.430	155944	1.40	10530	0.93	3.62
6	Linagliptin	6.444	156039	1.32	10847	0.90	3.71
Mean			155236.3				
Sum			2737.9				
% RSD			1.7				

SYSTEM SUITABILITY

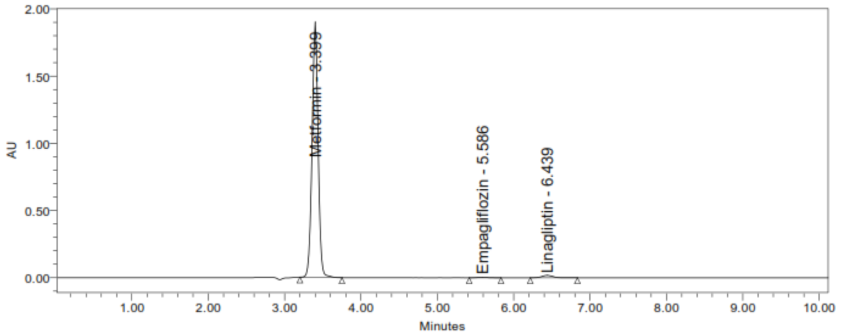


Figure 1: Chromatogram for system suitability.

	Peak Name	RT	Area	% Area	USP Plate Count	USP Resolution	USP Tailing
1	Metformin	3.399	10301139	98.05	8654		1.00
2	Empagliflozin	5.586	30802	0.29	11451	12.24	1.07
3	Linagliptin	6.439	174057	1.66	9657	3.57	1.02

Acceptance criteria

- Resolution between two drugs must not be less than 2.
- Theoretical plates must not be less than 2000.
- Tailing factor must not be more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

VALIDATION PARAMETERS

1. ASSAY

Standard and sample solution injected as described under experimental work. The corresponding chromatograms and results are shown below.

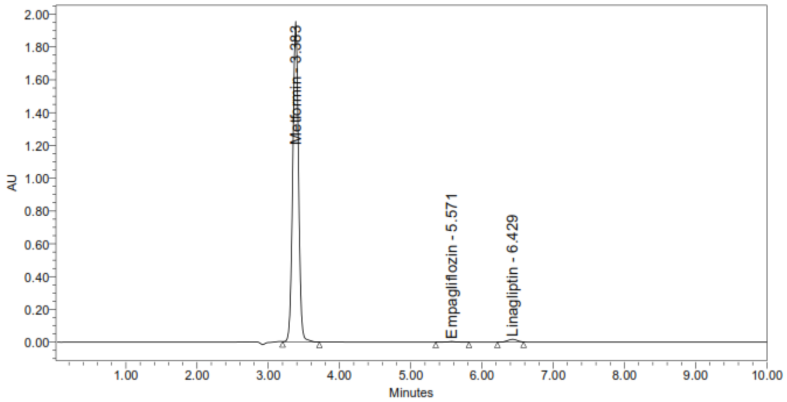


Figure 2: Chromatogram for Standard.

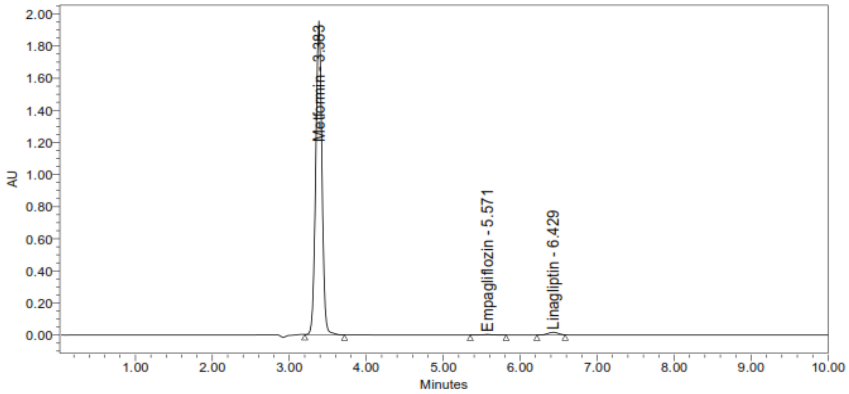


Figure 3: Chromatogram for Sample.

Table 11: Results of Assay for Metformin, Empagliflozin and Linagliptin.

S. No.	Samples
1	Metformin, Empagliflozin & Linagliptin Tablets 1000mg, 5 mg & 2.5 mg
2	Metformin, Empagliflozin & Linagliptin

Results

For Metformin

Assay	10896494	1000	0.3	1	25	10	1	1254	99.80	100	99.80
	10896494	25	10	1	1254	0.3	1	1000	100		

For Empagliflozin

Assay	33734	5	0.3	1	25	10	1	1254	99.80	100	102.33
	32901	25	10	1	1254	0.3	1	5	100		

For Linagliptin

Assay	155236	2.5	0.3	1	25	10	1	1254	99.80	100	99.80
	155236	25	10	1	1254	0.3	1	2.5	100		

Acceptance criteria

- **The Assay of the sample and standard are within the acceptance limit**

2. LINEARITY

The linearity range was found to lie from 600µg/ml to 1800 µg/ml of Metformin, 2µg/ml to 10µg/ml 0f Empagliflozin and 1µg/ml to 5µg/ml 0f Linagliptin chromatograms are shown below.

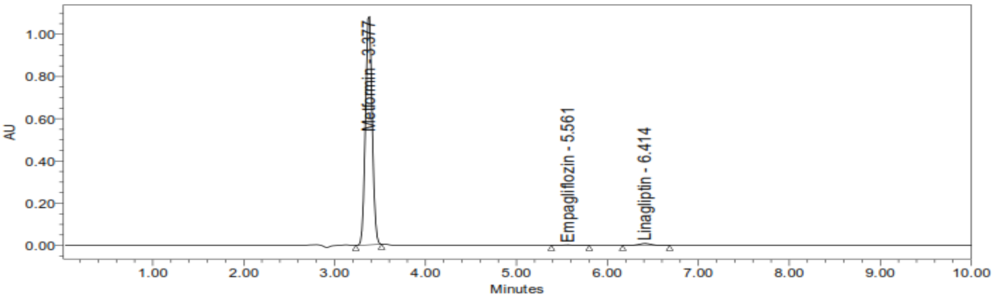


Figure 4: Chromatogram for linearity-1.

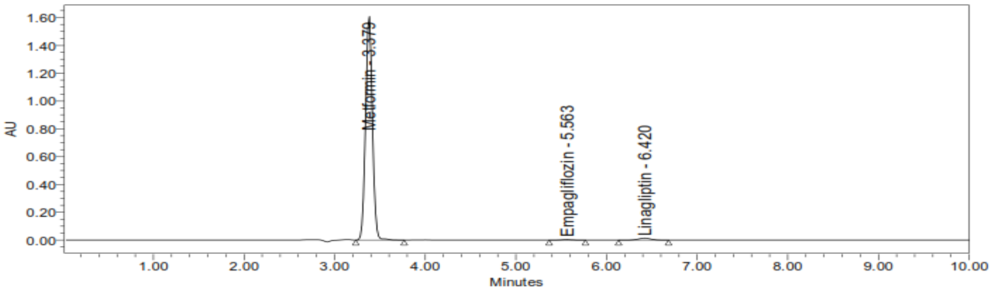


Figure 5: Chromatogram for linearity-2.

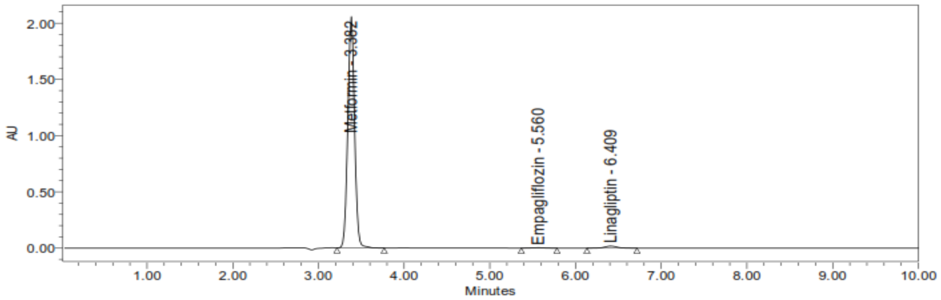


Figure 6: Chromatogram for linearity-3.

