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ESTIMATION OF GEMIGLIPTIN AND METFORMIN IN BULK AND ITS TABLET DOSAGE FORM

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ABSTRACT

Background: High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of metformin and gemigliptin was done by RP-HPLC. The Phosphate buffer was pH 3.0 and the mobile phase was optimized with consists of Methanol: Phosphate buffer mixed in the ratio of 70:30 % v/ v. Inertsil C18 column C18 (4.6 x 150mm, 5µm) or equivalent chemically bonded to porous silica particles was used as stationary phase. The detection was carried out using UV detector at 260 nm. The solutions were chromatographed at a constant flow rate of 0.8 ml/min. the linearity range of metformin and gemigliptin were found to be from 100500 µg/ml of metformin and 1-5µg/ml of gemigliptin. Linear regression coefficient was not more than 0.999.The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-102% of metformin and gemigliptin. LOD and LOQ were found to be within limit. The results obtained on the validation parameters met ICH and USP requirements .it inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision. Results: High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of metformin and gemigliptin was done by RP-HPLC. The Phosphate buffer was pH 3.0 and the mobile phase was optimized with consists of Methanol: Phosphate buffer mixed in the ratio of 70:30 % v/ v. Inertsil C18 column C18 (4.6 x 150mm, 5µm) or equivalent chemically bonded to porous silica particles was used as stationary phase. The detection was carried out using UV detector at 260 nm. The solutions were chromatographed at a constant flow rate of 0.8 ml/min. the linearity range of metformin and gemigliptin were found to be from 100500 µg/ml of metformin and 1-5µg/ml of gemigliptin. Linear regression coefficient was not more than 0.999. The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-102% of metformin and gemigliptin. LOD and LOQ were found to be within limit. The results obtained on the validation parameters met ICH and USP requirements .it inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

KEY WORDS: Inertsil C18 column, metformin and gemigliptin, RP-HPLC, Sensitivity.

INTRODUCTION

DEFINITIONS

- Analytical chemistry is the study of the separation, identification, and quantification of the chemical components of natural and artificial materials. It deals with methods for determining the chemical composition of samples of matter.
- A Qualitative Method yields information about the identity of atomic or molecular species or the functional groups in the sample.
- A Quantitative Method, in contrast, provides numerical information as to the relative amount of one or more of this component.
- Pharmaceutical analysis may be defined as a process or a sequence of processes to identify and/or quantify a substance or drug, the components of a pharmaceutical solution or mixture or the determination of the structures of chemical compounds used in the formulation of pharmaceutical product(2)

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Analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include but is not limited to: the sample, the reference standard and the reagents preparations, use of the apparatus, generation of the calibration curve, use of the formulae for the calculation, etc(2).

Classification of Chemical Analysis

Chemical analysis may be classified into four kinds:

- Proximate Analysis: It determines the amount of each element in a sample with no concern as to the actual compounds present.
- Partial Analysis: It deals with the determination of designated constituents in the sample.
- Trace Constituent Analysis: A specialized instance of partial analysis in which we are worried about the determination of specified components present in very minute quantity.
- Complete Analysis: It determines the proportion of each component of the sample.

On the basis of sample size, chemical analysis is often classified as following:

- Macro Analysis: The analysis deals with quantities of 0.1 gm or more.
- Meso (semimicro) Analysis: This analysis dealing with quantities ranging from g to g (0.01–0.1 gm).
- Micro Analysis: This analysis deals with quantities in the range g to g (0.001 – 0.01 gm).
- Sub-micro Analysis: This analysis deals with samples in the range g to g (0.0001 – 0.001 gm).
- Ultra-micro Analysis: This analysis deals with quantities below g (below 0.0001 gm) [1, 2].

Classification of Analytical Methods

Analytical methods can be separated into classical and instrumental methods.

Classical methods are also known as wet chemistry methods, where,

- Separation of analytes was performed by precipitation, extraction, or distillation.
- Qualitative analysis by reaction of analytes with reagents that yield products that could be recognized by their colors, boiling or melting points, solubilities, optical activities, or refractive indexes.
- Quantitative analysis by gravimetric or titrimetric techniques. In Gravimetry, the amount of a substance is determined by the mass of product generated by a chemical reaction. In Titrimetry, concentration is determined by the volume of a reagent needed to completely react with the analyte.
- These methods are highly accurate and precise but require a sufficient amount of sample, and a concentration of analyte in the sample of at least 0.1 percent. Furthermore these analyses require the constant attention of a trained scientist. Classical methods use separations such as precipitation, extraction, and distillation and qualitative analysis by color, odor, or melting point[3].
- Instrumental methods use an apparatus to measure physical quantities of the analyte such as light absorption, fluorescence, or conductivity. Highly efficient chromatographic and electrophoretic techniques began to replace distillation, extraction and precipitation for the separation of components of complex mixtures prior to their qualitative or quantitative determination. These newer methods for separating and determining chemical species are known collectively as Instrumental methods of Analysis.
- Thus, techniques employed in quantitative analysis are based upon —
- Chemical Properties: Measuring the amount of reagent necessary to complete the reaction, or ascertaining the amount of reaction product obtained.
- Electrical Properties: Appropriate electrical measurements (e.g. potentiometry).
- Optical Properties: The measurement of certain optical properties (e.g. absorption spectra). In some cases, a combination of optical or electrical measurements and quantitative chemical reaction (e.g. amperometric titration) may be used.

Introduction to Chromatography

- Chromatography is a technique in which the components of a mixture are separated based on the rates at which they are carried through a stationary phase by a gaseous or liquid mobile phase.
- Chromatography involves two phases chosen such that the components of the sample have differing affinities in each phase and a sample (or sample extract) being dissolved in a mobile phase (which may be a gas, a liquid or a supercritical fluid). The mobile phase is then forced through the stationary phase. A component with high affinity towards the stationary phase will take longer to travel through it than a component with low affinity towards the stationary phase and high affinity towards the mobile phase. As a result of these differences in mobilities, sample components will become separated from each other as they travel through the stationary phase[4].

CLASSIFICATION OF CHROMATOGRAPHIC METHODS

Chromatographic methods are of two types. In column chromatography, the stationary phase is held in a narrow tube and the mobile phase is forced through the tube under pressure or by gravity. In planar chromatography, the

stationary phase is supported on a flat plate or in the pores of a paper. Here the mobile phase moves through the stationary phase by capillary action or under the influence of gravity[5].

High Performance Liquid Chromatography

High-performance liquid chromatography also known as High-pressure or High price or High speed liquid chromatography, HPLC) is a form of column chromatography used frequently in analytical chemistry and biochemistry to identify, separate, and quantify compounds. It is a powerful tool in analysis. It is basically an improved form of column chromatography which has been optimized to provide rapid high resolution separations. Early LC used gravity fed open tubular columns with particles 100s of microns in size; the human eye was used for a detector and separations often took hours or even days to develop. HPLC as compared with the classical technique is characterized by:

- Small diameter (2-5 mm), reusable stainless steel columns without repacking & regeneration
- Column packings with very small (3, 5 and 10 µm) particles and the continual development of new substances to be used as stationary phases
- Relatively high inlet pressures and controlled flow of the mobile phase
- Precise sample introduction without the need for large samples
- Special continuous flow detectors capable of handling small flow rates and detecting very small amounts
- Automated standardized instruments
- Rapid analysis
- Greater reproducibility due to close control of the parameters affecting the efficiency of separation
- Capable of handling macro molecule & viscous solutions
- Efficient analysis of labile natural products
- Reliable handling of inorganic or other ionic species
- HPLC is probably the most universal type of analytical procedure. In addition HPLC also ranks as one of the most sensitive analytical procedures and is unique in that it easily copes with multi-component mixtures. Its application areas include quality control, process control, forensic analysis, environmental monitoring and clinical testing. It has achieved this position as a result of the constant evolution of the equipment used in LC to provide higher and higher efficiencies at faster and faster analysis times with a constant incorporation of new highly selective column packings

Principles of Separation

In general, three primary characteristics of chemical compounds can be used to create HPLC separations. They are:

- Polarity
- Electrical Charge
- Molecular Size

On the basis of separation chemistry, Liquid Chromatography is subdivided into the following techniques:

- Adsorption Chromatography functions via an adsorbent stationary phase. The degree to which a sample particle may adsorb to the stationary phase is determined by the relative polarity, or charge, that the particle may possess at a given time.
- Partition chromatography in which separation is based mainly on differences between the solubilities of the components in the mobile and stationary phases. Here, the two phases are liquids. The stationary phase liquid is present as thin film on an inert solid support.
- Ion-exchange chromatography where a resin coated solid is used as stationary phase which has ions (either cations or anions, depending on the resin) covalently bonded to it, and the solute ions of the opposite charge are electrostatically bound to the surface[6]. When the mobile phase (always a liquid) is eluted through the resin, the electrostatically bound ions are released as other ions are bonded preferentially.
- Ion-pair chromatography allows the separation of complex mixtures of polar and ionic molecules. The selectivity is determined by the mobile phase supplemented with a specific ion pairing reagent which large ionic molecules are having a charge opposite to the analyte of interest, as well as a hydrophobic region to interact with the stationary phase. The counter-ion combines with the ions of the eluent, becoming ion pairs in the stationary phase resulting in different retention times, thus facilitating separation of analytes.
- Size-exclusion chromatography, also called as Gel Permeation Chromatography uses gels of controlled pore-size distribution as stationary phase. Separation is based on the extent to which analyte molecules can penetrate the network of pores. This is used for large molecular weight compounds - proteins and polymers
- Large molecules excluded from pores - not retained, first eluted
- Intermediate molecules - retained, intermediate elution time.
- Small molecules permeate into pores - strongly retained, last eluted
- Chiral chromatography involves the separation of stereo isomers. To enable chiral separations to take place, either the mobile phase or the stationary phase must themselves be made chiral, giving differing affinities between the analytes.
- Affinity Chromatography is the most selective type of chromatography employed based on specific interactions in a lock-and-key paradigm between analytes and matrix-bound ligands. It utilizes the specific interaction between one kind of solute

molecule and a second molecule that is immobilized on a stationary phase.

Based on the modes of separation:

Normal-phase chromatography uses a polar stationary phase and a non-polar mobile phase. An adsorptive mechanism is used for the analysis of solutes. Typical stationary phases for normal-phase chromatography are silica or organic moieties with cyano and amino functional groups[7]. The mobile phase consists of a very nonpolar solvent like hexane or heptane mixed with a slightly more polar solvent like isopropanol, ethyl acetate or chloroform. In normal phase chromatography, the most nonpolar compounds elute first and the most polar compounds elute last. Retention increases as the amount of nonpolar solvent in the mobile phase increases.

This technique is still used, but is less common than reversed phase.

Reversed-phase chromatography uses a non-polar stationary phase and a polar mobile phase. This is the most common type of HPLC separation in use today. A partition mechanism is typically used for separations by non-polar differences. For reversed phase, alkyl hydrocarbons are the preferred stationary phase; octadecyl (C18) is the most common stationary phase, but octyl (C8) and butyl (C4) are also used in some applications. In reversed phase chromatography, the most polar compounds elute first with the most non-polar compounds eluting last.

Based on elution technique:

Isocratic:

Isocratic indicates constant composition.

Thus, it is a separation in which the mobile phase composition or polarity or elution strength remains constant throughout the process. The word was coined by Csaba Horvath who was one of the pioneers of HPLC

Gradient:

A separation in which the mobile phase composition is changed during the separation process i.e., gradually increasing the polarity or elution strength. Based on Scale of preparation:

Analytical:

Analytical modes are used only for separation of components and they cannot be recovered since sample taken is very low i.e., in micro grams. The column dimensions are also smaller when compared to that of preparative analysis.

Preparatory:

Separation and recovery of sample for reuse is possible since large amount of sample i.e., in grams is taken. Individual fractions of pure compounds can be collected using fraction collector and reused.

Based on the type of analysis:

Qualitative analysis:

This type of analysis is used to identify the compound, detect the presence of impurities, to find the number of components etc. Commonly used parameter is Retention time values.

Quantitative analysis:

This is used to determine the quantity of the individual or several components in a mixture. This is done by comparing the peak area of the standard & sample.

