A Rapid RP-HPLC Method Development and Validation for the Simultaneous Estimation of Alogliptin benzoate and Metformin HCl in Bulk and Pharmaceutical dosage form

Dissertation submitted to

The Tamilnadu Dr. M.G.R. Medical University, Chennai

In partial fulfillment of the requirements for the award of the
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Submitted by

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EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"A Rapid RP-HPLC Method Development and Validation for the Simultaneous Estimation of Alogliptin benzoate and Metformin HCl in Bulk and Pharmaceutical dosage form "** submitted by the student bearing **Reg. No. 261230203** to "The Tamil Nadu Dr. M.G.R. Medical University", Chennai, in partial fulfillment for the award of degree of **MASTER OF PHARMACY** in **PHARMACEUTICAL ANALYSIS** was evaluated by us during the examination held on ....

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This is to certify that the dissertation entitled **"A Rapid RP-HPLC Method Development and Validation for the Simultaneous Estimation of Alogliptin benzoate and Metformin HCl in Bulk and Pharmaceutical dosage form"** is a bonafied work done by **Mrs.S.REKA (Reg:No:261230203)**, J.K.K. Nattraja college of pharmacy, in part and fulfillment of the university rules and regulation for award of **Master of Pharmacy** in pharmaceutical analysis under my guidance and supervision during the academic year 2013-2014.

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DECLARATION

The work presented in this dissertation entitled **"A Rapid RP-HPLC Method Development and Validation for the Simultaneous Estimation of Alogliptin benzoate and Metformin HCl in Bulk and Pharmaceutical dosage form "** was carried out by me,under the direct supervision of **Mrs.S.JAYASEELAN, M.Pharm**., Assistant professor, Department of Pharmaceutical analysis, J.K.K. Nattraja College of Pharmacy, Kumarapalayam.

I further declare that, this work is original and has not been submitted in part for the award of any other degree or diploma in any other university.

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1. INTRODUCTION

Pharmaceutical chemistry deals with the chemistry of substances used as a therapeutic agent in medicine. It embraces the main branches of chemistry, radiochemistry analytical, physical, and organic chemistry. Its scope expands with development, in medicinal and allied studies, and the emphasis shifts as knowledge advances. On the other hand it is more narrowly concerned with isolation, determination of structure and synthesis of compounds (mainly organic), which may be used in medicine. It also involves the study of metabolism, mechanism of action of drugs and relationship between structure and biological activity [1].

Pharmaceutical analysis comprises those procedures necessary to determine the "identity, strength, quality and purity" of the drug. Again, it may be defined as the application of analytical procedures used to determine the purity, safety and quality of drugs and chemicals. It also deals with the analysis of raw materials and intermediates in the manufacture of drugs. The pharmaceutical analyst must therefore, have a firm background in basic organic analysis and in addition he should have special skills in the quality evaluation of drug products [2].

Pharmaceutical analysis includes both qualitative and quantitative analysis of drugs and pharmaceutical substances which starts from bulk drugs (starting materials to the finished dosage forms) which is applied for identifying or quantifying constituents in a sample [3].

- Qualitative analysis, which is concerned with the nature and the kind of material in the sample, without a specific interest in the exact amounts present while.

- Quantitative analysis, which is concerned with the amounts of various materials in the sample and the results of such analysis are expressible only in terms of numbers.

Modern pharmaceutical formulations are in multi-component dosage forms for the treatment of various diseases. An accurate estimation procedure for each ingredient in such multi-ingredient preparations is not an easy task, because of the complex nature of drug and excipients added and it becomes more difficult when active constituents are present in very small quantity.

The increasing emphasis on quality control and the complexities of pharmaceutical preparation have necessitated a pharmaceutical analyst to investigate and adapt newer techniques which not only yield accurate and precise result but also are rapid. Their requirement of analytical methods is usually met by instrumental methods and hence during recent year trend is to develop sensitive, specific and rapid method using various sophisticated methods of analysis.