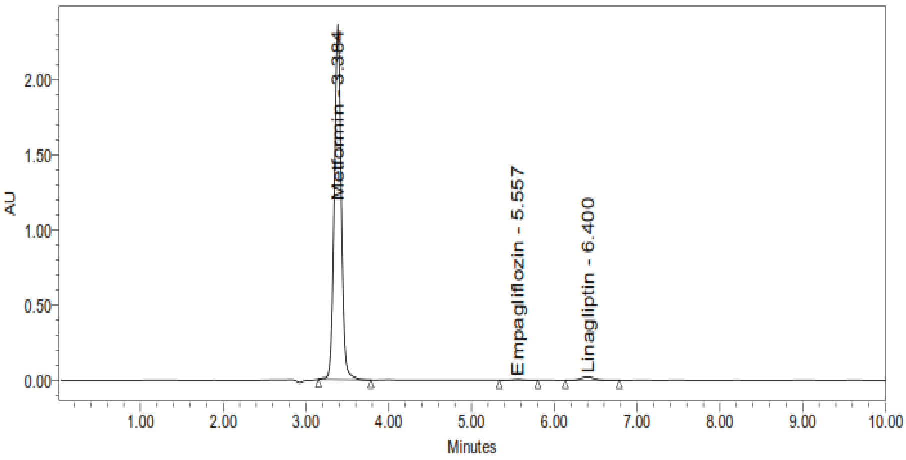


Figure 7: Chromatogram for linearity-4.

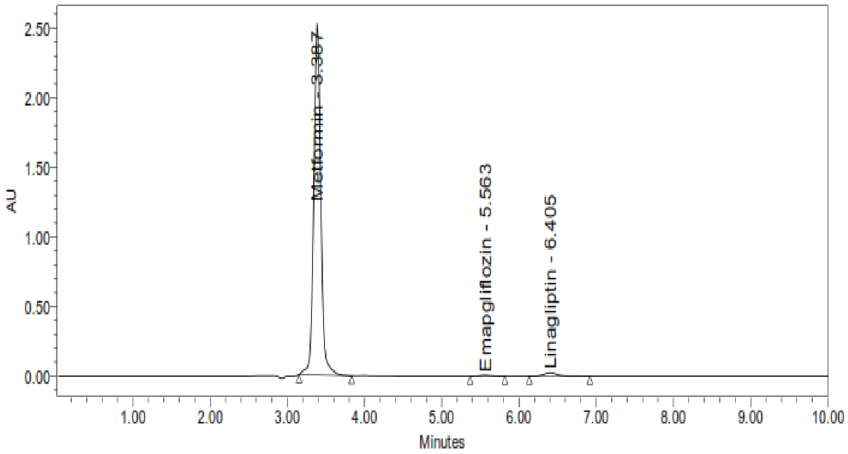


Figure 8: Chromatogram for linearity-5.

Table 12: Area of different concentrations of Metformin, Empagliflozin and Linagliptin.

S. No	Metformin		Empagliflozin		Linagliptin	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	600	5533712	2	17070	1	87438
2	900	8409615	4	24837	2	134113
3	1200	11221992	6	33671	3	181266
4	1500	13768045	8	42017	4	230245
5	1800	16898907	10	51023	5	273289

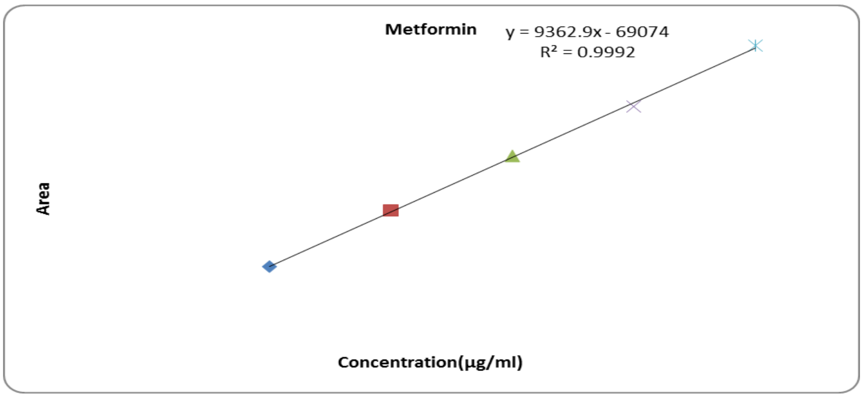


Figure 9: Calibration graph for Metformin

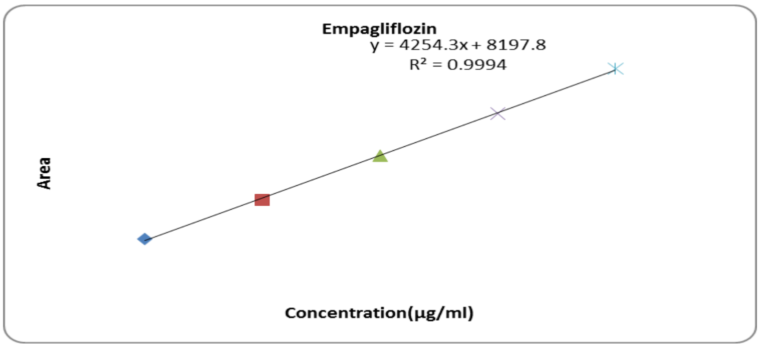


Figure 10: Calibration graph for Empagliflozin.

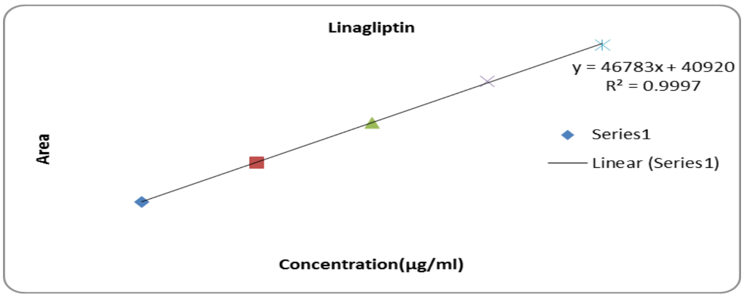


Figure 11: Calibration graph for Linagliptin

Acceptance criteria

Correlation coefficient (R^2) should not be less than 0.999

- The correlation coefficient obtained was 0.999 which is in the acceptance limit.

3. PRECISION

Precision of the method was carried out for both sample solutions as described under experimental work. The corresponding chromatograms and results are shown below.

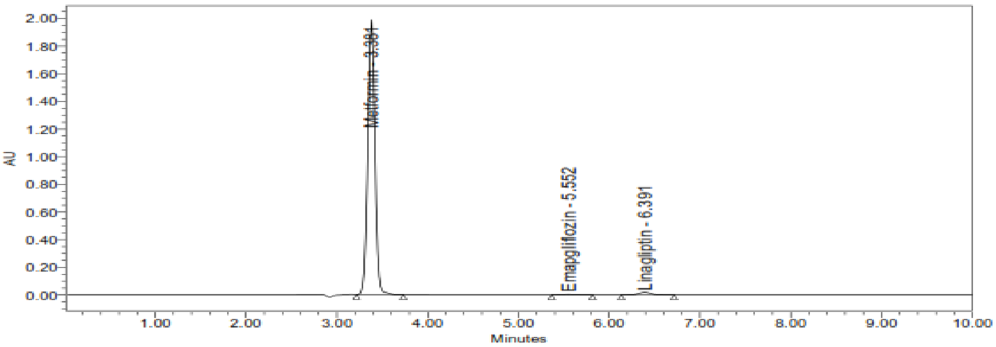


Figure 12: Chromatogram for Precision -1.

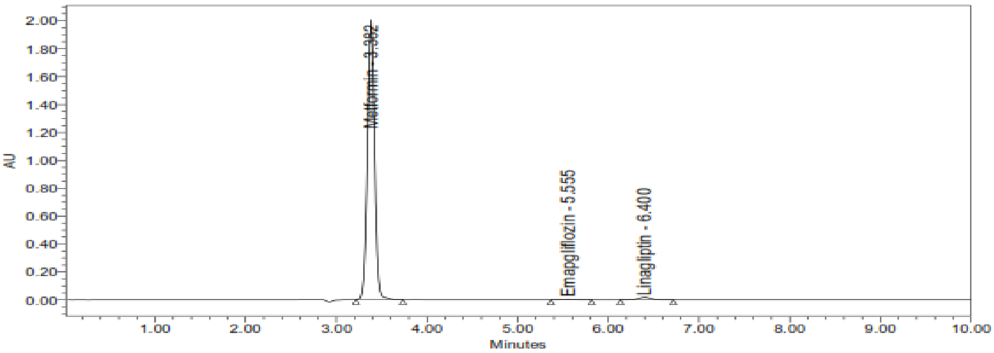


Figure 13: Chromatogram for Precision -2.