Instrumentation

LC can be (and has been) carried out using a glass tube hand-packed with powder through which a solvent is allowed to gravity-flow. So why do we need all the complicated high-tech equipment? There are many aspects to the answer:

Speed:

A single analysis by "Classical" LC can take anywhere from 2 to 12 hours to carry out. HPLC allows an equivalent analysis to be done in 2 to 12 minutes.

Reproducibility:

A classical column must be freshly packed for each analysis, increasing the chance of errors. A single HPLC column can be used for hundreds or thousands of samples.

Quantitation:

Classical LC was (and still is) an excellent technique for sample preparation or purification, but requires additional collection and analysis steps to be useful as a tool for quantitative analysis. HPLC uses on-line detectors that provide quantitative information during the course of the separation.

Sensitivity:

Classical LC used relatively large columns and correspondingly large volumes of mobile phase solvents. The resulting dilution limited the sensitivity of the technique. HPLC uses miniaturized columns to keep sample dilution to a minimum.

- The starting point is the column. Decreasing the average particle size of the column packing minimizes band broadening. A corresponding decrease in the size of the column minimizes solvent consumption and dilution of the sample.
- Gravity alone cannot make liquid flow at a reasonable velocity through a column packed with very small particles, so we must use a constant-flow pump.
- Flow resistance in the column can generate a considerable back-pressure, so the entire system, including the connecting tubing and fittings must be

made of pressure-resistant material such as stainless steel or specially formulated plastics.

- A reservoir must be provided to assure a consistent supply of mobile phase.
- Because the system is pressurized, some provision must be made for introducing samples from the outside world into the system. This is most commonly done using a rotary injection valve.
- Collecting and hand-analyzing fractions is tedious, so some type of flow-through detector is used to monitor for the presence of sample compounds in the column effluent.
- Finally, a data system is required to collect and interpret the results.
- The end result is an HPLC system composed of six basic modules (reservoir, pump, injector, column, detector, and data system) connected by appropriate tubing and fittings. Thus, today's HPLC requires basic & special modules as follows:

1. Solvent reservoir with a Degassing system.
2. Extremely precise gradient mixers (optional).
3. HPLC high pressure pumps with very constant flow.
4. Unique high accuracy, low dispersion, HPLC sample valves.
5. Very high efficiency HPLC columns with inert packing materials
6. High sensitivity low dispersion HPLC detectors
7. High speed data acquisition system.
8. Low dispersion connecting tubes for valve to column and column to detector.

Mobile Phase

Mobile phase is the phase which moves in a definite direction. The mobile phase consists of the sample being separated/ analyzed and the solvent that moves the sample through the column. In the case of HPLC the mobile phase consists of a non-polar solvent(s) such as hexane in normal phase or polar solvents in reverse phase chromatography and the sample being separated. The elutropic series lists solvents in order of their polarity which is useful when selecting a solvent for a particular separation where more polar compounds will normally require a more polar solvent [8].

High Polarity

Water > Acetic acid > Methanol > Ethanol > 1-Propanol > Acetonitrile > Ethyl acetate > Acetone > Dichloromethane > Chloroform > Diethyl ether > Toluene > Cyclohexane > n-Hexane

Solvent Reservoir

The reservoir that holds the mobile phase is often no more than a glass bottle. Examples of mobile phase reservoirs range from standard laboratory glassware such as beakers or flasks covered with aluminum foil through larger vessels such as media bottles, solvent jugs, or carboys, to purpose-made glassware that includes built-in provision for stirring and degassing. The requirements for a solvent reservoir are simple:

- Solvents should not be stored in plastic containers.
- Glass containers should not be used for aqueous mobile phase with pH > 8.
- The reservoir and its attachment to the pump should be made of materials that will not contaminate the mobile phase: Teflon, glass, or stainless steel.
- In addition, a 10-micron frit or inlet filter called as "sinker frit" should be connected to the end of the inlet line that dips into the reservoir.

Degassing System

Mobile phase accounts for 70 % or more of all the problems in chromatography. Presence of particles in mobile phase may cause non producible flow rate, decrease in selectivity, spurious peaks, increased back pressure, irreversible adsorption and decrease in life of column. Hence, 0-20 micron size filters are being used for filtration. Degassing is one of the most effective measures to eliminate these problems. Benefits are:

- Stability in baseline, enhanced sensitivity
- Reproducible retention times for eluting peaks
- Reproducible injection volumes for quantitation. Stable pump operation

The methods for Degassing include:

Sonication:

When pure solvents are mixed to make up the mobile phase, excess dissolved gas escapes to form bubbles. If the mobile phase reservoir is placed in an ultrasonic bath, the sound waves promote the coalescence of small bubbles which can escape more easily. Sonication alone will degas a gallon of solvent approximately in 20 minutes.

Vacuum or offline degassing:

vacuum reduces pressure of gases on the surface of the solvent which reduces the mass of gas in the solution. Advanced system called the Inline vacuum degassers are developed where the mobile phase is passed through gas permeable tubing enclosed in a vacuum chamber such that the air free mobile phase directly enters the pump without any re-saturation of air.

Helium sparge:

A stream of helium bubbles will sweep dissolved air out of liquids (helium is virtually insoluble in most HPLC solvents, so very little helium replaces the air). Helium sparging is very effective & is suitable for use with on-line mixing systems. This removes background absorbance on UV detectors and the quenching phenomenon caused by the dissolved oxygen on a fluorescent detector.

Heat or offline degassing method:

This method increases the vapor pressure of the solution which proportionately decreases the partial pressure of the gas in the solution. This will prevent the further absorption of the gas into the solution. Heat may reduce the solubility of the gas in solution. But, this method is not suitable for organic solvents.

On-line membrane degassing:

The mobile phase or solvent flows through a hollow fiber made of a semi-permeable membrane. A partial vacuum is maintained on the outside of the membrane. Because air can diffuse through the membrane while solvent vapor cannot, dissolved air is removed from the solvent before it reaches the pump [4,8].

The Gradient Programmer

In High Pressure Programmer, predetermined volume of solvents pass from each solvent reservoir directly to a pump and then to a mixing manifold where the solvents gets mixed which then passes to the sample valve and column. In Low pressure programmer, predetermined volume of solvent from each reservoir passes to an oscillating valve, the output from which is connected to a mixing manifold which mixes solvents from each of the programmed valves which is then pumped by a single pump to the column[9].

Solvent Pumping System

This is very important in HPLC, since its performance directly affects the retention time, reproducibility and detector sensitivity.

Pumps work on two basic principles:

1. Constant Pressure Pumps - Pneumatic Pump
   They produce a pulse less flow
2. Constant Displacement Pumps - Reciprocating & Syringe Pump
3. They maintain a constant flow rate through the column irrespective of changing column conditions.

Pneumatic Pump

- The pneumatic pump has a much larger flow capacity than the piston type pumps but, nowadays, is largely used for column packing and not for general analysis because this pump can deliver high pressures instantly by means of an on/off valve which drives the slurry or column packing material into the column.
- These pumps work by introduction of high pressure gas into the pump and the gas in turn forces the solvent from the pump chamber into the column.

Reciprocating Pump

- The single piston reciprocating pump was the first of its type to be used with high efficiency LC columns (columns packed with small particles) and is still very popular today. These types of pumps operate by using a reciprocating piston. The liquid enters a stainless steel pumping chamber via an inlet valve and is pushed out via an outlet valve by the action of the piston or diaphragm mostly made of synthetic sapphire driven by a stainless steel motorized cam or crank. Pulse dampeners are incorporated into the system like a triple-headed pump
- A more efficient way to provide a constant and almost pulse free flow is the use of dual-headed reciprocating pumps. Here, both pump chambers are driven by the same motor through a common eccentric cam; this common drive allows one piston to pump while the other is refilling. As a result, the two flow-profiles overlap each other significantly reducing the pulsation downstream of the pump; this is visualized below.

Syringe Type Pumps

- This works on the principle of solvent displacement by a piston mechanically driven at constant rate in a piston chamber of about 250-500ml capacity with a generation of pulse less flow with high pressure capabilities (200-475 atm).
- Syringe pumps are now mostly used for SFC and micro column chromatography. This type of pump is still occasionally used for the mobile phase supply to microbore columns that require small volumes of mobile phase to develop the separation.

Sample Injecting System

A good injection system should be:

- Chemically inert with eluent and sample
- Able to operate at high pressure (up to the 4000 psi) with high reproducibility
- Smallest possible contribution to peak broadening should provide the possibility of injecting 0.1 to 100 ml volume of liquid sample

Amount of sample to be injected depends on:

- Sensitivity of the detector
- Extent of dilution undergone in the column
- Rheodyne injector

Because of their superior characteristics, valves are now used almost to the exclusion of syringe injection[10]. With these sampling valves, samples can be introduced reproducibly into pressurized columns without significant interruption of flow, even at elevated temperature

Position (A) shows the inject position. Injection in the front port causes the sample to flow into the sample loop. The tip of the needle passes through the rotor seal and, on injection, is in direct contact with the ceramic stator face.

After injection, the valve is rotated to position (B) and the mobile phase flushes the sample directly onto the column. The sample is actually forced out of the beginning

of the loop so it does not have to flow through the entire length of the loop

Auto samplers

Most modern HPLC systems use autosamplers. With the automatic sampling devices, large numbers of samples can be routinely analyzed by LC without operator intervention[11].

The actual injection valve is the same as the manual valves described above, but the sample is introduced by an automated syringe drawing samples from vials in a motorized tray. Most autosamplers work by penetrating the capped sample vial with a needle attached to a length of flexible tubing. Sample is then withdrawn from the vial into the loop of a sample injector by suction (or positive displacement in some cases). The injector then rotates into the inject position at the right time for the next sample injection into the LC. While this sample is being separated and analyzed, the needle and injector are washed out with rinse fluid, and the next sample vial on the turntable rotates into position for penetration by the needle. All of these operations (injection, rotation, etc.) are controlled by timers in either the LC or the autosampler.

Columns

- ✓ Heart of the chromatogram.
- ✓ Should be rust free and hence made of steel/ polyethylene/PEEK Analytical column:
- ✓ 4mm (id) × 15 or 25cm
- ✓ 6.0mm (id) × 15 or 25 cm
- ✓ 8.0mm (id) × 30 cm
- ✓ Preparative column: 20 or 30 or 50mm(id) × 25 c
- ✓ Fast LC: 4.6mm × 5cm
- ✓ Small bore: 2.5mm (id) ×15cm
- ✓ Microbore: 1.0mm × 25cm or 50 cm
- ✓ Standard size column: 4.0mm 0r 4.6 mm (id) ×15cm or 25 cm or 30 cm
    - A PRECOLUMN is used to precondition the mobile phase in order to minimize the chemical attack by the mobile phase on the column packing of the analytical column. The precolumn is packed loosely with ordinary silica, which does not have to be of HPLC-quality.
    - IN-LINE FILTER is to catch particulate matter that could plug the column frit or column, causing poor separation or high column pressure - or both. These in-line filters consist of a low volume frit-holder and a 0.5-micron or 2-micron frit.
    - The GUARD COLUMN is a short column packed with material similar to that contained in the analytical column. The job of the guard column is to pick up or retain sample impurities that could be irreversibly adsorbed onto the analytical column & also filters the mobile phase and sample that enters the analytical column, but the combination of an in-line filter plus guard column is even more effective.

Detectors

- The detector is the component that emits a response due to the eluting sample compound and subsequently signals peak on the chromatogram. It is positioned immediately posterior to the stationary phase in order to detect the compound as elute from the column. Criteria for Detectors include:
- High sensitivity, high linear dynamic range
- Applicable to most of the solutes
- Does not contribute to band broadening
- Non-destructive
- Faster response
- A large number of LC detectors have been developed over the past thirty years based on a variety of different sensing principles. However, only about twelve of them can be used effectively for LC analyses and, of those twelve, only four are in common use which are employed in over 95% of all LC analytical applications. The four dominant detectors used in LC analysis are:
- UV detector (fixed and variable wavelength)
- Electrical conductivity detector Fluorescence detector and
- Refractive index detector
- All the detectors so far developed come under these categories:
- Bulk Property Detectors: They specifically measure the difference in some physical property of the solute present in the mobile phase in comparison to the individual mobile phase.
- Refractive Index Detector
- Conductivity Detector
- Viscometer
- Polarimeter
- Solute Property Detectors: measures the solute properties like Fluorescence, resistance.
- UV\VISIBLE Detectors
- Fluorescence Detectors
- Electrochemical
- IR
- ELSD
- Radioactivity (4,10,12)
- De-solvation Detectors: Flame ionization etc.
- LC-MS Detectors Reaction Detectors

Applications of HPLC

Preparative HPLC refers to the process of isolation and purification of compounds.