There are various analytical techniques used for quantitative analysis and qualitative analysis of mixtures. Various pharmaceutical analytical techniques, which are being used can be categorized as follows [4]:

Spectral methods:

Where we use light absorption or emission characteristics of drugs (eg) UV–VIS Spectroscopy, IR Spectroscopy, NMR Spectroscopy, Flourimetry, etc.

- **Chromatographic methods:** Where we use affinity or partition coefficient differences between drugs (e.g.) Thin Layer Chromatography (TLC). High pressure liquid Chromatography (HPLC), Paper Chromatography, etc.

- **Electro analytical techniques:** Based on the electrochemical property of drugs (e.g.) Potentiometry, Conductometry, Polarography, Amperometry, Paper electrophoresis, etc.

- **Biological and microbiological methods:** Where we use either animals or microorganisms for analysis (eg) Biological assay of some vitamins, microbiological assay of antibiotics and vitamins.

- **Radioactive methods:** Like Radio Immuno assay and related techniques.

- **Physical methods:** Where we measure some physical characteristics of drugs. Eg. Differential Thermal Analysis (DTA), Differential Scanning Calorimetry (DSC). Thermo Mechanical Analysis (TMA). Thermo Gravimetric Analysis (TGA), etc.

- **Miscellaneous techniques:** Like conventional titrimetric methods, polarimetric methods etc.

1.1. Chromatography [5, 6]

Chromatography is a method of chemical analysis and processing that is rapidly replacing some of the more traditional techniques of sample identification and purification. Modern pharmaceutical formulations are complex mixtures containing one or more therapeutically active ingredients, to a number of inert materials like diluents, disintegrates, colors and flavors. In order to ensure quality and stability of the final product, the pharmaceutical analyst must be able to separate the mixtures into individual components prior to quantitative analysis.

History of chromatography

Chromatography was first developed and defined by the Russian botanist Mikhail Tswett (1892-1917) in 1903. He produced a colorful separation of plant pigments using a column of calcium carbonate (Chalk). The word chromatography comes from the Greek words for color "Chroma" and writes "graphein". So chromatography means to write with colour. Some people state that mikahil Tswett, which invented the term 'chromatography', named the process after him because 'Tswett' is the Russian word for 'color'.

Definitions for chromatography

- Tswett gave the first definition of chromatography. Chromatography is a method in which the compounds of a mixture are separated on an adsorbent column in a flowing system.

- It is the spatial separation of the components of a complex molecular mixture based on their differing affinities for distinct phase in relative motion. The various forms of chromatography differ mostly on the type of phase that are being used and, resulting from that, the different sorts of affinities (ion exchange, size exclusion, use of antibodies – immunosorbend) that are encountered.

- Chromatography is a separation method in which a mixture is applied as a narrow initial zone to a stationary, porous absorbent and the components are caused to undergo differential migration by the flow of the mobile phase, a liquid or a gas. It does not fit for all chromatographic techniques and it is not as concise as the previous definition, but it highlights the most important concepts and is easy to understand.

- One of the most widespread definitions is, of course, the one from the Commission on Analytical Nomenclature of IUPAC: chromatography is a physical method of separation in which the compound to be separated are distributed between two phases. One of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction (IUPAC, 1993).

1.1.2. Chromatography methods

General chromatography techniques require that a solute undergo distribution between two phases, one of them fixed (Stationary phase), the other moving (Mobile phase). Chromatography involves a sample being dissolved in the mobile phase (can be gas, liquid or even supercritical field). This mobile phase is then forced, often under Pressure, through an “immobilized” stationary phase. Solutes are separated by a dynamic differential migration process in a system consisting of two phases, the individual

substance exhibit different mobilities by reasons of differences in adsorption, partition, solubility, vapour pressure, molecular size or ionic charge (Table 1).

Table - 1: Different chromatography methods

Name	Mechanism	Stationary Phase	Mobile Phase
Paper	Partition	Liquid	Liquid
Thin Layer	Adsorption	Solid	Liquid
Gas	Adsorption / Partition	Liquid / solid	Gas
Column	Adsorption / Partition	Liquid / solid	Liquid

Liquid chromatography (LC) [7, 8]

It is analytical chromatography techniques that are useful for separating ions or molecules that are dissolved in a solvent. If the sample solution is in contact with a second solid or liquid phase, the different solutes will interact with the other phase to differing degrees due to differences in adsorption, iron exchange, partitioning, or size. These differences allow the mixture components to be separated from each other by using these differences to determine the transit time of the solutes through a column.