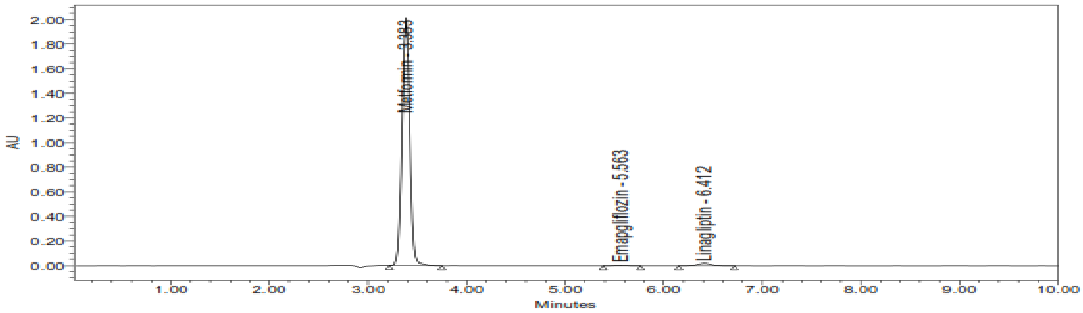


Figure 14: Chromatogram for Precision -3.

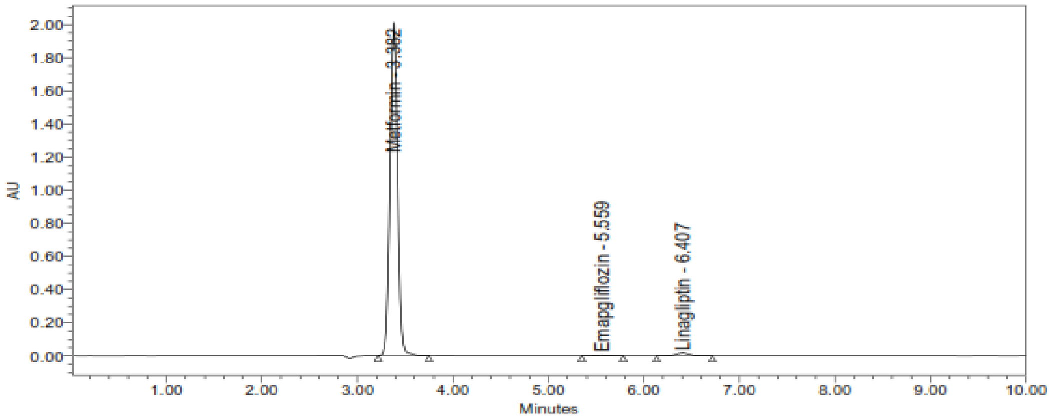


Figure 15: Chromatogram for Precision -4.

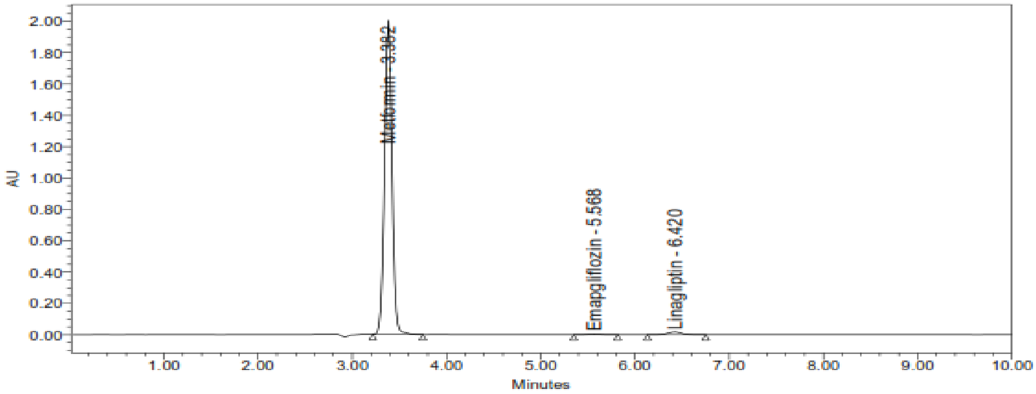


Figure 16: Chromatogram for Precision -5.

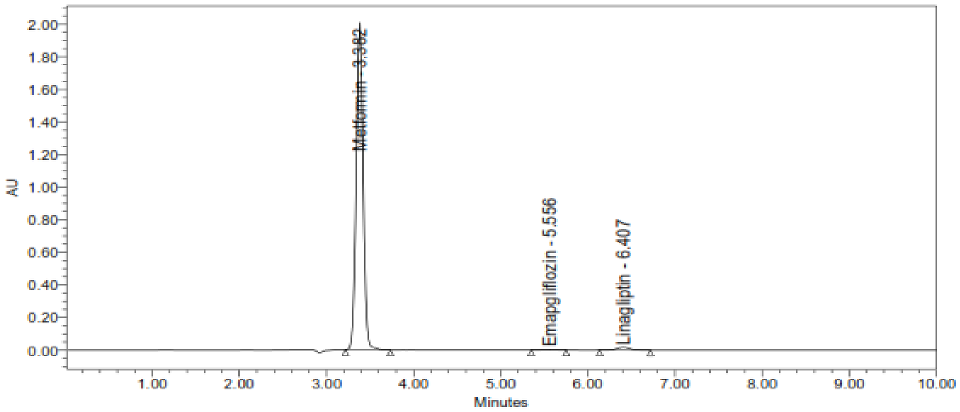


Figure 17: Chromatogram for Precision -6.

Table 13: Results of Precision for Metformin, Empagliflozin and Linagliptin.

Injection	Area for Metformin	Area for Empagliflozin	Area for Linagliptin
Injection-1	10884334	32186	176519
Injection-2	10990504	32232	176202
Injection-3	11000201	32493	178059
Injection-4	10989075	32894	178530
Injection-5	10926237	33063	176142
Injection-6	10952941	33164	178382
Average	10957215.4	32671.8	177305.7
Standard Deviation	65743292.3	196031.0	1063834.2
%RSD	0.4	1.3	0.6

Acceptance criteria

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

4. INTERMEDIATE PRECISION (ruggedness).

There was no significant change in assay content and system suitability parameters at different conditions of ruggedness like day to day and system to system variation.

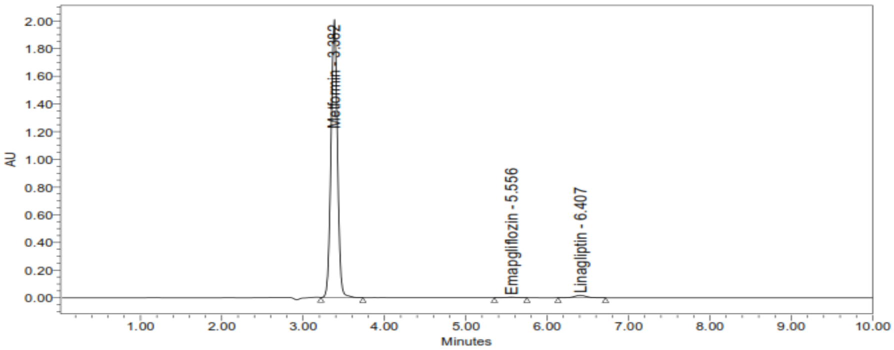


Figure 18: Chromatogram for ID Precision -1.

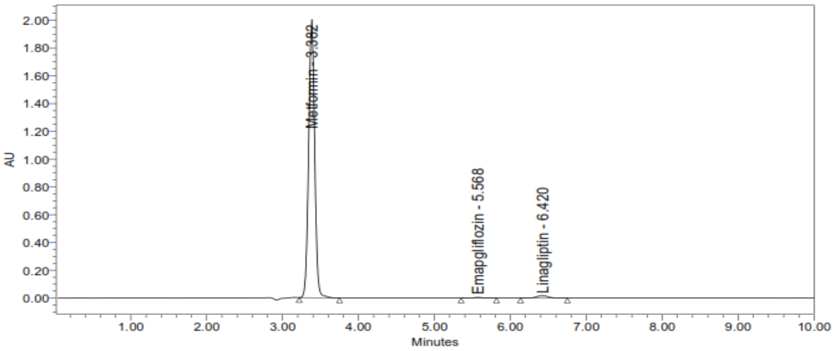


Figure 19: Chromatogram for ID Precision -2.