Chemical separations can be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates for a given set of column and mobile phase.
Purification refers to the process of separating or extracting the target compound from other (possibly structure related) compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic condition[12]. The migration of the compounds and contaminants through the column need to differ enough so that the pure desired compound can be collected or extracted without incurring any other undesired compound.
Identification of the compounds by HPLC is a crucial part of any HPLC assay. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from the extraneous peaks at the detection levels in which the assay would be performed[13].
Quantification of compounds by HPLC is the process of determining the unknown concentration of a compound in a solution. It involves injecting a series of known concentration of the standard compound solution onto the HPLC for detection. The chromatograph of these known concentrations will give a series of peaks that correlate to the concentration of the compound injected[14].

SYSTEM SUITABILITY TEST (SST)

Prior to the analysis of samples of each day, the operator must establish that the HPLC system and procedure are capable of providing data of acceptable quality. This is accomplished with system suitability experiments, which can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The key to achieving system suitability is an optimal instrumentation and an efficient analytical column[15]. A few commonly used system suitability parameters are retention factor, repeatability, resolution, tailing factor, theoretical plates.

Acceptance criteria

SPECIFICITY

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

Inject sample as well as other related compounds like solvents, intermediates, degradation products.

Inject separately different components of the matrix of the sample (like tablets).

Subject sample to degradation studies to produce 10-30% of degradation of analyte[16].

Confirm peak purity with DAD, IR, NMR and MS.

Change the chromatographic conditions no evidence of additional compounds.

Acceptance criteria

Chromatograms should not show interference due to any peaks at the retention times of the drugs of interest.

Resolution between the main peak and interfering peak, if any, should be ≥ 1.5.

Main peaks in the chromatogram should pass the system suitability criteria[17].

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. This is sometimes termed trueness. There are three ways to determine accuracy:

- ✓ Comparison to a reference standard
- ✓ Recovery of the analyte spiked into blank matrix
- ✓ Standard addition of the analyte

Accuracy is calculated as the percentage of recovery by the assay of known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with the confidence intervals.
ICH documents recommend that accuracy should be assessed using a minimum of nine determinations i.e., three replicates at three concentrations across the specified range of the procedure.

Acceptance criteria

The mean % recovery of pyrimethamine & sulphodoxine at each spiked level should be not less than 98% & not more than 102%.

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. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility[18].

a. Repeatability

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

b. Intermediate precision

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

c. Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies usually applied to standardization of methodology).

ICH documents recommend that repeatability should be assessed using a minimum of nine determinations

covering the specified range of the procedure (i.e., three replicates of three concentrations) or using a minimum of six determinations at 100% of the test concentration.

Acceptance criteria
% RSD of the peak areas & %assay of six injections should be NMT 2.0%.

LIMIT OF DETECTION (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value. Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental[19].

- Based on Visual Evaluation
- Based on Signal-to-Noise
- Based on Standard Deviation of the Response and the Slope LOD can be expressed as:

$LOD = 3.3\sigma/S$

Where,

σ = Standard deviation of intercepts of calibration curve S= Slope of the calibration curve

The slope S may be calculated from the calibration curve of the analyte.

LIMIT OF QUANTIFICATION (LOQ)

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. Several approaches for determining the Quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

- Based on Visual Evaluation
- Based on Signal-to-Noise
- Based on Standard Deviation of the Response and the Slope LOQ can be expressed as:

$LOQ = 10\sigma/S$

Where,

σ = Standard deviation of intercepts of the calibration curve S= Slope of the calibration curve

The slope S may be calculated from the calibration curve of the analyte.

LINEARITY

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Linearity studies should cover the range of 0-150% of the expected level of the analyte. The data is then processed using the method of least squares regression. The resulting plot, slope, intercept and correlation coefficient provide the desired information on linearity. ICH recommends that, for the establishment of linearity, a minimum of five concentrations should normally be used.

Acceptance criteria
Correlation coefficient should be NLT 0.999

RANGE

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) 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 of the analytical procedure is validated by verifying that the analytical procedure provides acceptable precision, accuracy and linearity when applied to the samples containing analytes at the extremes of the range as well as within the range[20].

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. A good practice is to vary important parameters in the method systematically and measure their effect on separation. The variable method parameters may involve temperature (±50C), buffer pH (±0.5), ionic strength of buffers, level of additives to MP, flow rate (±0.2ml/min), wavelength (±2nm).

Acceptance criteria

- Main peaks in the chromatogram should pass the system suitability criteria.
- %RSD of peak areas & %assay of main peaks should be not more than 2.0%

Aim and Objective

Literature review reveals that there is no analytical method reported for the analysis of Metformin HCL and Gemigliptin by simultaneous estimation by RP-HPLC. Spectrophotometer, HPLC and HPTLC are the reported analytical methods for compounds either individually or in combination with other dosage form. Hence, it was felt that, there is a need of new analytical method development for the simultaneous estimation of Metformin HCL and Gemigliptin in pharmaceutical dosage form.

Present work is aimed to develop a new, simple, fast, rapid, accurate, efficient and reproducible RP-HPLC method for the simultaneous analysis of Metformin HCL and Gemigliptin. The developed method will be validated according to ICH guidelines.

Objective of the work:

- The analytical method for the simultaneous estimation of Metformin HCL and Gemigliptin will be developed by RP-HPLC method by optimizing the chromatographic conditions.

- The developed method is validated according to ICH guidelines for various parameters specified in ICH guidelines, Q2 (R1).

Chemical Data:

-
- IUPAC Name : 1-carbamimidamido-N,N-dimethylmethanimidamide
- Chemical formula: C4H11N5
- Molecular weight : 129.1636 g/mol
- CAS No : 657-24-9
- pKa : 12.4 K
-

Physical Data:

- Description: Metformin is a biguanide anti-hyperglycemic 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 hypoglycenic effects of sulfonylureas and insulin. It's main side effects are dyspepsia, nausea and diarrhea.
- Solubility: Freely soluble in water, slightly soluble in alcohol, practically insoluble in acetone and in methylene chloride.
- Melting Point: - 222 deg C. to 226 deg C.
- Category: Anti-diabetic drug.
- 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.

Chemical Data:

IUPAC Name: (3S)-3-amino-4-(5,5-difluoro-2-oxopiperidino)-1-[2,4-di(trifluoromethyl)- 5,6,7,8-tetrahydropyrido[3,4-d]pyrimidin-7-yl]butan-1-one
Chemical formula: C18H19F8N5O2
Molecular weight : 489.36 g/mol
CAS No : 911637-19-9

Physical Data:

Description: White
Solubility: It is very slightly soluble in water (0.9 mg/mL). Soluble in methanol (ca. 60 mg/mL), sparingly soluble in ethanol (ca. 10 mg/mL), very slightly soluble in isopropanol (< 1 mg/mL), and very slightly soluble in acetone (ca. 1 mg/mL).
Category: Antidiabetic Agents.

Mechanism of Action:

DPP-4 is a serine protease located on the cell surfaces throughout the body. In plasma, DPP-4 enzyme rapidly inactivates incretins including GLP-1 and GIP which are produced in the intestine depending on the blood glucose level and contribute to the physiological regulation of glucose homeostatis. Active GLP-1 and GIP increase the production and release of insulin by pancreatinc beta cells. GLP-1 also reduces the scretion of glucacon by pancreatic alpha cells, thereby resulting in a decreased hepatic glucose production. However these incretins are rapidly cleaved by DPP-4 and their effects last only for a few minutes. DPP-4 inhibitors block the cleavage of the gliptins and thus lead to an increasee insulin level and a reduced glucagon level in a glucose-dependent way. This results in a decrease of fasting and postprandial glycemia, as well as HbA1c levels. [(2)]

Plan of Work

To develop a new analytical method for the simultaneous estimation of Metformin HCL and Gemigliptin by RP-HPLC.

The dissertation work has been carried out in the following steps:

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 potassium dihydrogen phosphate with buffer (pH 3.0), Methanol in proportion 30: 70 v/v respectively.

Wave length selection:

UV spectrum of 10 µg / ml Metformin and Gemigliptinin diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 260. At this wavelength both the drugs show good absorbance.

Optimization of Column:

The method was performed with various columns like C18 column, hypersil column, lichrosorb, and inertsil ODS column. Inertsil ODS (4.6 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 0.8ml/min flow.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Instrument used : Waters HPLC with auto sampler and PAD or detector.
Temperature : Ambient
Column : Inertsil ODS (4.6 x 150mm, 5µm)

Buffer : 6.8 grams of potassium dihydrogen ortho phosphate in1000 ml water pH adjusted with ortho phaosparic acid.
pH : 3.0
Mobile phase : 30% buffer 70% Methanol
Flow rate : 1 ml per min
Wavelength : 260 nm
Injection volume : 10 µl
Run time : 10min.

PREPARATION OF BUFFER AND MOBILE PHASE:
Preparation of Phosphate buffer:

Accurately weighed 6.8 grams of KH2PO4 was taken in a 1000ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted to pH 3.0 with Orthophosphoric acid.

Preparation of mobile phase:
Accurately measured 300 ml (30%) of above buffer and 700 ml of Methanol HPLC (70%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:
The Mobile phase was used as the diluent.
Preparation of the metformin &gemigliptin standard & sample solution:
Standard Solution Preparation:

Accurately weigh and transfer 10 mg of Metformin and Gemigliptin10mg of working standard into a 10Ml & 10 ml clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.
(Stock solution)

Further pipette 3ml& 0.3ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to10 mg of Metformin and Gemigliptin (marketed formulation) sample into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.
(Stock solution)

Further pipette 3 ml of Metformine and Gemigliptinof the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

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

SYSTEM SUITABILITY:

Tailing factor for the peaks due to Metformin and Gemigliptinin Standard solution, should not be more than 2.0
Theoretical plates for the Metformin and Gemigliptin peaks in Standard solution should not be less than 2000.
6.5.1. Calculation: (For Metformin)
Assay % =

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

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 Metformine mg/ml.
Calculation: (For Gemigliptin)

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

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 Gemigliptin mg/ml.
Sample and Standard Details

S.No	Samples
1.	Metformin & Gemigliptin Tablets 50mg & 0.5mg
2.	Metformin & Gemigliptin Working standards

METHOD VALIDATION SUMMARY:
PRECISION:
Preparation of stock solution:

Accurately weigh and transfer 25 mg of Metformin and Gemigliptin working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.
(Stock solution)
Further pipette 3 ml of Metformin &Gemigliptin of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.
Procedure:
The standard solution was injected for five times and measured the area for all five Injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.
Acceptance Criteria:

The % RSD for the area of five 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 day by using different make column of same dimensions.

Preparation of stock solution:

Accurately weigh and transfer 25 mg of Metformin and 10mg of Gemigliptin working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

(Stock solution)

Further pipette 3ml of Metformin &Gemigliptin of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

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

Acceptance Criteria:

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

ACCURACY:

Preparation of Standard stock solution:

Accurately weigh and transfer 10 mg of Metformin andGemigliptin10mg of working standard into a 10mL& 100ml clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

(Stock solution)

Further pipette 3ml& 0.3ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation Sample solutions:

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

Accurately weigh and transfer 5mg of Metformin and 5.3mg of Gemigliptin working standard into a 10mL and 100 ml 0f clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock Solution).

Further pipette 3 ml of Metformine & 0.3 ml of Gemigliptin of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

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

Accurately weigh and transfer 10 mg of Metformin and 10 mg of Gemigliptin working standard into a 10mL and 100 ml 0f clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

(Stock Solution)

Further pipette 3 ml of Metformin & 0.3 ml of Gemigliptin of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

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

Accurately weigh and transfer 14.4mg of Metformin and 14.5mg of Gemigliptin working standards into a 10mL and 100ml of clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

(Stock solution).