HPLC

High-performance liquid chromatography (HPLC) is a form of liquid chromatography to quantify and analyze mixtures of chemical compounds.

Types of HPLC

HPLC is subdivided on the basis of separation chemistry. All of these techniques can be used the same instrumentation.

Reversed - Phase chromatography

In this type uses a non-polar (hydrophobic) stationary phase and a polar (usually including some water) mobile phase. This is the most common type of HPLC separation in use today.

Normal - phase chromatography

In this type uses a polar (hydrophilic) stationary phase and a non-polar (usually with no water) mobile phase. This was the type of separation to which the term "Chromatography" was first applied.

Ion - exchange chromatography

Uses a stationary phase support which has been derivatized permanently bind charged groups to the surface. The mobile phase is typically an aqueous buffer. This technique is used primarily for the analysis of ions such as strong acids or bases or for separation of large molecules such as nucleic acids, proteins, or large peptides.

Ion - pair chromatography

Ion pair chromatography is a "hybrid" technique in which charge groups are temporarily bound to the surface of a "reversed-phase" type of column packing. This technique is often used for the analysis of small, weak-acid or weak-base compounds.

Size - exclusion chromatography

Size - exclusion chromatography uses a stationary phase of controlled pore size distribution. Separation is based on the extent to which analyte molecules can penetrate the network of pores.

Affinity chromatography

Affinity chromatography operates using immobilized biochemical's that have a specific affinity to the compound of interest. Separation occurs as the mobile phase and sample pass over the stationary phase. The sample compound or compounds of interest are retained as the rest of the impurities and mobile phase through.

Reverse phase chromatography

Mechanism

The separation mechanism in reverse phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilized hydrophobic ligand, i.e. the stationary phase. Reversed phase chromatography is an adsorptive process by experimental design, which relies on a partitioning mechanism to effect separation. The solute molecules partition (i.e. An equilibrium is established) between the mobile phase and the stationary phase.

Stationary phase

Any inert, non-polar substance that achieves sufficient packing can be used for reversed-phase chromatography (Figure 1). The most popular column is an octadecyl carbon chain (C18) bonded silica (USP classification L1) with 297 columns commercially available This is followed by C8 bonded silica (L7 - 166 columns), pure silica (L3 - 88 columns), cyano bonded silica (L10 - 73 columns) and phenyl bonded silica (L11 - 72 columns). Note that C18, C8 and phenyl are dedicated reversed phase packings while cyano columns can be used in a reversed phase mode depending on analyte and mobile phase conditions. It should be noted at this point that not all C18 columns have identical retention properties. Surface functionalization of silica can be performed in a monomeric or a polymeric reaction with different short-chain organosilanes used in a second step to cover remaining silanol groups (end-capping).

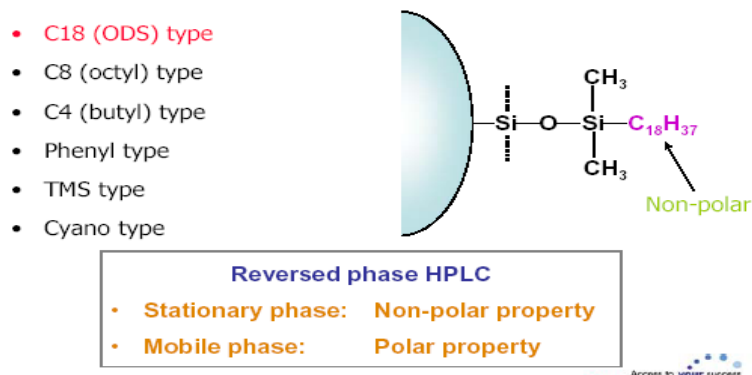


Figure - 1: Schematic diagram of stationary phase column

Mobile phase

Mixtures of water or aqueous buffers and organic solvents are used to elute analytes from a reversed phase column. The solvents have to be miscible with water and the most common organic solvents used are acetonitrile, methanol or tetrahydrofuran (THF). Other solvents can be used such as ethanol, 2-propanol (iso-propyl alcohol). Elution can be performed isocratic (the water-solvent composition does not change during the separation process) or by using a gradient (the water-solvent composition does change during the separation process).