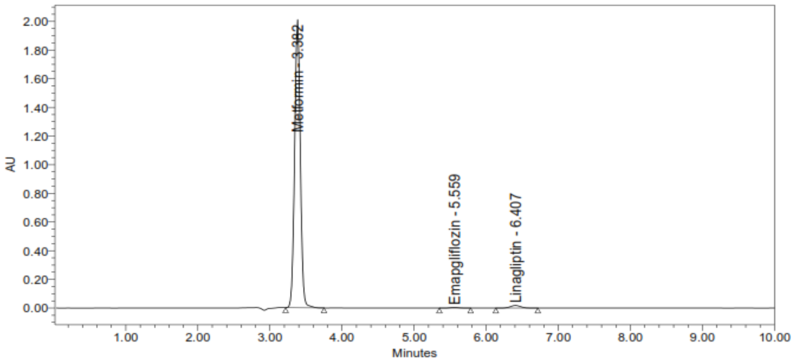


Figure 20: Chromatogram for ID Precision -3.

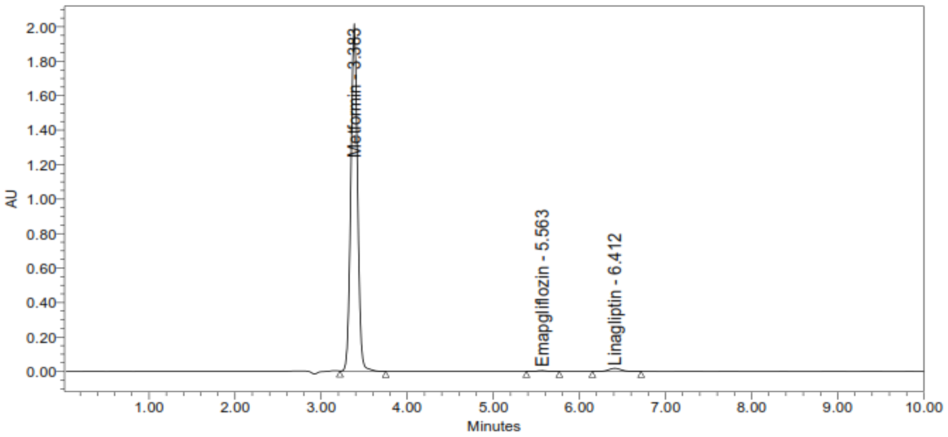


Figure 21: Chromatogram for ID Precision -4.

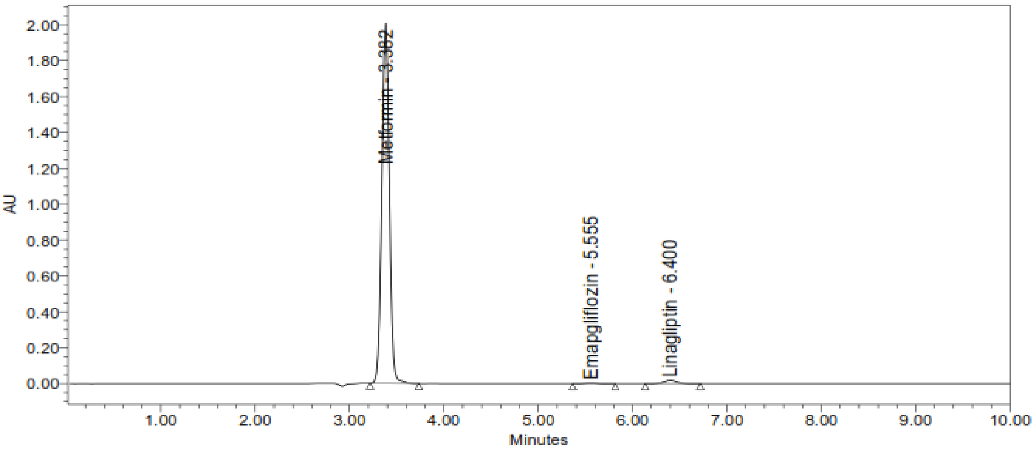


Figure 22: Chromatogram for ID Precision -5.

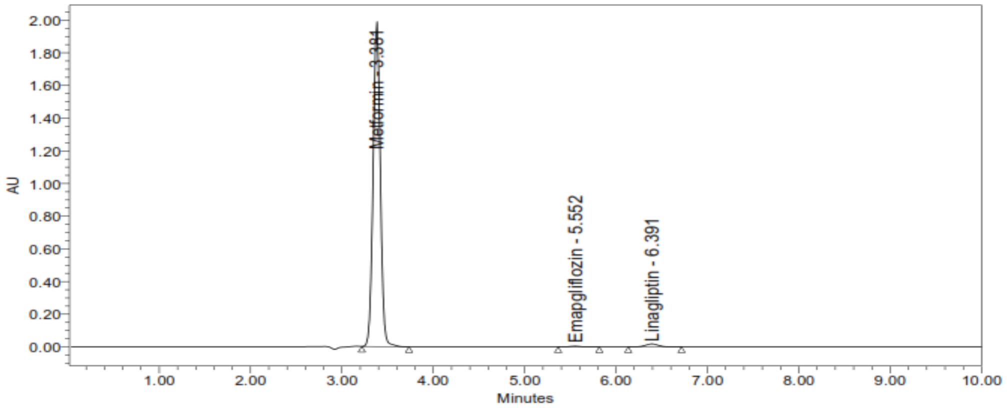


Figure 23: Chromatogram for ID Precision -6.

Table 14: Results of Intermediate precision for Metformin, Empagliflozin & Linagliptin

Injection	Area for Metformin	Area for Empagliflozin	Area for Linagliptin
Injection-1	10884334	32186	176519
Injection-2	10990504	32232	176202
Injection-3	11000201	32493	178059
Injection-4	10989075	32894	178530
Injection-5	10926237	33063	176142
Injection-6	10952941	33164	178382
Average	10957215.4	32671.8	177305.7
Standard Deviation	65743292.3	196031.0	1063834.2
%RSD	0.4	1.3	0.6

Acceptance criteria

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is rugged.

ACCURACY

Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated.

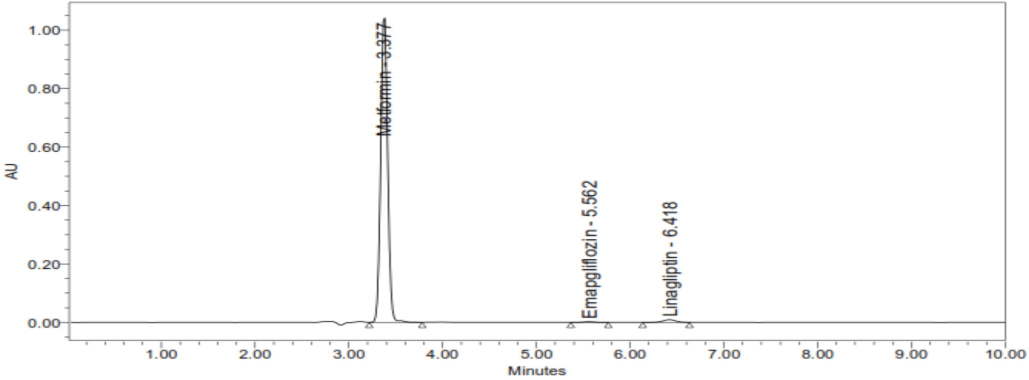


Figure 24: Chromatogram for Accuracy 50%-1.

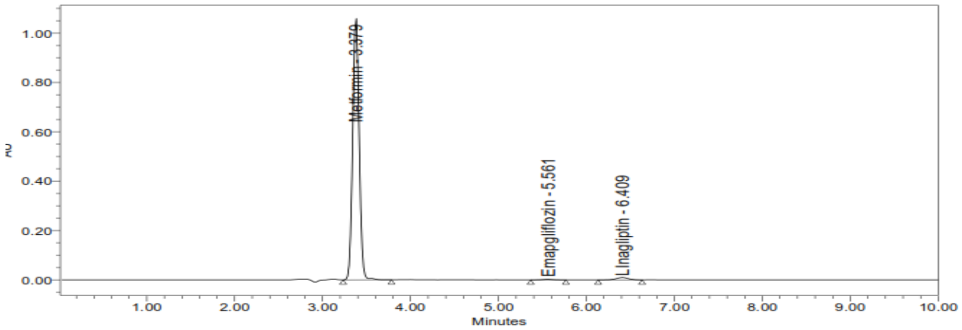


Figure 25: Chromatogram for Accuracy 50%-2.