Further pipette 3 ml of Metformin & 0.3 ml of Gemigliptin of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

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

Calculate the Amount found and Amount added for Metformin & Gemigliprin 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 10 tablets crush in mortor and pestle and transfer equivalent to 10 mg of Metformin and Gemigliptin (marketed formulation) sample into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

(Stock solution)

Preparation of Level – I (100ppm of Metformin &1ppm of Gemigliptin): 1ml and 0.1 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – II (200ppm of Metformin &2ppm of Gemigliptin): 2ml and 0.2 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – III (300ppm of Metformin &3ppm of Gemigliptin): 3ml and 0.3 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – IV (400ppm of Metformin &4ppm of Gemigliptin): 4ml and 0.4 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – V (500ppm of Metformin &5ppm of Gemigliptin): 5ml and 0.5 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute 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 X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Acceptance Criteria:

• Correlation coefficient should be not less than 0.999.

LIMIT OF DETECTION:

Limit of Detection: (For Metformin):

Preparation of 300µg/ml solution:

Accurately weigh and transfer 10 mg of Metformin working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

(Stock solution)

Further pipette 3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 0.12µg/ml solution):

Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent

Pipette 0.4mL of 1µg/ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank: 52µV

Signal Obtained from LOD solution: 152 µV

S/N = 152/52 = 2.9

Acceptance Criteria:

• S/N Ratio value Shall be 3 for LOD solution.

6.6.5.2. Limit of Detection: (For Gemigliptin) Preparation of 3µg/ml solution:

Accurately weigh and transfer 10mg of Gemigliptin working standard into a 100ml clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

(Stock solution)

Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 0.015µg/ml solution):

Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Further pipette 0.5ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank: 52 µV

Signal Obtained from LOD solution: 156 µV

S/N = 156/52 = 3.0

Acceptance Criteria:

• S/N Ratio value shall be 3 for LOD solution.

LIMIT OF QUANTIFICATION:

Limit of Quantification (for Metformin HCL)

Preparation of 300µg/ml solution:

Accurately weigh and transfer 10 mg of Metformin working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

(Stock solution)

Further pipette 3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 0.42µg/ml solution):

Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Pipette 1.0mL of above solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

Pipette 1.4 mL of above solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank: 52 µV

Signal Obtained from LOQ solution: 522µV

S/N = 522/52 = 10.03

Acceptance Criteria:

• S/N Ratio value shall be 10 for LOQ solution.

Limit of Quantification: (for Gemigliptin) Preparation of 3µg/ml solution:

Accurately weigh and transfer 10mg of Gemigliptin working standard into a 100mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

(Stock solution)

Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 0.05µg/ml solution):

Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Pipette 1.7mL of above solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank: 52 µV

Signal Obtained from LOQ solution: 524µV

S/N = 524/52 = 10.

ROBUSTNESS:

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

a) The flow rate was varied at 0.8 ml/min to 1.2ml/min.

Standard solution 300ppm of Metformin &3ppm of Gemigliptin was prepared and analysed using the varied flow rates along with 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 by change in the flow rate ±10%.

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

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

Standard solution 300 µg/ml of Setraline & 3µg/ml of Gemigliptin was 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 by change in the Mobile phase ±10

* Results for actual Mobile phase composition (55:45 Methanol: Buffer (ph-2.8) has been considered from Accuracy stand.

RESULTS AND DISCUSSION

Optimized chromatogram is obtained by following conditions:

Trial 1:

Mobile phase : Water: Methanol (50:50%v/v) Column : Thermosil C18 (4.6*150mm) 5µm
Flow rate : 1.0 ml/min
Wavelength : 260 nm
Column temp : Ambient
Sample Temp : Ambient
Injection Volume : 10 µl

(a) Trial chromatogram for Metformin and Gemigliptin

From the above chromatogram it was observed that the Metformin peak was splitted

Trial 2:

Mobile phase : Phosphate buffer
pH 4 : Methanol (40:60%v/v)
Column : Termosil C18 (4.6*150mm) 5µm
Flow rate : 1.0 ml/min
Wavelength : 260 nm
Column temp : Ambient
Sample Temp : Ambient
Injection Volume : 10 µl

From the above chromatogram it was observed that the Metformin and Gemigliptin peaks are splitted

Trial 3:

Mobile phase : Phosphate buffer (0.05m)pH 4.0: Methanol (40:60%v/v)
Column : Symmetry C18 5µm (4.6*250mm) Make; waters
Flow rate : 0.8 ml/min
Wavelength : 260 nm
Column temp : Ambient
Sample Temp : Ambient
Injection Volume : 10 µl

Trial chromatogram for Metformin and Gemigliptin

From the above chromatogram it was observed that the Metformin and Gemigliptin peaks are splitted

Trial 4:

Mobile phase : Phosphate buffer (0.05M) pH 3.6: ACN (40:60%v/v)
Column : Symmetry C18 5µm (4.6*250mm) Make; waters
Flow rate : 0.8 ml/min
Wavelength : 260 nm
Column temp : Ambient
Sample Temp : Ambient
Injection Volume : 10 µl

Trial chromatogram for Metformin and Gemigliptin

From the above chromatogram it was observed that the Metformin and Gemigliptin peaks are splitted

Trial 5:

Mobile phase : Phosphate buffer pH 3.0: Methanol (30:70%v/v)
Column : Inertsil C18 5µm (4.6*250mm)
Flow rate : 0.8 ml/min
Wavelength : 260 nm
Column temp : Ambient
Sample Temp : Ambient
Injection Volume : 10 µl

Trial chromatogram for Metformin and Gemigliptin

From the above chromatogram it was observed that the Metformin and Gemigliptin peaks are splitted

Chromatogram for Metformin and Gemigliptin

Column :Inertsil C18 (4.6 x 250mm, 5µm)
Buffer pH : 3.0.
Mobile phase : 30% buffer 70% Methanol
Flow rate : 1.0ml per min
Wavelength : 260 nm
Temperature : ambient.
Run time : 10min.

Figure 7.2 chromatogram for system suitability

7.2.1. Calculation: (For Metformin)

Assay %=

AT WS DT P Avg. Wt
-----x ------x ----- x ------x----------X 100
AS DS WT 100 Label Claim

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 METFORMINE mg/ml.

RESULTS:

System Suitability Results:

1). Tailing factor Obtained from the standard injection is 1.3

2). Theoretical Plates Obtained from the standard injection is 4668.7

Assay Results:

Weight of 10 tablets: 1.25 grams
Average Weight : 0.125grams

1314011 10 3 10 10 99.8 125
= ----------x-----x-----x------x-----x-------x------- X100 = 99.95%
1311967 10 10 25 3 100 50

7.2.3. Calculation: (For Gemigliptin)

Assay % =

AT WS DT P Avg. Wt.
-------- x ----------x --------- x ----------x----------X100
AS DS WT 100 Label Claim

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 GEMIGLIPTIN mg/ml.

RESULTS:

System Suitability Results:

1). Tailing factor Obtained from the standard injection is 1.3
2). Theoretical Plates Obtained from the standard injection is 6090.3

Assay Results:

Weight of 10 tablets: 1.25 grams
Average Weight : 0.125grams

125260 10 0.3 10 10 99.7 125
= ----------x-----x-----x------x-----x-------x------- X100 = 99.95%
124581 10 10 25 3 100 0.5

Acceptance criteria:

• Resolution between two drugs must be not less than 2
• Theoretical plates must be not less than 2000
• Tailing factor must be not less than 0.9 and not more than 2.
• It was found from above data that all the system suitability parameters for developed method were within the limit.

VALIDATION PARAMETERS:

PRECISION:

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

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.

INTERMEDIATE PRECESSION (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.

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.

Acceptance Criteria:

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

LINEARITY:

The linearity range was found to lie from 100µg/ml to 500µg/ml of Metformin, 5µg/ml to 25µg/ml 0f Gemigliptin and chromatograms are shown below.

Acceptance criteria:

• Correlation coefficient (R2) should not be less than 0.999

• The correlation coefficient obtained was 0.999 which is in the acceptance limit. The linearity was established in the range of 10% to 50% of Metformin and 5% to 25% of Gemigliptin.

Table.1.1. Classification of Instrumental methods based on various Analytical signals.

The table above lists the names of instrumental methods that are based upon various analytical signals.

Signal	Instrumental Methods
Emission of radiation	Emission spectroscopy (X-ray, UV, visible, electron, Auger); fluorescence, phosphorescence, and luminescence
Absorption of radiation	Spectrophotometry and photometry (X- ray, UV, visible, IR); photoacoustic spectroscopy; nuclear magnetic resonance and electron spin resonance spectroscopy
Scattering of radiation	Turbidimetry; nephelometry; Raman Spectroscopy
Refraction of radiation	Refractometry; interferometry
Diffraction of radiation	X-Ray and electron diffraction methods
Rotation of radiation	Polarimetry; optical rotary dispersion; circular dichroism
Electrical potential	Potentiometry; chronopotentiometry
Electrical charge	Coulometry
Electrical current	Polarography; amperometry
Electrical resistance	Conductometry
Mass-to-charge ratio	Mass spectrometry
Thermal properties	Thermal conductivity and enthalpy
Radioactivity	Activation and isotope dilution methods

Table.1.2. Classification of Column Chromatographic method

General Classification	Specific Method	Stationary Phase	Type of Equilibrium
Liquid chromatography (LC) (mobile phase: liquid)	Liquid-liquid, or partition	Liquid adsorbed on a solid	Partition between immiscible liquids
	Liquid-bonded phase	Organic species bonded to a solid surface	Partition between liquid and bonded surface
	Liquid-solid, or adsorption	Solid	Adsorption
	Ion exchange Size exclusion	Ion-exchange resin Liquid in interstices of a polymeric solid	Ion exchange Partition/sieving
Gas chromatography (GC) (mobile phase: gas)	Gas-liquid	Liquid adsorbed on a solid	Partition between gas and liquid
	Gas-bonded phase	Organic species bonded to a solid surface	Partition between liquid and bonded surface
	Gas-solid	Solid	Adsorption
Supercritical-fluid chromatography (SFC) (mobile phase: supercritical fluid)		Organic species bonded to a solid surface	Partition between supercritical fluid and bonded surface

Table.1.3. Desired parameters for Pumps used in HPLC (4)

Pressure (psi)	3000-5000
Flow range(ml/min)	0.5-2ml (analytical) 0.0-10ml preparative)
Accuracy	±5%
Reproducibility	±1%
Solvent storage	200-500ml

Gradient elution	optional
Capacity	For routine analysis, but suitable for method development
Pulse-free delivery of MP	Essential

Table.1.4. Advantages and disadvantages of various pumps used in HPLC

Type of Pump	Advantages	Disadvantages
Syringe pump (Positive Displacement)	Pulse free delivery at high pressure Flow rate independent of viscosity of MP	Limited solvent capacity Change of solvent is inconvenient
Reciprocating pump (Diaphragm type)	Constant flow rate Independent of viscosity of solvents Suitable for continuous operations	Detection noise due to pulsating output
Pneumatic pump	Rugged, inexpensive, easy to operate Pulse-free	Flow rate dependent on the viscosity of MP Change of solvent is inconvenient Gradient operation difficult

Table.1.5. Analyte and Column choice for various HPLC methods

LC MODE	ANALYTES SEPARATTED	COLUMN CHOICE
NORMAL PHASE	Non-ionic, non-polar to medium polar substances	Silica, alumina, Amine, cyano, nitro
REVERSED PHASE	Non-ionic & ion forming non-polar to medium polar substances	C18, C8, C2, $[CH2]_n$-CN,Phenyl, [CH2]n-Diol Columns
REVERSED PHASE ION PAIR	Ionic substances with poor retention in Reverse Phase	C18, C8 and Cyano (CN) Columns
ION EXCHANGE	Ion forming substances: Inorganic ions, organic acids & bases, proteins & nucleic acids	Strong anion and cation exchangers