The pH of the mobile phase can have an important role on the retention of an analyte and can change the selectivity of certain analytes. Charged analytes can be separated on a reversed phase column by the use of ion-pairing (also called ion-interaction). This technique is known as reversed phase ion-pairing chromatography.

Reverse phase chromatography is widely in use due to the following advantages

Many compounds such as biologically active substance, have limited solubility in non-polar solvent that are employed in normal phase chromatography (Table 2). Ionic or high polar compounds have high heats of adsorption on straight silica or alumina columns and therefore can elute as a tailing peaks. Column deactivation from polar

modifiers is a problem in liquid – solid chromatography, which frequency can lead to irreducibility in chromatography systems.

Table - 2: Comparison of Normal Phase and Reverse Phase HPLC

Properties	Normal phase	Reverse phase
Polarity of stationary phase	High	Low
Polarity of mobile phase	Low to medium	Low to high
Sample elution order	Leader polar first	Most polar first
Retention will be increased by	Increasing surface of the stationary phase Increasing surface of n-alkyl chain length of stationary phase Decreasing Polarity of Mobile phase Increasing Polarity of Sample molecules	Increasing surface of stationary surface Increasing polarity of mobile phase. Deceasing polarity of sample molecule

1.1.3. Instrumentation of HPLC

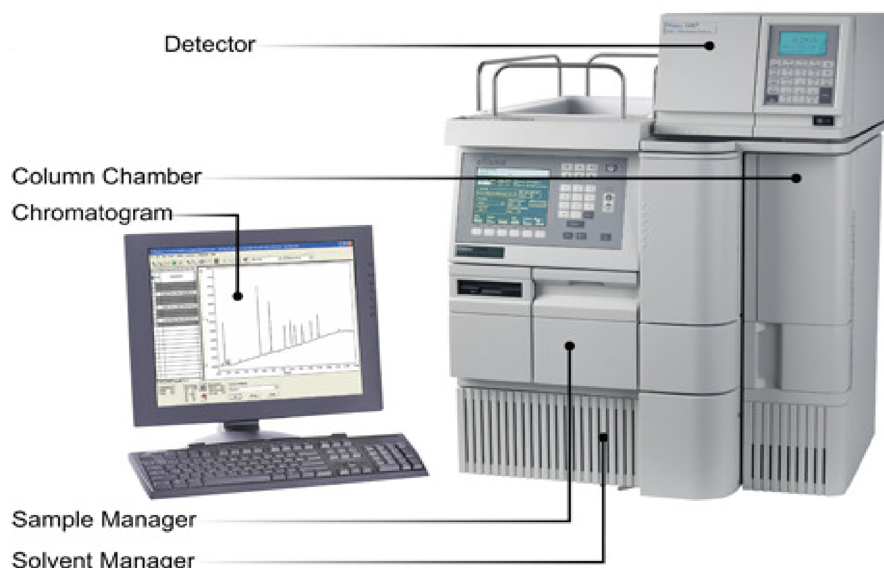


Figure – 2: Schematic diagram of HPLC (waters instrumentation)

Solvent Reservoir

The reservoir that holes the mobile phase is often no more than a glass bottle. The solvent is delivered from the reservoir to the pump means of Teflon tubing – called the "inlet line" to the pump. Some HPLC systems have additional features that allow the mobile phase to be degassed and isolated from contact with air.

Injectors for HPLC

Samples are injected into the HPLC via an injection port. The injection port of an HPLC commonly consists of an injection valve and the sample loop. The sample is typically dissolved in the mobile phase before injection into the sample loop. The sample is then drawn into a syringe and injected into the loop via the injection valve. A rotation of the valve rotor closes the valves and opens the loop in order to inject between 10mcg/ml to over 500mcg/ml. In modern HPLC systems, the sample injection is typically automated.