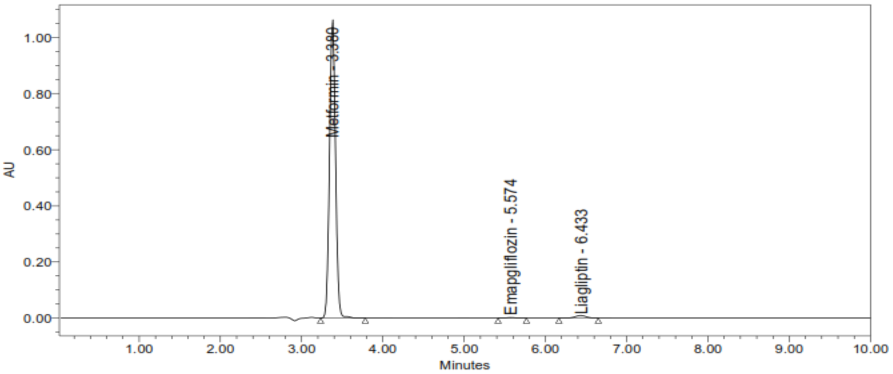


Figure 26: Chromatogram for Accuracy 50%-3.

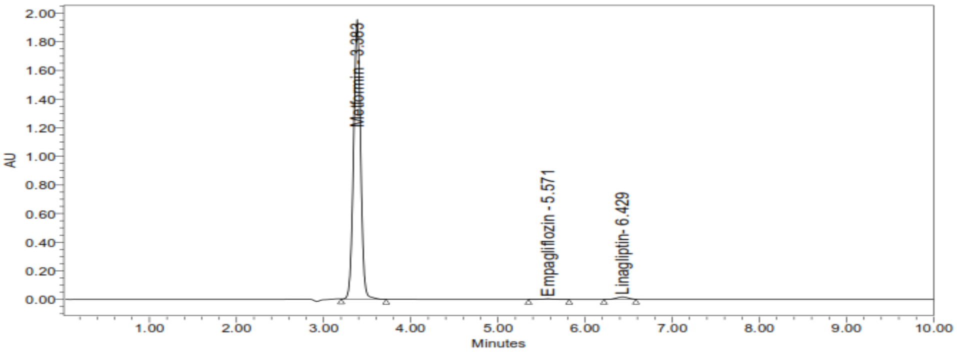


Figure 27: Chromatogram for Accuracy 100%-1.

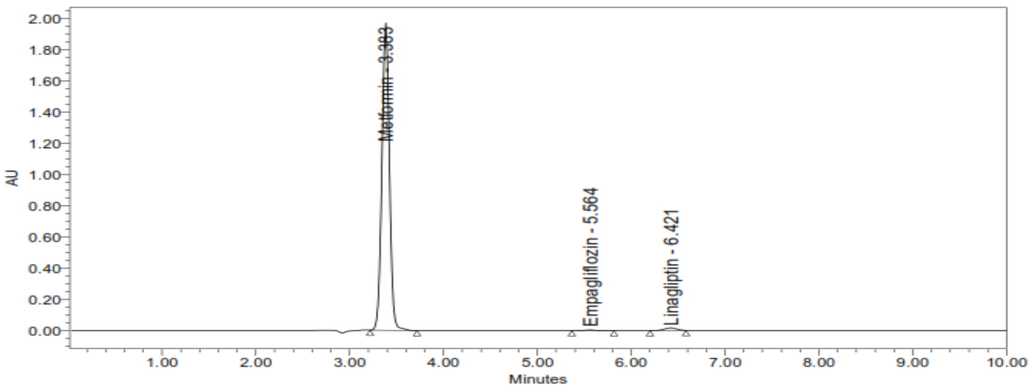


Figure 28: Chromatogram for Accuracy 100%-2.

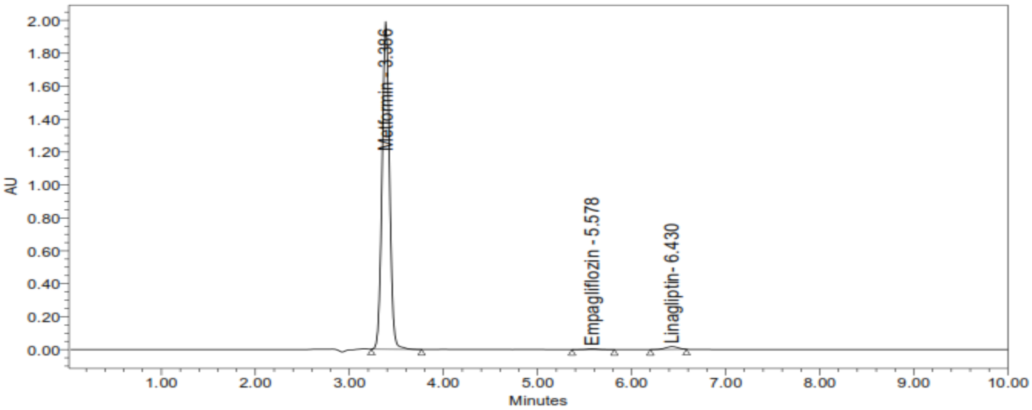


Figure 29: Chromatogram for Accuracy 100%-3.

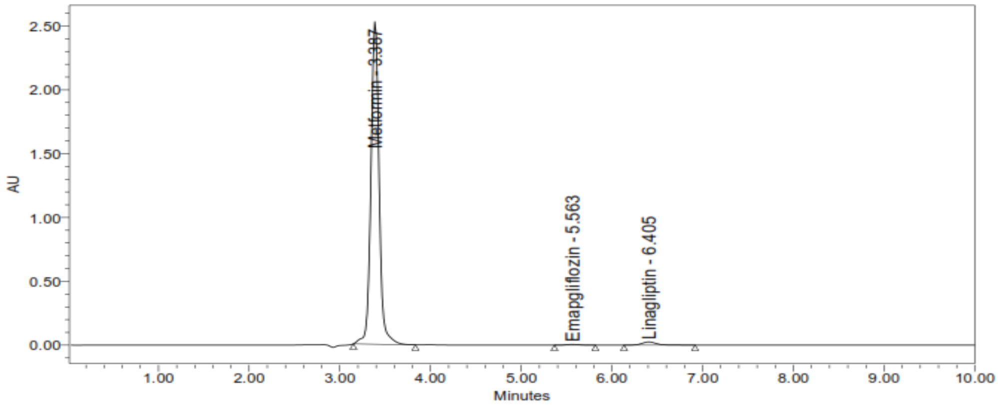


Figure 30: Chromatogram for Accuracy 150%-1.

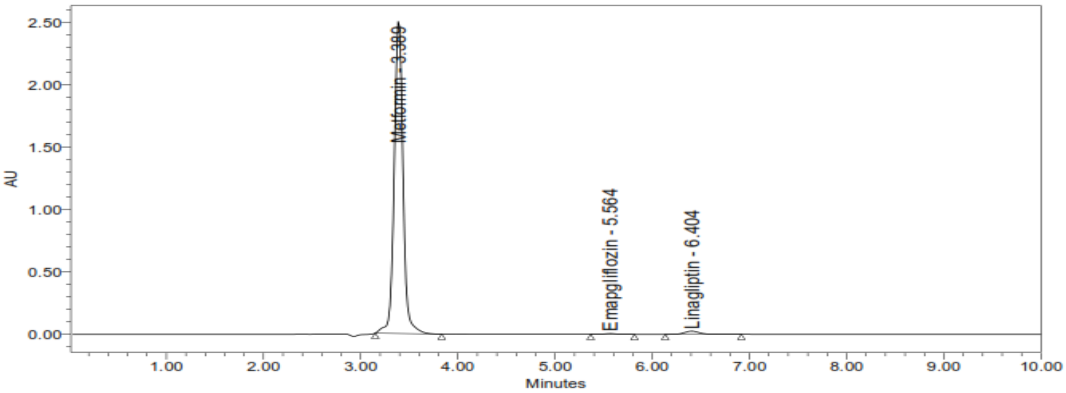


Figure 31: Chromatogram for Accuracy 150%-2.

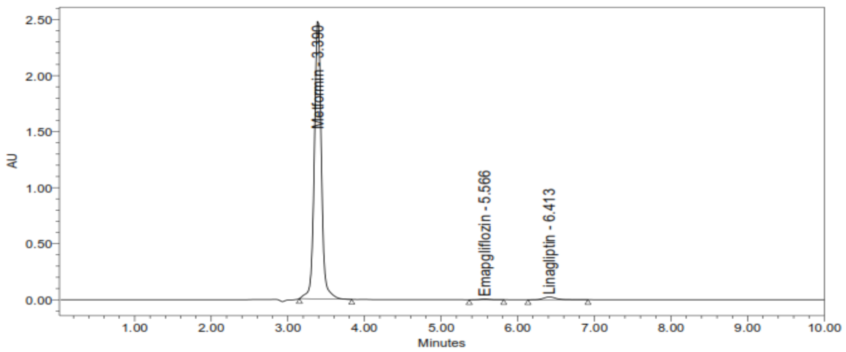


Figure 32: Chromatogram for Accuracy 150%-3.