Table.1.6. Characteristics and applications of Detectors used in HPLC

Detector	**Nature ofanalytes**	**Solvent requirements**	**Comments**
UV/VIS	Compounds with chromophores	UV grade non- UV absorbingsolvents	Has a degree of selectivity & has many HPLC applications
Fluorescence	Fluorescentcompounds	UV grade non-UV absorbing solvents	Highly selective & sensitive. Often used to analyze derivatized compounds.
Refractive Index	Compounds with a different RI to the MP	Not suitable for Gradient runs	Universal detector but has limited sensitivity

Conductivity	Charged or polar compounds	Needs conducting MP	Highly suitable for Ion Exchange methods
Electrochemical	Readily oxidized or reduced compounds	Needs conducting MP	Very selective & sensitive, for biological samples
ELSD	Virtually all compounds	Must use volatile solvents & volatile buffers	Universal detector, highly sensitive but not selective
MS	Broad range of compounds	Must use volatile solvents & volatile buffers	Highly sensitive Powerful 2D analytical tool & many modes available
Radio-activity	For radio labelled samples	UV grade non-UV absorbing solvents	Tritium or C-14 used for radiolabelling & used in metabolite research
IR	Limited primarily to the analysis of synthetic polymers	IR transparent organic solvents only used	Speed, ease of sample preparation, non-consumption of sample
Low angle laser light	Used with synthetic & biopolymers	Uses gels likedextran etc.,	Used in SEC analysis Measures analyte scattering, molecular weight without calibration curve even at low detection limit
Optical activity	To detect enantiomers	Solvent should not interfere with the measurable property	Used for analysing enantiomers
Viscometer	Used with synthetic & biopolymers	Solvent should not interfere with the measurable property	Used for analyzing high molecular weight substances

Table.1.11. Acceptance criteria for System suitability Test (4)

System suitability parameter	**Acceptance criteria**
Retention factor (k)	The peaks of interest should be well resolved from other peaks & the void volume, generally k should be > 2.
Repeatability	An RSD of ≤ 1%
Resolution (Rs)	Rs should be > 2 between peak of interest & the closest eluted interferences
Tailing factor	T should be ≤ 2.
Theoretical plates (N)	In general N should be > 2000.

Table 6.1: Instruments used

S.no	**Instrument**	**Model**
1.	HPLC	WATERS software, Empower 2695 separation module, PDA detector
2.	UV/VIS Spectrophotometer	LABINDIA UV 3000+
3.	pH meter	Adwa – AD 1020
4.	Weighing machine	Afcoset ER – 200A
5.	Pipettes and Burettes	Borosil
6.	Beakers	Borosil

CHEMICALS USED:

Table 6.2: Chemicals used

S.No	Instrument	Brand
1.	Metformin	Mylon
2.	Gemigliptin	Cipla
3.	KH_2PO^4	FINER chemical LTD
4.	Water and Methanol for HPLC	LICHROSOLV (MERCK)
5.	Acetonitrile for HPLC	MOLYCHEM
6.	Ortho phosphoric Acid	MERCK

Figure 1.1. Schematic diagram of HPLC basic Instrumentation(8)

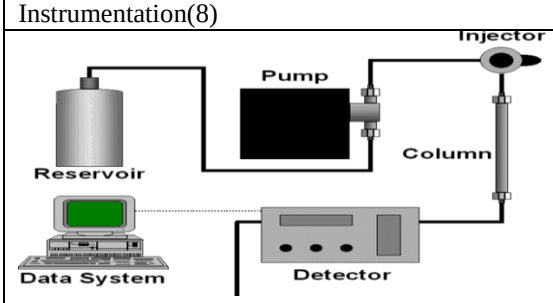


Figure 1.2. Schematic diagram of a six-port Rheodyne valve

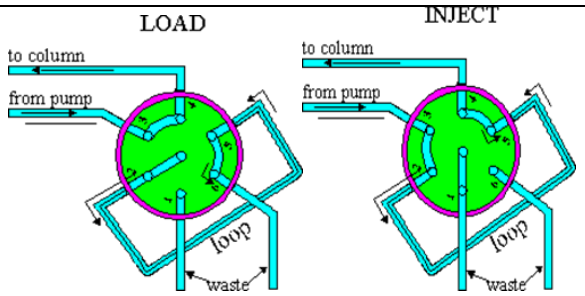
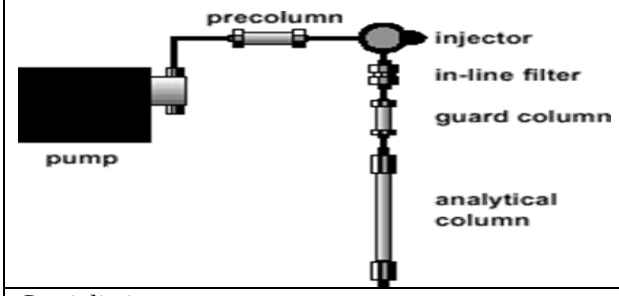
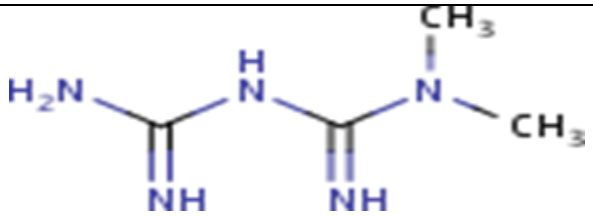


Figure.1.3. Schematic diagram of columns

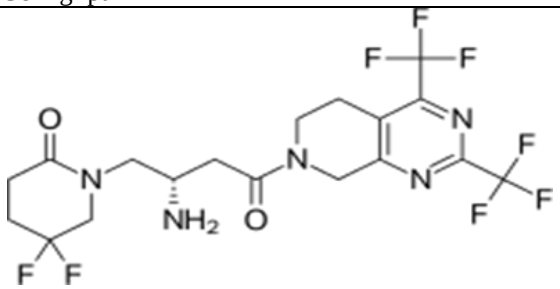


4. Drug Profile

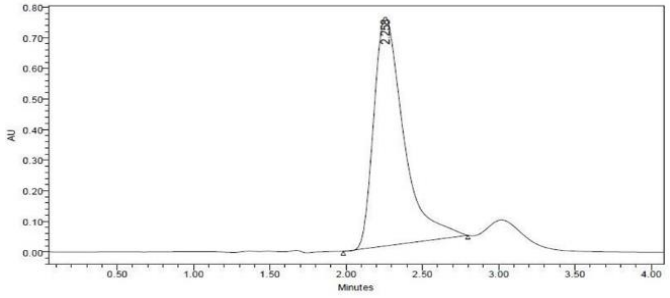
Metformin



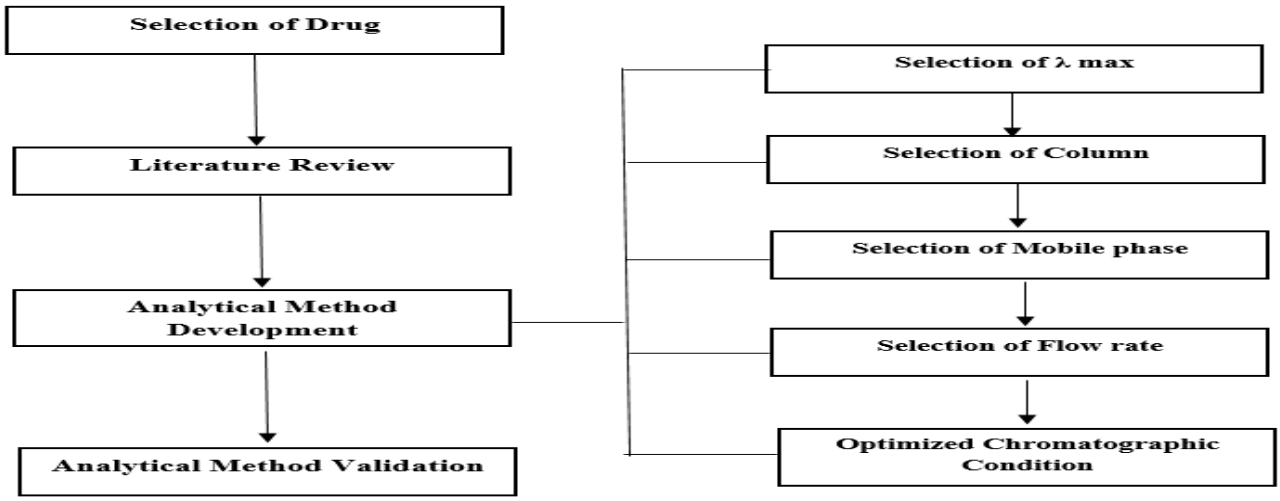
Gemigliptin



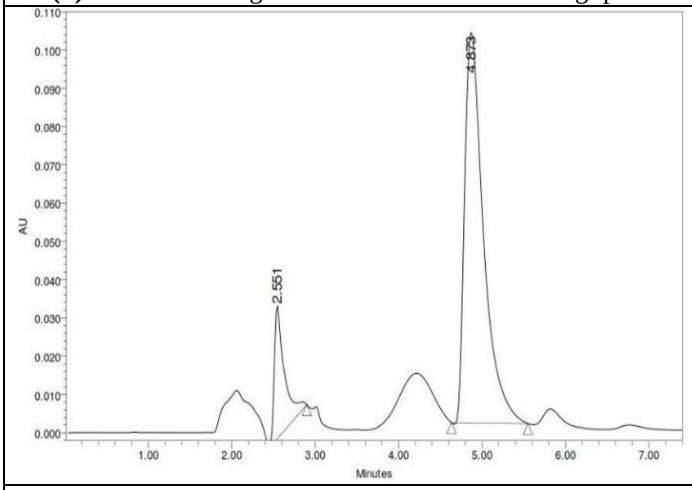
7.1(a) Trial chromatogram for Metformin and Gemigliptin



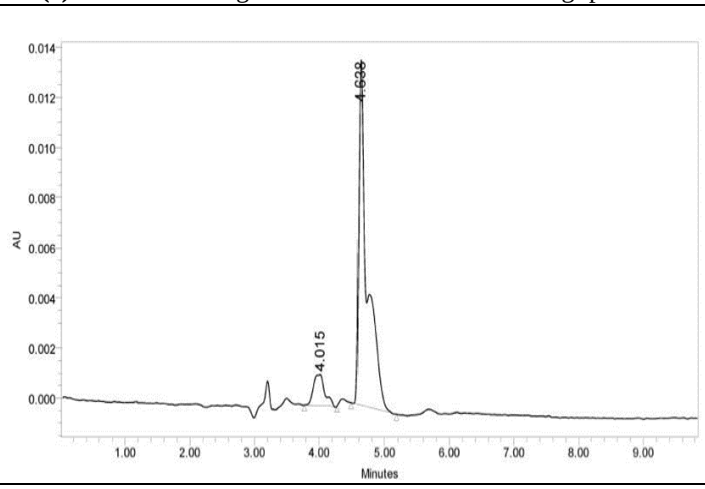
Plan of Work



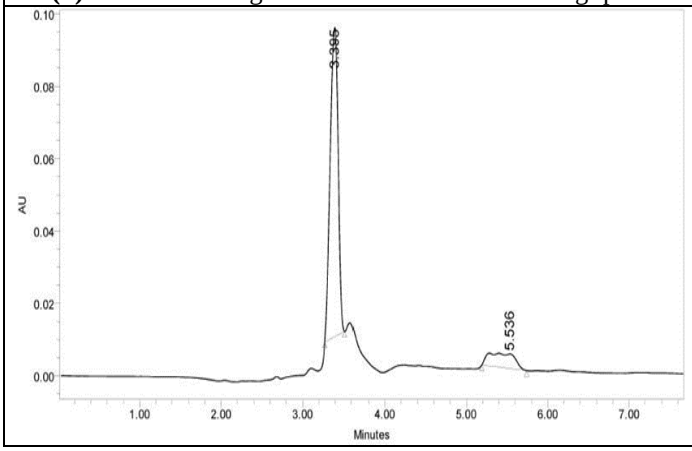
7.1(b) Trial chromatogram for Metformin and Gemigliptin