Stopped flow injection (Figure 3 and 4) is a method whereby the pumps are turned off, allowing the injection port or attain atmospheric pressure. The syringe containing the sample is then injected into the valve in the usual manner, and the pump is turned on. For syringe type and reciprocation pumps, flow in the column can be brought to zero and rapidly resumed by diverting the mobile phase by means of a three-way valve placed in front of the injector. This method can be used up to very high pressures.

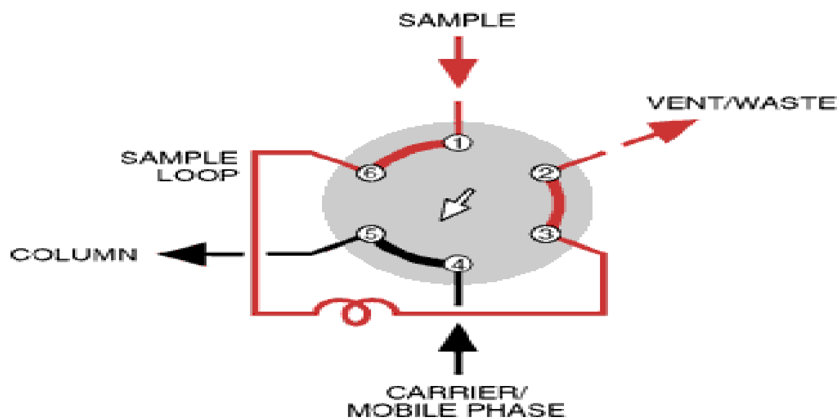


Figure – 3: Representative figure of sample load position

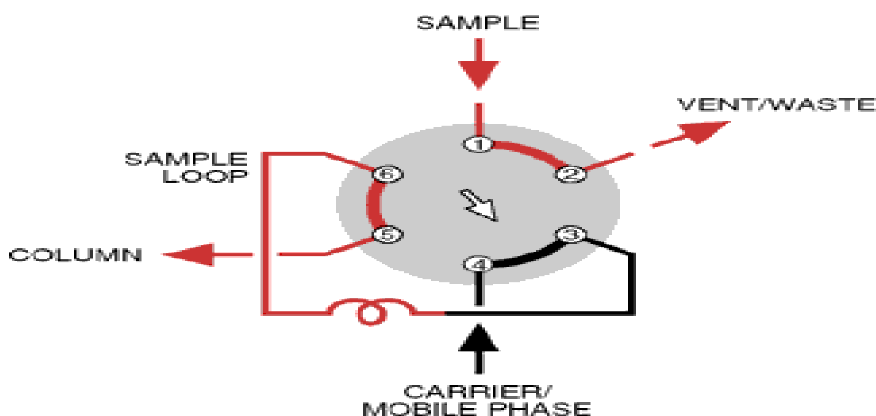


Figure - 4: Representative figure of sample inject position

HPLC Pumps

There are several types of pumps available for use with HPLC analysis.

Pumping system

The function of the pump in HPLC is to pass mobile phase through the column at a controlled flow rate. Features of an ideal pumping system include:

- Generating pressure from 6000 psi to 10000 psi.
- Pulse free output.
- Flow rates ranging from 0.1 to 10 ml/min.
- Flow control and reproducibility of 0.5% relative or better.
- Corrosion resistant components.

Classification of pumps

HPLC pump can be classified in to the following groups according to the manner in which they operate:

Constant flow rate pump (or) constant displacement pump

Reciprocating piston pumps

Reciprocating Piston pumps are consist of a small motor driven piston, which moves rapidly back and forth in a hydraulic chamber that may vary from 35-400mcg/ml in volume. With the backstroke, the separation column valve is closed, and the piston pulls is solvent from the mobile phase reservoir. On the forward stroke, the pump pushes solvent out of the column from the reservoir. A wide range of flow rates can be attained by altering the piston volume during each cycle, or by altering the stroke frequency. A dual and triple head pump consists of identical piston chamber units, which operate at 180 or 120 degrees out of phase. This type system is significantly smoother because one pump is filled while the other is in the delivery cycle.