Table 15: Accuracy (recovery) data for Metformin.

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	5420274	500	496.44	99.29	99.12
100%	10905235	1000	998.80	99.88	
150%	16080832	1500	1472.8	98.19	

***Average of three determinations**

Table 9: Accuracy (recovery) data for Empagliflozin.

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	16604	2.5	2.52	100.73	100.48
100%	33389	5	5.06	101.28	
150%	49166	7.5	7.46	99.42	

***Average of three determinations.**

Table 10: Accuracy (recovery) data for Linagliptin.

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	78504	1.25	1.26	100.94	100.75m
100%	157002	2.5	2.52	100.94	
150%	234215	3.75	3.76	100.38	

***Average of three determinations**

Acceptance Criteria

- The percentage recovery was found to be within the limit (97-103%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

6. LIMIT OF DETECTION FOR METFORMI, EMPAGLIFLOZIN AND LINAGLIPTIN

Based on the visualization method

Detection limit is determined by the analysis of sample with known concentration of the analyte and by establishing the minimum level at which the analyte can be reliably detected.

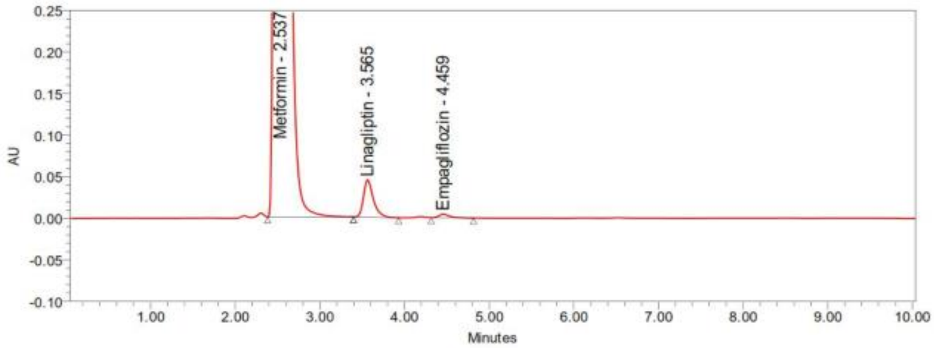


Figure 33: Chromatogram of Metformin, Empagliflozin and Linagliptin showing LOD by Visualization method

Observation: The lowest concentration of the sample can be detected.

7. LIMIT OF QUANTIFICATION FOR METFORMI, EMPAGLIFLOZIN AND LINAGLIPTIN

Based on the visualization method

Quantitation limit is determined by the analysis of sample with known concentration of the analyte and by establishing the minimum level at which the analyte can be reliably quantitated.

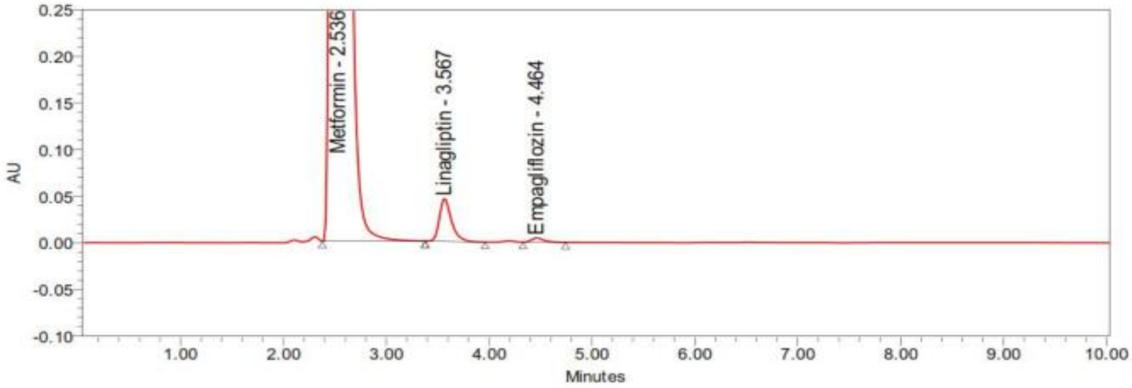


Figure 34: Chromatogram of Metformin, Empagliflozin and Linagliptin showing LOQ by Visualization method. Observation: The lowest concentration of the sample can be quantified.

8. ROBUSTNESS

The standard and samples of Metformin, Linagliptin and Empagliflozin were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Variation in flow

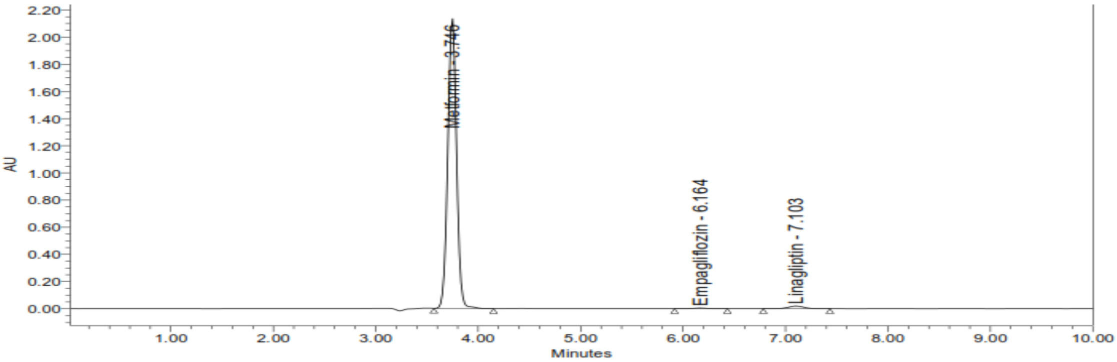


Figure 35: Chromatogram showing less flow.

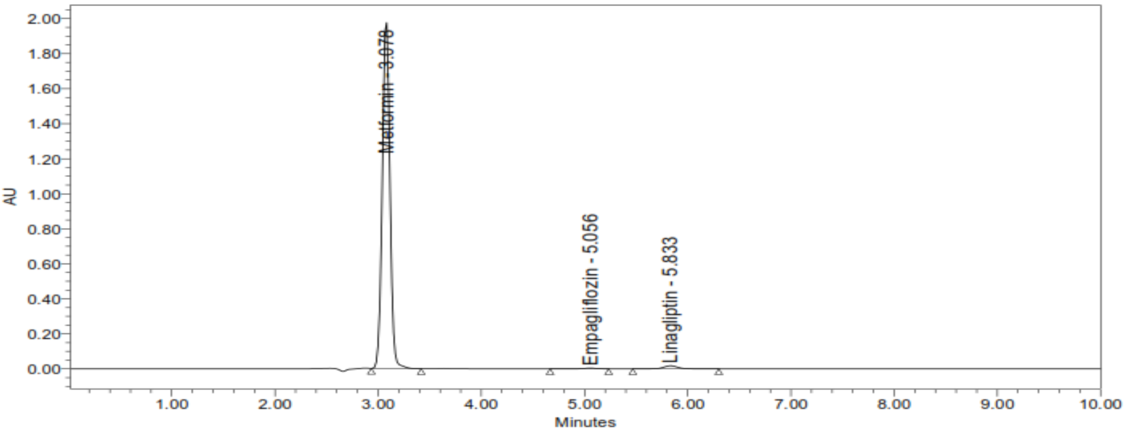


Figure 36: Chromatogram showing more flow.

Variation of mobile phase organic composition

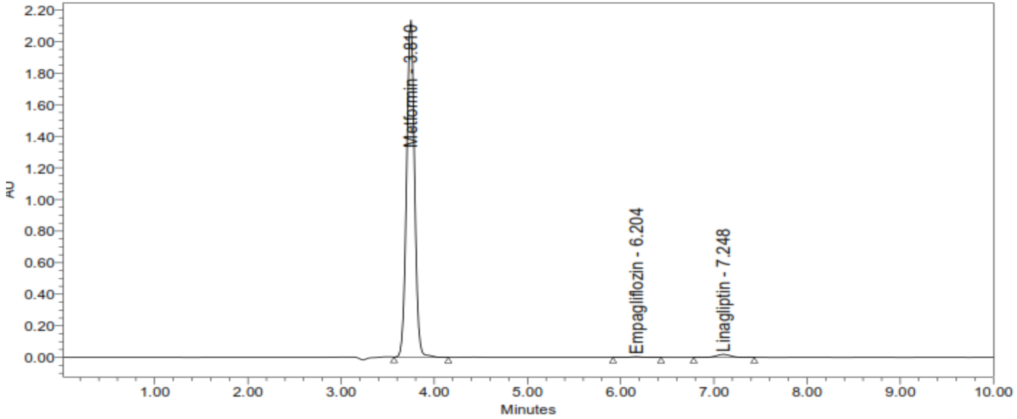


Figure 37: Chromatogram showing less organic composition.