7.1(c) Trial chromatogram for Metformin and Gemigliptin



7.1(d) Trial chromatogram for Metformin and Gemigliptin



7.1(e) Trial chromatogram for Metformin and Gemigliptin

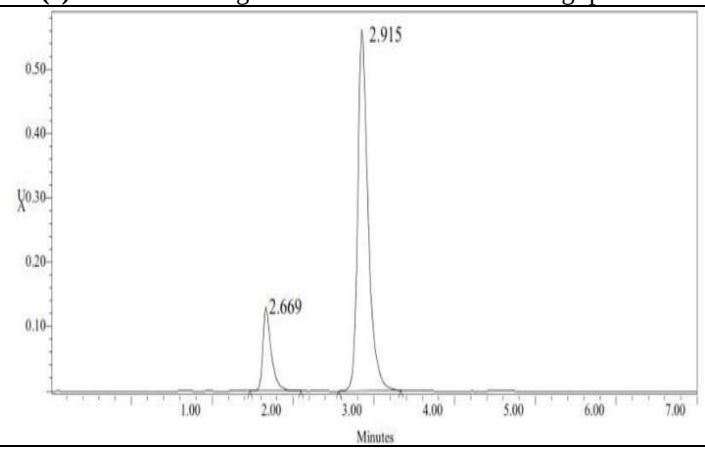
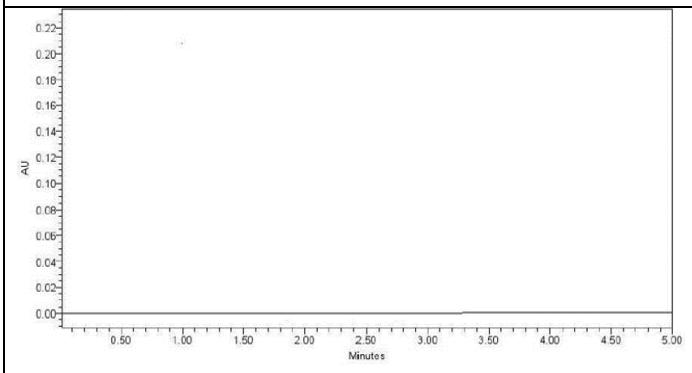


Figure 7.1(h) chromatogram for blank



7.1(h) Chromatogram for Metformin and Gemigliptin Standard Preparation

7.1(g) Chromatogram for Metformin and Gemigliptin sample Preparation

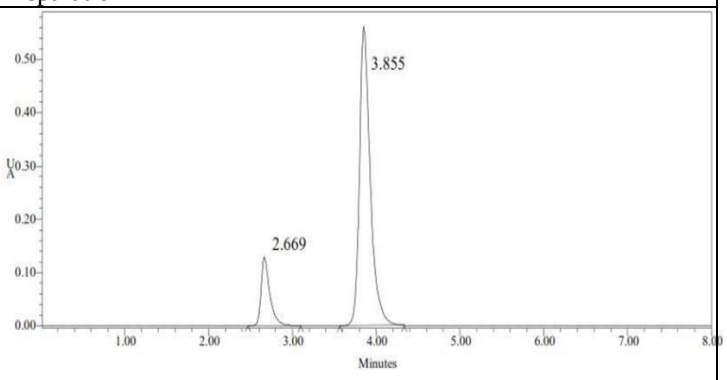


Figure 7.2 chromatogram for system suitability

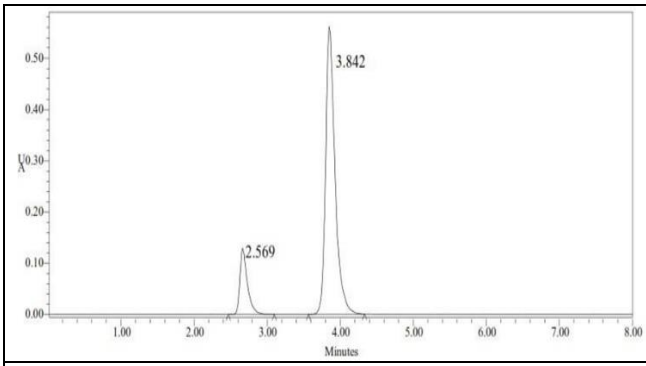


Figure no 7.3.1 (a) chromatogram for standard injection -1

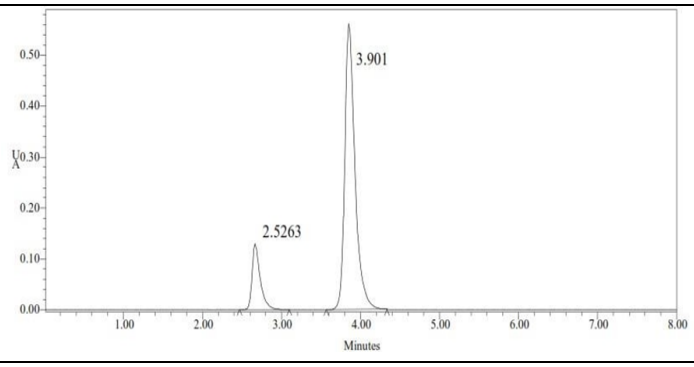


Figure no 7.3.1(b) chromatogram for standard injection-2

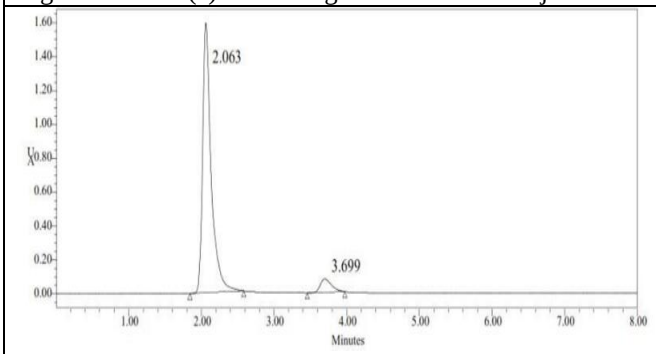


Figure no 7.3.1 (c) chromatogram for standard injection-3

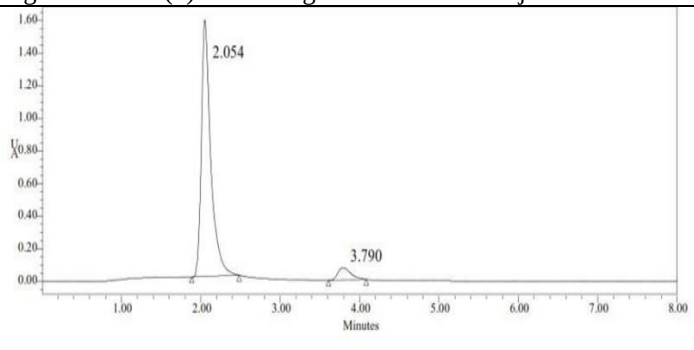
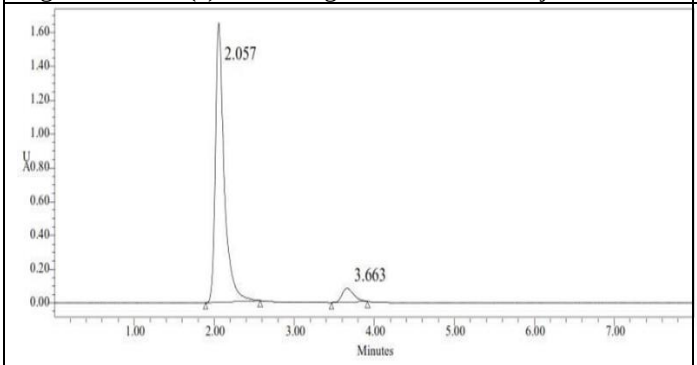


Figure no 7.3.1 (d) chromatogram for standard injection-4



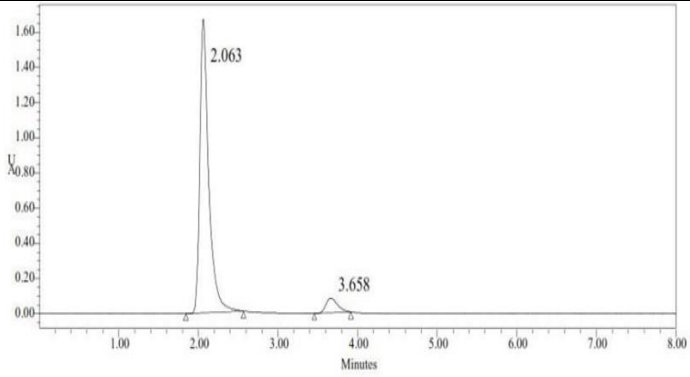
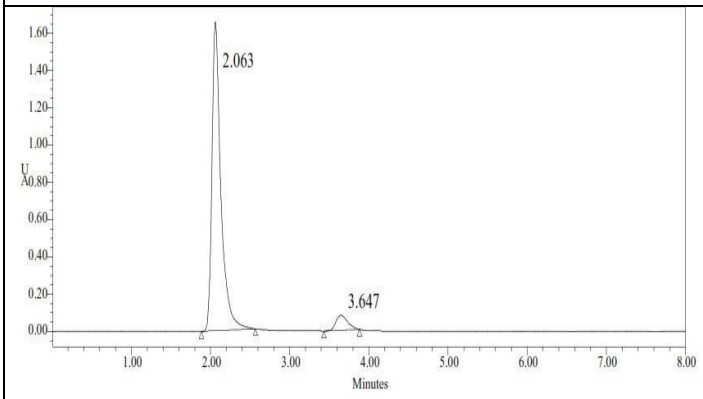


Figure 7.3.2 (a) chromatogram for sample injectiocn-1

Figure 7.3.2 (b) chromatogram for sample injection-2



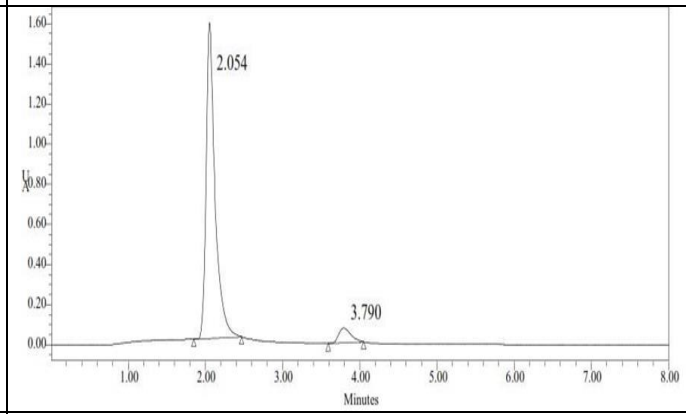


Figure 7.3.2 (c) chromatogram for sample injection-3

Figure 7.3.2 (d) chromatogram for sample injection-4

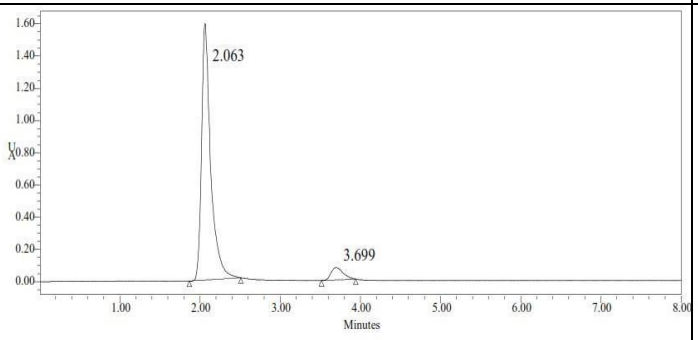
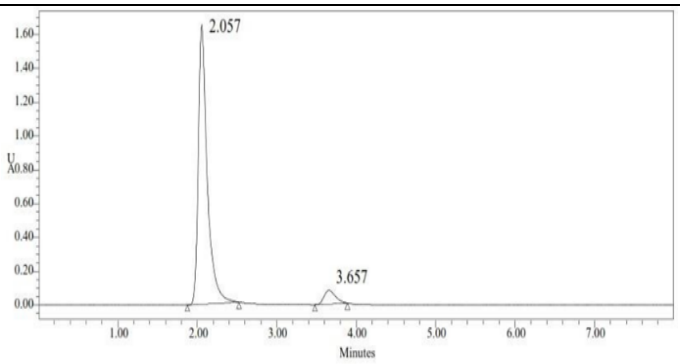