Syringe type pumps

Syringe type pumps are most suitable for small-bore columns because this pump delivers only a finite volume of mobile phase before it has to be refilled.These pumps have volume between 250 to 500ml. The pump operates by a motorized lead screw that delivers mobile phase to the column at a constant rate. Changing the voltage on the motor controls the rate of solvent delivery.

Constant pressure pumps

In this pump the mobile phase is driven through the column with the use of pressure from a gas cylinder. A low-pressure gas source is needed to generate high liquid pressures. The valuing arrangement allows the rapid refill of the solvent chamber whose capacity is about 70ml. This provides continuous mobile phase flow rates.

Displacement pump

Displacement pumps usually consist of large syringe like chambers equipped with a plunger that is activated by a screw driven mechanism powered by stepping motors. Displacement pumps also produce a flow that tends to be independent of viscosity and backpressure. In addition, the output is pulse free. Disadvantages include limited solvent capacity (250 ml) and considerable inconvenience when solvents must be changed.

Pneumatic pumps

In pneumatic pumps, the mobile phase is contained in a collapsible container housed in a vessel that can be pressurized with a compressed gas. Pumps of this kind are inexpensive and pulse free. They suffer from limited capacity, pressure output, dependence of flow rate on solvent viscosity and column backpressure. In addition, they are not amenable to gradient elution and are limited to pressures less than about 2000 psi.

Sample injection system

The limiting factor in the precision of LC measurements lie in reproducibility with which samples are introduced into the column packing. The earliest and simplest means of sample introduction was syringe injection through a self-sealing electrometric septum. In stop flow injections, the flow of solvent is stopped momentarily, and fitting at column head is removed and the sample is injected directly into the head of the column packing. After replacing the fitting the system is again pressurized. Commercial chromatographs use valves for sample injection. With these devices, sample is first transferred at atmospheric pressure from a syringe into a sample loop. Turning the valve from load to inject position connects the sample loop into the high-pressure mobile Phase stream, whereby the contents of the sample loop are transferred on to the column. In Rheodyne 7125 valve, sample from a micro liter syringe is loaded into the needle port, filling the sample loop, which is a small piece of stainless steel tube connected between ports. Any excess goes to waste from another port. On turning to 'inject', the loop contents are flushed on to the column. A variety of loop volumes are available, commonly 10-50 µl.

Columns

The column (Figure 5) is one of the most important components of the HPLC chromatograph because the separation of the sample components is achieved when those components pass through the column. Normally, columns are filled with silica gel because its particle shape, surface properties, and pore structure help to get a good separation. Silica is wetted by nearly every potential mobile phase, is inert to most compounds and has a high surface activity which can be modified easily with water and other agents. Silica can be used to separate a wide variety of chemical compounds, and its chromatographic behaviors generally predictable and reproducible.

There are various columns that are secondary to the separating column or stationary phase. They are guard (Figure 6), derivatizing, Capillary, and preparatory columns