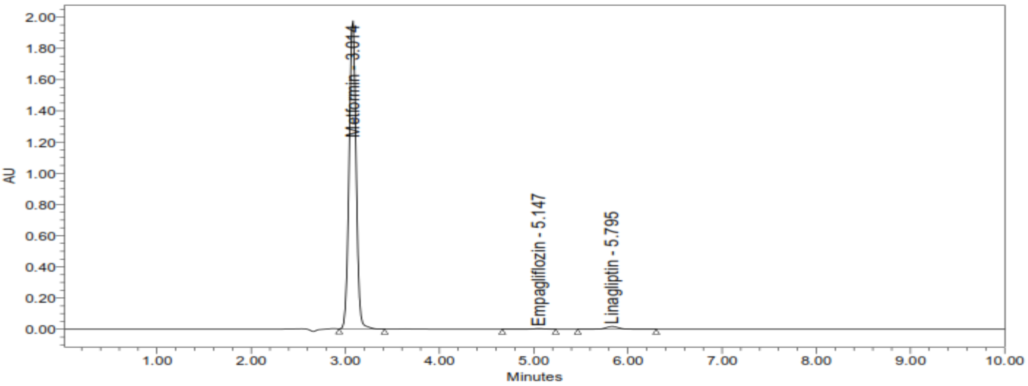


Figure 38: Chromatogram showing more organic composition.

Table 16: Results for variation in flow for Metformin.

S. No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	9160	1.03
2	1.0	8586.2	1.02
3	1.1	8548	1.02

Table 17: Results for variation in flow for Empagliflozin.

S. No	Flow Rate (ml/min)	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	0.9	11774	1.02	12.38
2	1.0	11213.5	1.06	12.22
3	1.1	11089	0.98	11.96

Table 18: Results for variation in flow for Linagliptin

S. No	Flow Rate (ml/min)	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	0.9	10168	1.01	3.63
2	1.0	10847.2	0.96	3.71
3	1.1	9183	1.04	3.49

*Results for actual flow (1.0ml/min) have been considered from Assay standard.

Table 19: Results for variation in mobile phase composition for Metformin.

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	9160	1.03
2	*Actual	8586.2	1.02
3	10% more	8548	1.02

Table 20: Results for variation in mobile phase composition for Empagliflozin.

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	10% less	11774	1.08	12.38
2	*Actual	11213.5	1.06	12.22
3	10% more	11089	1.03	11.95

Table 21: Results for variation in mobile phase composition for Linagliptin.

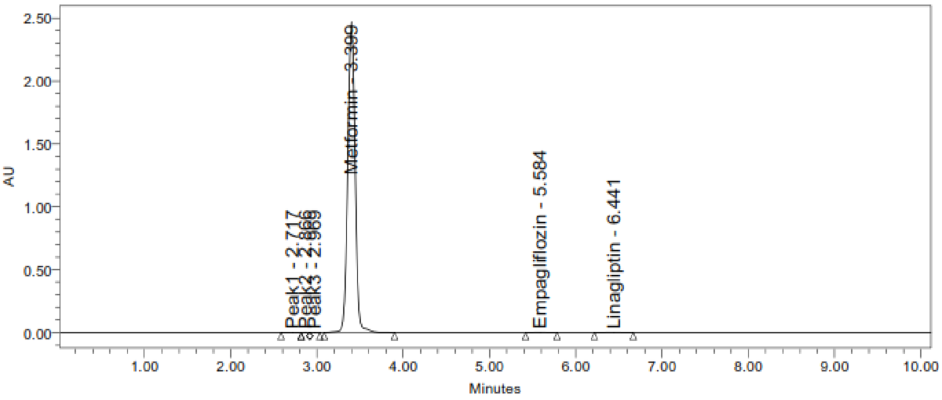
S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	10% less	10168	1.01	3.63
2	*Actual	10847.2	0.96	3.71
3	10% more	9183	1.04	3.89

*Results for actual Mobile phase composition have been considered from Accuracy standard.

Acceptance criteria

The Retention time, USP plate count, USP tailing factor obtained for change of flow rate, variation in mobile phase was found to be within the acceptance criteria. Hence the method is robust.

FORCED DEGRADATION STUDIES



	Peak Name	RT	Area	% Area	USP Plate Count	USP Resolution	USP Tailing
1	Peak1	2.717	3330	0.02	1392		0.88
2	Peak2	2.866	2788	0.02	17108	0.91	
3	Peak3	2.969	1868	0.01			
4	Metformin	3.399	11443065	99.81	8273		1.00
5	Empagliflozin	5.584	34860	0.06	11016	11.86	1.02
6	Linagliptin	6.441	160868	0.08	9785	3.50	1.08

Fig. 39: Acid Degradation.

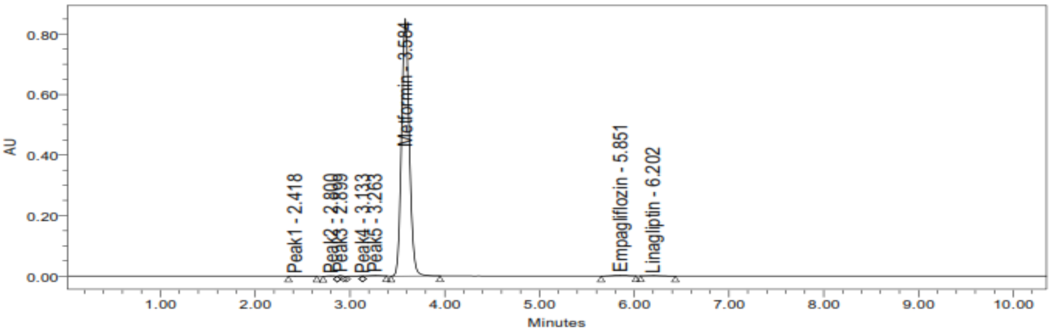
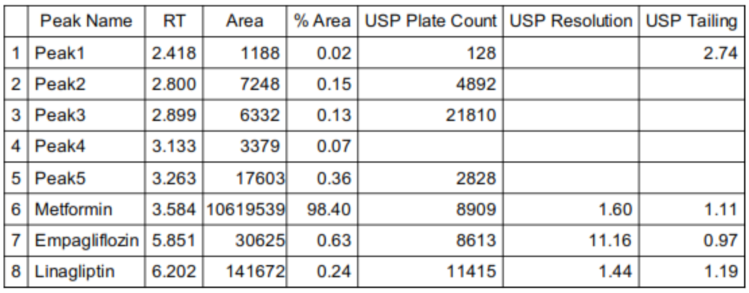
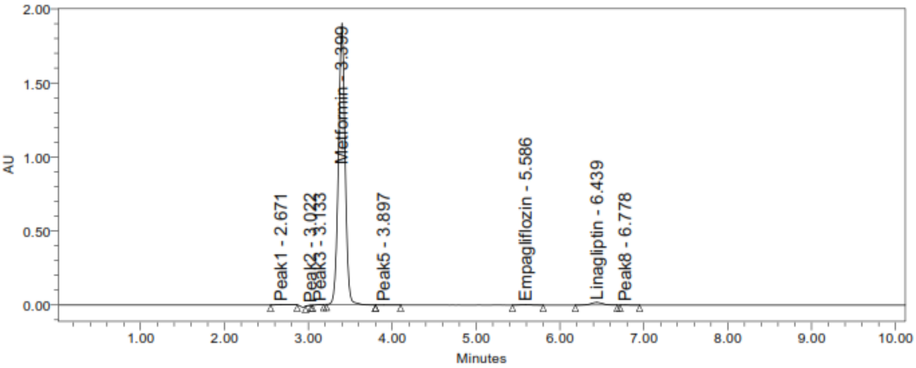


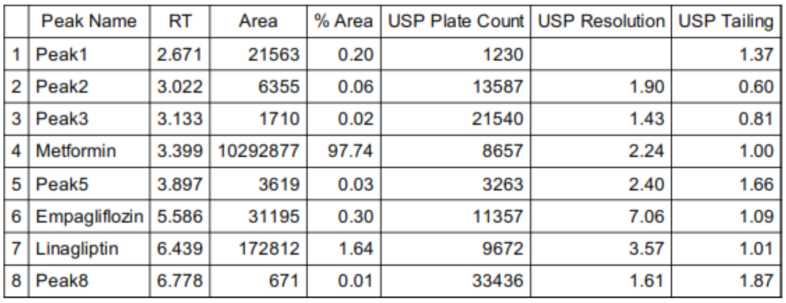
Fig 40: Base Degradation.