Figure 7.3.2 (e) chromatogram for sample injection-5



7.3.3.1. Chromatograms For Sample Concentration-50%:

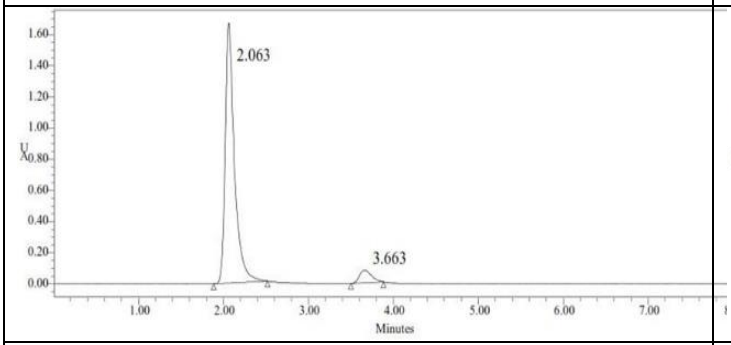


Figure no 7.3.3.1 (a) chromatogram for sample concentration-50%

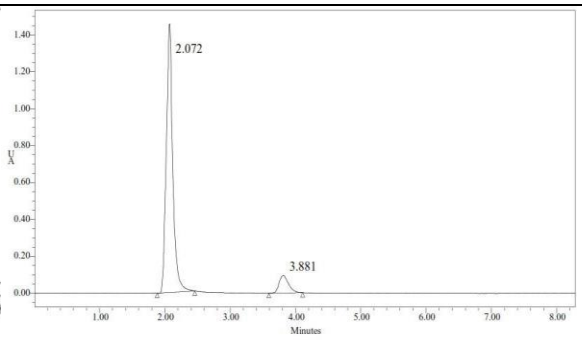
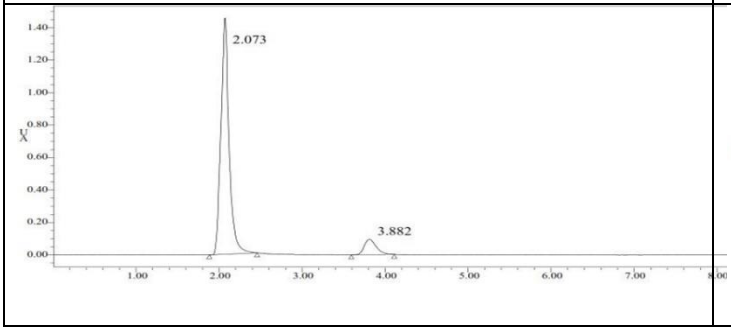


Figure no 7.3.3.1 (b) chromatogram for sample concentration-50%



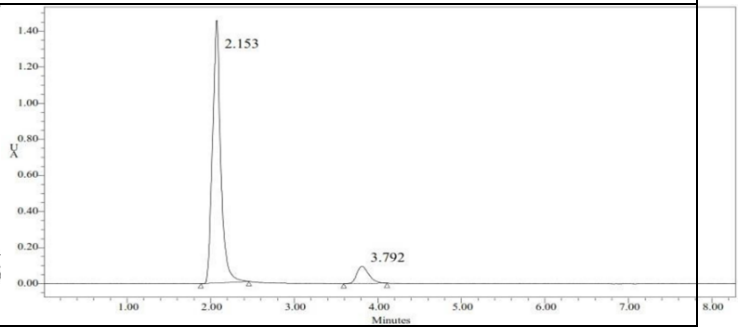


Figure no 7.3.3.1 (c) chromatogram for sample concentration-50%

7.3.3.2. Chromatograms For Sample Concentration-100%:

Figure no 7.3.3.2 (a) chromatogram for sample concentration-100%

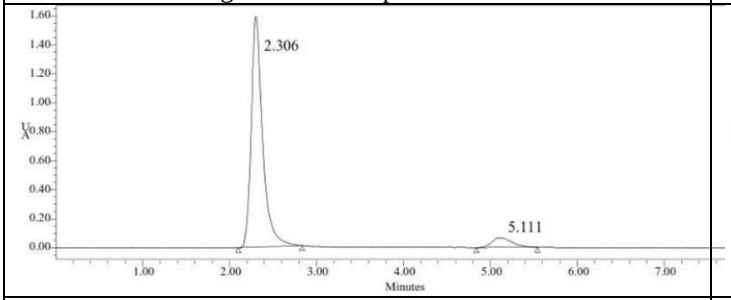


Figure no 7.3.3.2 (b) chromatogram for sample concentration-100%

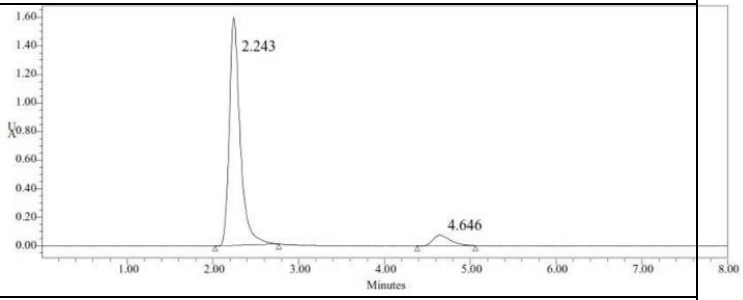


Figure no 7.3.3.2 (c) chromatogram for sample concentration-100%

7.3.3.3. Chromatograms For Sample Concentration-150%:

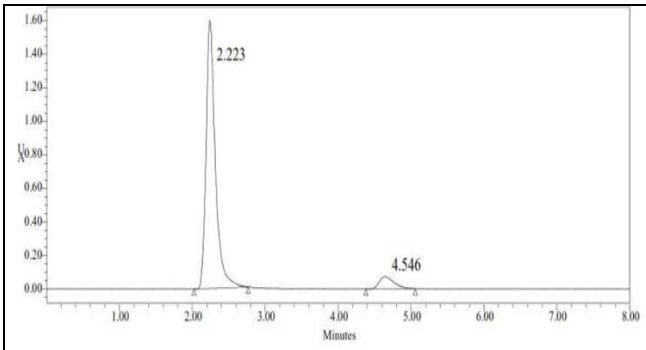


Figure no 7.3.3.3 (a) chromatogram for sample concentration-150%

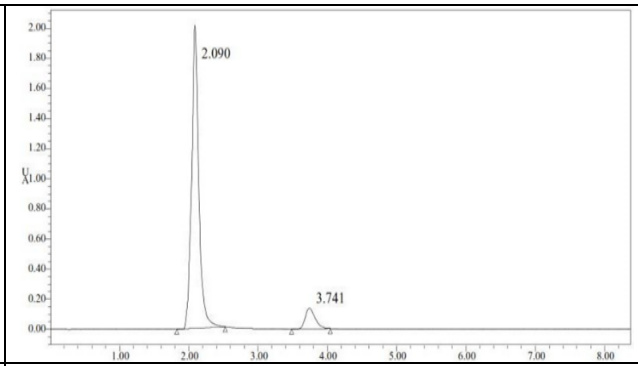


Figure no 7.3.3.3 (b) chromatogram for sample concentration-150%

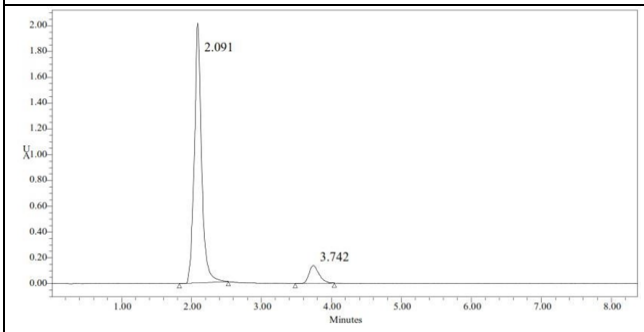


Figure no 7.3.4 (a) chromatogram for linearity concentration-100µg/ml of Metformin & 5 µg/ml of Gemigliptin

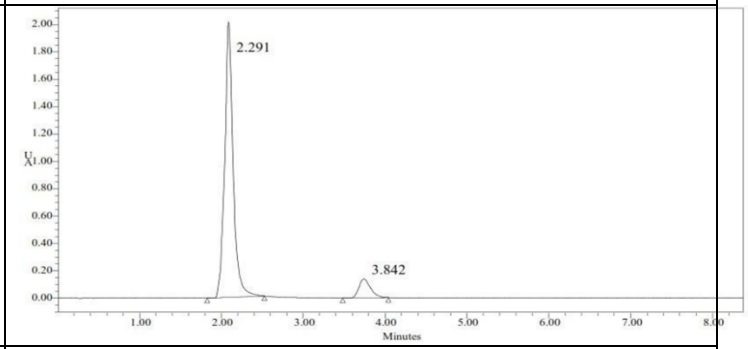


Figure no 7.3.4 (b) chromatogram for linearity concentration-200 µg/ml of Metformin & 10 µg/ml of Gemigliptin

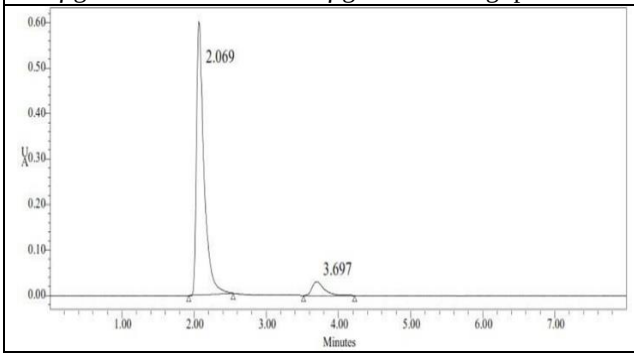


Figure no 7.3.4 (c) chromatogram for linearity concentration--300 µg/ml of Metformin & 15 µg/ml of Gemigliptin

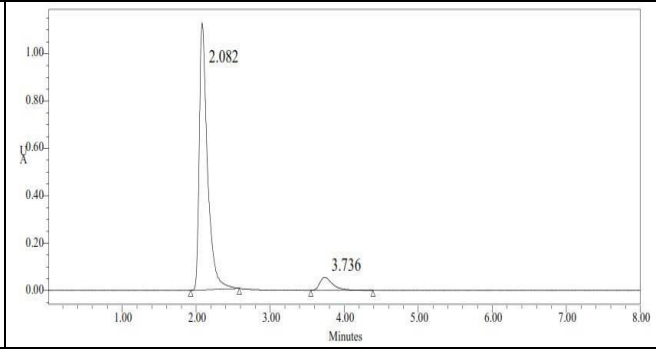


Figure no 7.3.4 (d) chromatogram for linearity concentration-400ppm of Metformin & 20ppm of Gemigliptin