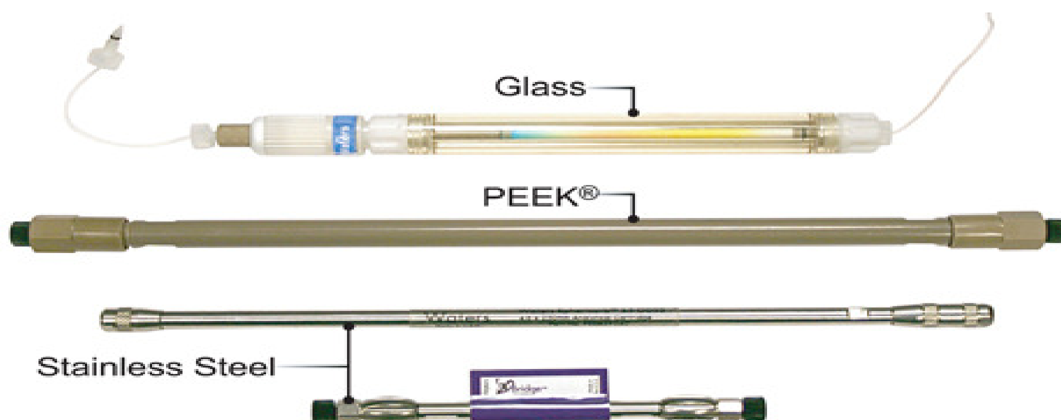


Figure - 5: Representative figure of HPLC column

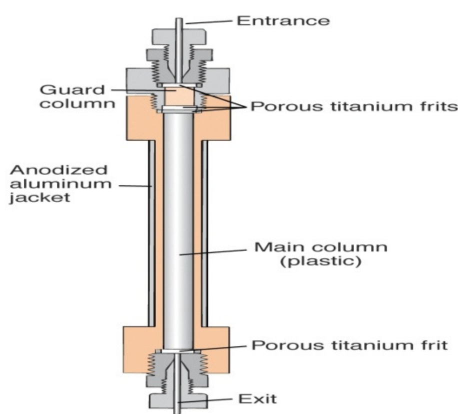


Figure – 6: Representative figure of Guard columns of HPLC

Guard columns are placed anterior to the separating column. This serves as a protective factor that prolongs the life and usefulness of the separation column.

They are dependable columns designed to filter or remove.

1. Particles that clog the separation column.

2. Compounds and ions could ultimately cause “baseline drift”, decreased resolution, decreased sensitivity, and create false peaks.

3. Compounds that may cause precipitation upon contact with the stationary or mobile phase.

4. Compound that might co-elute and cause extraneous peaks and interfere with detection and / or quantification.

These columns must be changed on a regular basis in order to optimize their protective function. The size of the packing varies with the type of protection needed (Table 3 and 4).

Derivatizing columns

Pre or post primary column derivatization can be an important aspect of the sample analysis. Reducing or altering the parent compound to a chemically related daughter molecule or fragment elicits potentially tangible data, which may complement other results or prior analysis. In a few cases, the derivatization step to cause data to become questionable, which is one reason why HPLC was advantageous over gas chromatography. Because GC requires volatile, thermally stable, or nonpolar analytes, derivatization were usually required for those samples, which did not contain these properties, Acetylation, Silylation, or concentrated acid hydrolysis are a few derivatization techniques.

Capillary columns

Advances in HPLC led to smaller analytical columns. Also known as micro columns, capillary columns have a diameter much less than a millimeter and there are three types: open – tubular, partially packed, and tightly packed. They allow the user to work with nanonliter sample volumes, decreased flow rate, and decreased solvent volume usage which may lead to cost effective.

Microbore and small columns

Bore Columns are also analytical and small volumes assays. A typical diameter of a small – bore column is 1-2mm. Like capillary columns, instruments must usually be modified to accommodate these smaller capacity columns (i.e. Decreased flow rate).

Fast columns

One of the primary reasons for using these columns is to obtain improved sample throughput (amount of compound per unit time). Fast columns are designed to decrease time for the chromatographic analysis without forsaking significant deviations in results. These columns have the same internal diameter but much shorter length than most other columns, and they are packed with smaller particles that are typically 3 mcg/ml in diameter. Advantages include increased sensitivity, deceased analysis time, decreased mobile phase usage, and increased reproducibility.

Preparative Columns

These columns are utilized when the objective its prepares bulk (milligrams) of sample for laboratory reparatory applications. A facilitate large volume injection into the HPLC system. Accessories important to mention are the back-pressure regulator and the fraction collector. The back-pressure regulator is placed immediately posterior to HPLC detector. The fraction collector is an automated device that collects uniform increment of the HPLC output.

Some bonded stationary phases for HPLC and column dimensions were presented in table 3 and 4.