	Peak Name	RT	Area	% Area	USP Plate Count	USP Resolution	USP Tailing
1	Peak1	2.418	1188	0.02	128		2.74
2	Peak2	2.800	7248	0.15	4892		
3	Peak3	2.899	6332	0.13	21810		
4	Peak4	3.133	3379	0.07			
5	Peak5	3.263	17603	0.36	2828		
6	Metformin	3.584	10619539	98.40	8909	1.60	1.11
7	Empagliflozin	5.851	30625	0.63	8613	11.16	0.97
8	Linagliptin	6.202	141672	0.24	11415	1.44	1.19

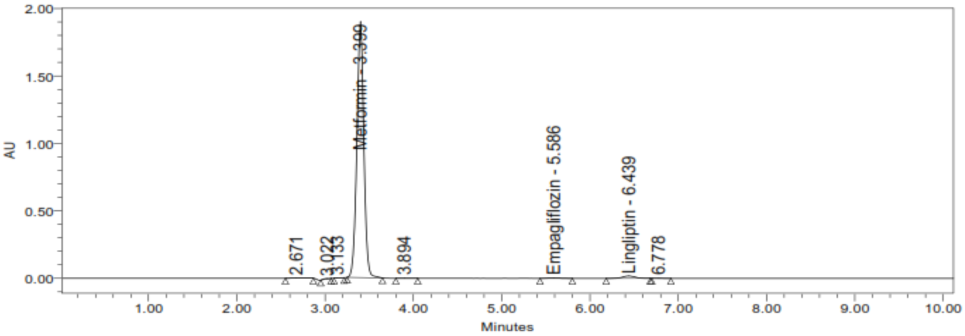
Base Degradation

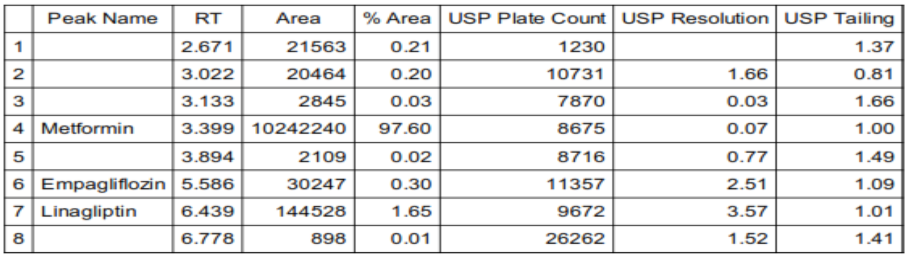




	Peak Name	RT	Area	% Area	USP Plate Count	USP Resolution	USP Tailing
1	Peak1	2.671	21563	0.20	1230		1.37
2	Peak2	3.022	6355	0.06	13587	1.90	0.60
3	Peak3	3.133	1710	0.02	21540	1.43	0.81
4	Metformin	3.399	10292877	97.74	8657	2.24	1.00
5	Peak5	3.897	3619	0.03	3263	2.40	1.66
6	Empagliflozin	5.586	31195	0.30	11357	7.06	1.09
7	Linagliptin	6.439	172812	1.64	9672	3.57	1.01
8	Peak8	6.778	671	0.01	33436	1.61	1.87

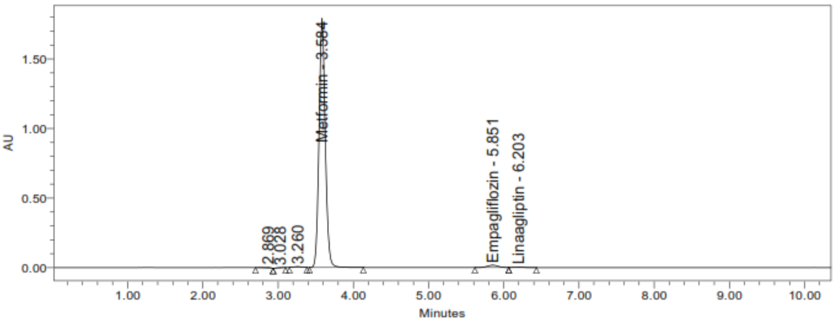
Fig. 41: Peroxide Degradation.





	Peak Name	RT	Area	% Area	USP Plate Count	USP Resolution	USP Tailing
1		2.671	21563	0.21	1230		1.37
2		3.022	20464	0.20	10731	1.66	0.81
3		3.133	2845	0.03	7870	0.03	1.66
4	Metformin	3.399	10242240	97.60	8675	0.07	1.00
5		3.894	2109	0.02	8716	0.77	1.49
6	Empagliflozin	5.586	30247	0.30	11357	2.51	1.09
7	Linagliptin	6.439	144528	1.65	9672	3.57	1.01
8		6.778	898	0.01	26262	1.52	1.41

Fig. 42: Thermal Degradation.



	Peak Name	RT	Area	% Area	USP Plate Count	USP Resolution	USP Tailing
1		2.869	39569	0.38	2620		0.69
2		3.028	10650	0.10	6359	0.86	0.90
3		3.260	41869	0.40	3829	1.28	1.06
4	Metformin	3.584	10206693	97.31	8758	1.79	1.10
5	Empagliflozin	5.851	30491	1.57	7947	10.86	1.00
6	Linagliptin	6.203	145872	0.25	11764	1.43	1.26

Fig. 43: Photo Degradation.

Table 22: Showing Metformin Degradation

	METFORMIN			
	Standard	**Sample**	**% Assay**	**%Degradation**
ACID	10896494	11443065	105.016	5.02
BASE	10896494	10619539	97.45831	2.54
PEROXIDE	10896494	10292877	94.46045	5.54
THERMAL	10896494	10242240	93.99574	6.00
PHOTO	10896494	10206693	93.66951	6.33

Table 23: Showing Empagliflozin Degradation.

	Empagliflozin			
	Standard	**Sample**	**% Assay**	**%Degradation**
ACID	32901	34860	105.9542	5.95
BASE	32901	30625	93.08228	6.92
PEROXIDE	32901	31195	94.81475	5.19
THERMAL	32901	30247	91.93338	8.07
PHOTO	32901	30491	92.67499	7.33

Table 24: Showing Linagliptin Degradation

	Linagliptin			
	Standard	**Sample**	**% Assay**	**%Degradation**
ACID	155236	160868	103.628	3.63
BASE	155236	141672	91.26234	8.74
PEROXIDE	155236	172812	111.3221	11.32
THERMAL	155236	144528	93.10212	6.90
PHOTO	155236	145872	93.96789	6.03

SUMMARY

The estimation of Metformin, Empagliflozin and Linagliptin was done by RP-HPLC. The assay of Metformin, Linagliptin and Empagliflozin was performed with tablets and the % assay was found to be 99.80, 102.33 and 99.80, which shows that the method is useful for routine analysis.

The linearity of Metformin, Empagliflozin and Linagliptin was found to be linear with a correlation coefficient of 0.999, 0.999 and 0.999, which shows that the method is capable of producing good sensitivity.

The acceptance criteria of precision is RSD should not be more than 2.0%, and the method showed precision 0.4, 1.3 and 0.6 for Metformin, Empagliflozin and Linagliptin, which exhibited that the method is precise.

The acceptance criteria of intermediate precision are that RSD should not be more than 2.0%, and the method showed precision 0.4,1.3 and 0.6 for Metformin, Empagliflozin and Linagliptin, which exhibited that the method is repeatable when performed on different days also.

The accuracy limit is the percentage recovery that should be in the range of 97.0% - 103.0%. The total recovery was found to be 99.12%, 100.48% and 100.75 for Metformin, Linagliptin and Empagliflozin. The validation of the developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility.

The LOD and LOQ for Metformin, Empagliflozin and Linagliptin were performed by the visualisation method, and the chromatographs exhibited good detectability and were quantified, respectively.

The robustness limit for mobile phase variation and flow rate variation are well within the limit, which shows that the method has good system suitability and precision under the given set of conditions.

CONCLUSION

A simple, accurate, precise method was developed for the simultaneous estimation of Metformin, Empagliflozin and Linagliptin in tablet dosage form by using RP-HPLC.

In forced degradation studies for the peroxide value of Linagliptin was found to be 11.23. Hence special packaging material which will prevent oxidation should be used or it may be advisable to add small amounts of the antioxidants to avoid oxidation.

Hence this method can be applied for the estimation of Metformin Hydrochloride, Empagliflozin and Linagliptin in drugs in testing laboratories and pharmaceutical industries.

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