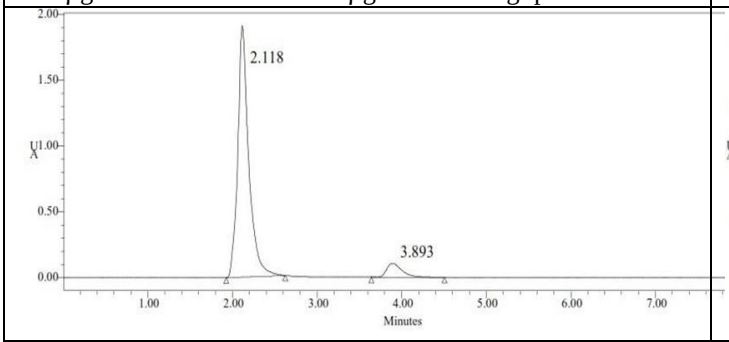


Figure 7.3.4 (f) calibration graph for Metformin at 225 nm

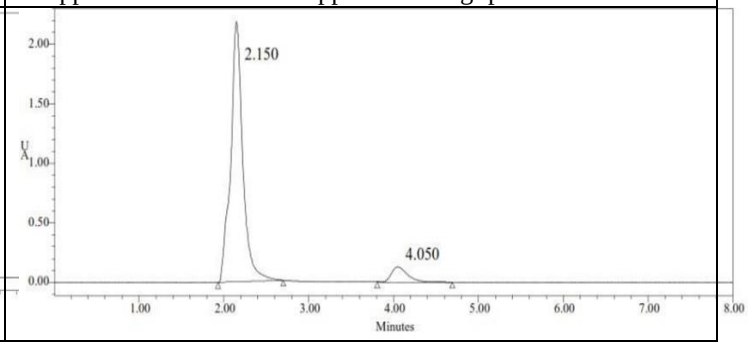


Figure 7.3.4 (f) calibration graph for Gemigliptin at 225 nm

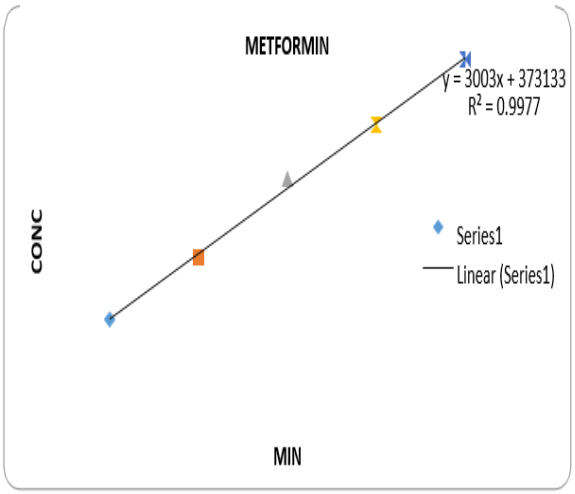


Figure no 7.3.5 chromatogram of Metformin & Gemigliptin showing LOD

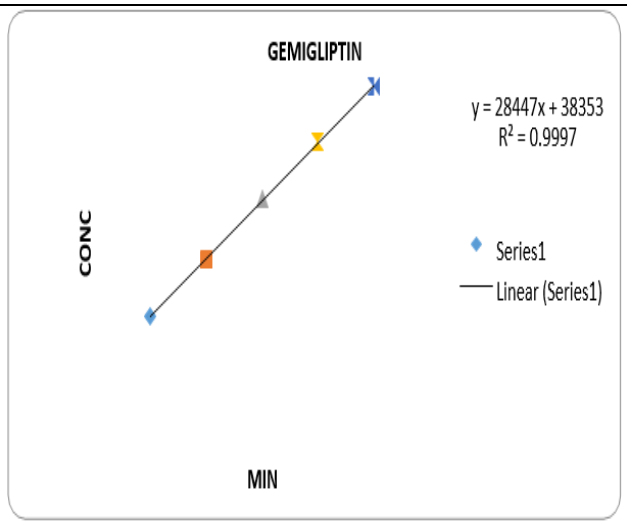
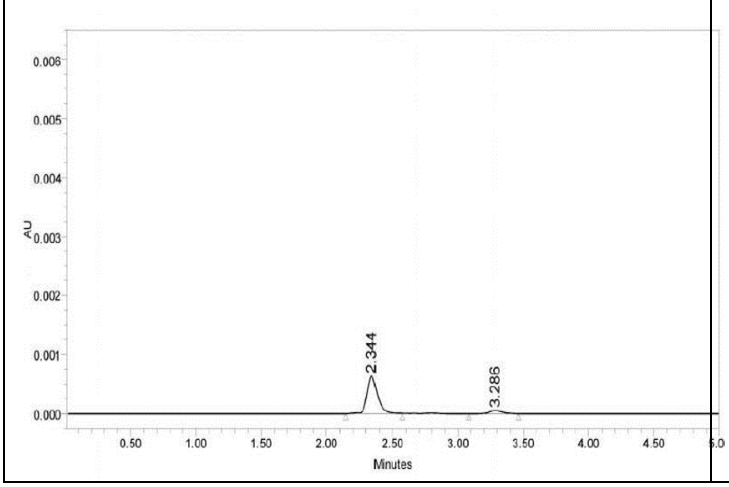


Figure no 7.3.6 (a) chromatogram of Metformin & Gemigliptin showing LOQ



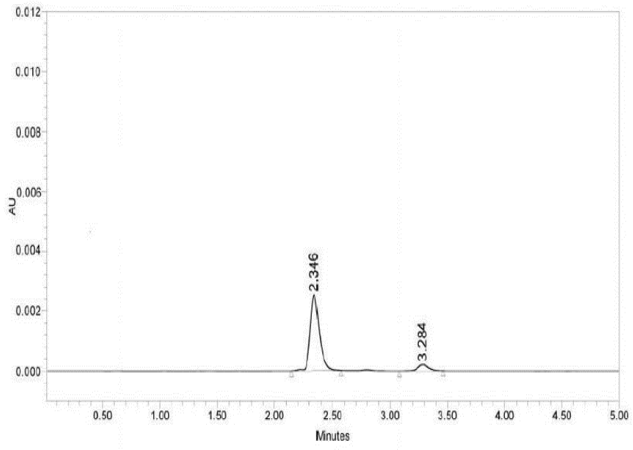


Figure no 7.3.7.2 (a) chromatogram showing less organic composition

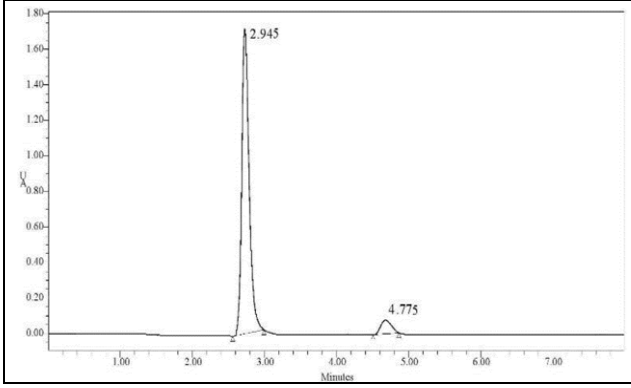


Figure no 7.3.7.2 (a) chromatogram showing less organic composition

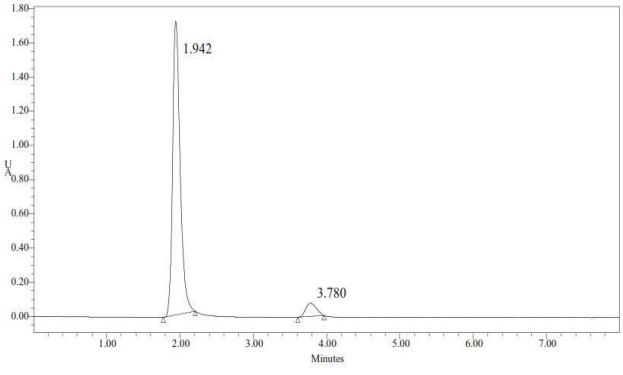


Table 1: Results of system suitability parameters for Metformin and Gemigliptin

S.No	Name	Retention time(min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1.	Metformin	2.5	124505	213642		1.2	4673.4
2.	Gemiglipti n	3.9	1308495	154566	6.0	1.2	6090.3

Table 2: Results of method precession for Metformin:

Injection	**Area**
Injection-1	1302729
Injection-2	1302947
Injection-2	1303236
Injection-2	1303977
Injection-5	1309759
Average	1304529.8
Standard deviation	2961.1
% RSD	0.2

Table 3: Results of method precession for Gemigliptin:

Injection	**Area**
Injection-1	123149
Injection-2	123766
Injection-3	124271
Injection-4	124691
Injection-5	124956
Average	124162.7
Standard deviation	725.6
% RSD	0.6

Table 4: Results of Intermediate precision for Metformin

Injection	**Area**
Injection-1	1300148
Injection-2	1304520
Injection-3	1305937
Injection-4	1306476
Injection-5	130871
Average	1305070.2
Standard deviation	3061.8
% RSD	0.2

Table 5: Results of Intermediate precision for Gemigliptin

Injection	**Area**
Injection-1	122487
Injection-2	122626
Injection-3	122632
Injection-4	122702
Injection-5	122962
Average	122681.8
Standard deviation	174.8
% RSD	0.1

Table 6: Accuracy (recovery) data for Metformin

%Concentration (at	**Area**	**Amount Added (mg)**	**Amount Found (mg)**	**% Recovery**	**Mean Recovery**

specification Level)					
50 %	656659.5	5.0	5.036	100.7%	
100 %	1304258	10.0	10.003	100.0%	99.84 %
150 %	1854608	14.4	14.224	98.780 %	

Table 7: Accuracy (recovery) data for Gemigliptin

%Concentration (at specification Level)	**Area**	**Amount Added (mg)**	**Amount Found (mg)**	**% Recovery**	**Mean Recovery**
50 %	65800	5.3	5.34	100.8%	
100 %	124353	10	10.10	100.01%	
150 %	177940	14.2	14.45	9968%	100.51%

Table 8: Area of different concentration of Metformin

S.No	**Linearity Level**	**Concentration**	**Area**
1.	I	100 ppm	668934
2.	II	200 ppm	956781
3.	III	300 ppm	1313873
4.	IV	400 ppm	1563458
5.	V	500 ppm	1867084
Correlation Coefficient			0.999

Table 9: Area of different concentration of Gemigliptin

S.No	**Linearity Level**	**Concentration**	**Area**
1.	I	1 ppm	66510
2.	II	2 ppm	94701
3.	III	3 ppm	124802
4.	IV	4 ppm	152731
5.	V	5 ppm	179732
Correlation Coefficient			0.999

Table 10: Analytical performance parameters of Metformin and Gemigliptin

Parameters	**Metformin**	**Gemigliptin**
Slope (m)	66574	12529
Intercept (c)	53592	50245
Correlation coefficient (R^2)	0.999	0.999

Table 11: Results of LOD

Drug name	**Baseline (µV)**	**Signal obtained (µV)**	**S/N ratio**
Metformin	52	152	2.9
Gemigliptin	52	156	3

Table 12: Results of LOQ

Drug name	**Baseline (µV)**	**Signal obtained (µV)**	**S/N ratio**
Metformin	52	522	10.03
Gemigliptin	52	524	10.1

Table 13: Flow Rate (ml/min) data for Metformin

S.no	**Flow rate (ml/min)**	**System Suitability Results**	
		USP Plate Count	**USP Tailing**
1.	0.6	5339.9	1.4
2.	0.8	4673.4	1.3
3.	1.0	5216.0	1.4

Table 14: Flow rate (ml/min) data for Gemigliptin

S.no	Flow rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1.	0.8	7063.3	1.3
2.	1.0	6090.3	1.2
3.	1.2	6998.0	1.3

Table 15: Change in Organic Composition in the Mobile Phase for Metformin

S.no	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1.	10 % less	4508.4	1.3
2.	*Actual	4673.4	1.4
3.	10 % more	4318.1	1.3

Table 16: Change in Organic Composition in the Mobile Phase for Gemigliptin

S.no	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1.	10 % less	6387.7	1.2
2.	*Actual	6090.3	1.2
3.	10 % more	6232.5	1.2

LIMIT OF DETECTION FOR METFORMIN AND GEMIGLIPTIN

The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio

Acceptance criteria:

• Signal to noise ratio shall be 3 for LOD solution
• The result obtained is within the limit.

LIMIT OF QUANTIFICATION (LOQ):

The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio.

Acceptance criteria:

• Signal to noise ratio shall be 10 for LOQ solution
• The result obtained is within the limit.

ROBUSTNESS:

The standard and samples of Metformin and Gemigliptin 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.

Acceptance criteria:

• Percentage RSD should be below 2.

The % RSD obtained for change of flow rate, variation in mobile phase was found to be below 1, which is within the acceptance criteria. Hence the method is robust

Summary and Conclusion

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of metformin and gemigliptin was done by RP-HPLC. The Phosphate buffer was pH 3.0 and the mobile phase was optimized with consists of Methanol: Phosphate buffer mixed in the ratio of 70:30 % v/ v. Inertsil C18 column C18 (4.6 x 150mm, 5µm) or equivalent chemically bonded to porous silica particles was used as stationary phase. The detection was carried out using UV detector at 260 nm. The solutions were chromatographed at a constant flow rate of 0.8 ml/min. the linearity range of metformin and gemigliptin were found to be from 100500 µg/ml of metformin and 1-5µg/ml of gemigliptin. Linear regression coefficient was not more than 0.999.

The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-102% of metformin and gemigliptin. LOD and LOQ were found to be within limit.

The results obtained on the validation parameters met ICH and USP requirements .it inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

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