Table - 3: Shown Bonded Stationary Phases for HPLC

Stationar y	Functional group	Application
Silica	Si-OH	Normal phase (Pesticides, alkaloids)
C_{18}	Octadeyl	Reverse phase (Fatty acid PAH Vitamins)
C_8	Octyl	Reverse-phase and ion pair (Peptides proteins)
C_6H_5	Phenyl	Reverse phase (polar aromatic fatty acid)
CN	Cyano	Normal and reverse phase (polar compound)
NO_2	Nitro	Normal and Reverse-phase (PAH, Aromatic)
NH_2	Amino	Normal phase (weak ion exchange carbohydrates, Organic acid, chlorinated pesticides)

Table - 4: Column dimensions

Type	Internal Diameter (mm)	Length (cm)	Particle size (µm)
Analytical	0.3 - 0.46	3-28	3-10
Semimicro	0.1 – 0.21	10 – 25	3 – 18
Semipreparative	0.8 – 1.0	10 – 25	5 – 10
Preparative	2.0 – 5.0	10 – 25	10 – 20

Detectors

The detector for an HPLC is the compound that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram. It is positioned immediately posterior to the stationary phase in order to detect the compounds as they elute from the column. The bandwidth and height of the peaks may usually be adjusted using the coarse and fine tuning controls, and the detection and sensitivity parameters may also be controlled (in most cases). There are many types of detectors that can be used with HPLC.

Refractive Index (RI) detectors

RI detectors measure the ability of sample molecules to bend or refract light. This property for each molecule or compound is called its refractive index. For most RI detectors, light proceeds throug33h a bi-modular flow-cell to a photo detector. One channel of the flow-cell directs the mobile phase passing through the column while the other directs only the mobile phase. Detection occurs when the light is bent due to the samples eluting from the column, and this is read as a disparity between the two channels.

Ultraviolet (UV) detectors

UV detectors measure the ability of a sample to absorb light. This can be accomplished at one or several wavelengths:

- ❖ **Fixed Wavelength** measures at one wavelength, usually 254 nm.
- ❖ **Variable Wavelength** measures at one wavelength at a time, but can detect over a wide range of wavelengths.
- ❖ **Diode Array** measures a spectrum of wavelengths simultaneously.

Fluorescence detectors

Fluorescence detectors are measuring the ability of a compound to absorb then re-emits light at given wavelengths. Each compound has a characteristic fluorescence. The excitation source passes through the flow-cell to a photo detector while a monochromatic measures the emission wavelengths. Has a sensitivity limit of 10^{-9} to 10^{-11} gm/ml

Radiochemical detection

Involves the use of radio labeled material, usually tritium (^{3}H) or carbon-14 (^{14}C). It operates by detection of fluorescence associated with beta-particle ionization, and it is most popular in metabolite research. It is two types of detector as follows:

- ❖ **Homogeneous**- Where addition of scintillation fluid to column effluent causes fluorescence.
- ❖ **Heterogeneous**- Where lithium silicate and fluorescence caused by beta-particle emission interaction with the detector cell. Has a sensitivity limit up to 10^{-9} to 10^{-10} gm/ml.

Electrochemical detectors

Electrochemical detectors are measure compounds that undergo oxidation or reduction reactions. Usually accomplished by measuring the gain or loss of electrons from migrating samples as they pass between electrodes at a given difference in electrical potential. Has sensitivity of 10^{-12} to 10^{-13} gm/ml.

Mass Spectroscopy (MS) Detectors

The sample compound or molecule is ionized, it is passed through a mass analyzer, and the ion current is detected. There are various methods for ionization

- **Electron Impact (EI)** - An electron current or beam created under high electric potential is used to ionize the sample migrating off the column.

- **Chemical Ionization (CI)** - A less aggressive method which utilizes ionizing gas to remove electrons from the compounds eluting from the column.

- **Fast Atom Bombardment (FAB)** - Xenon atoms are propelled at high speed in order to ionize the effluents from the column. Has a detection limit of 10^{-8} to 10^{-10} gm/ml.

Nuclear Magnetic Resonance (NMR) Detectors

Certain nuclei with odd- numbered masses, including H and ^{13}C, spin about an axis in a random fashion. However, when placed between the poles of a strong magnet, the spins are aligned either parallel or anti-parallel to the magnetic field, with the parallel orientation favored since it is slightly lower in energy. The nuclei are then irradiated with electromagnetic radiation, which is absorbed and places the parallel nuclei into a higher energy state; consequently, they are now in "resonance" with the radiation. Each H or C will produce different spectra depending on their location and adjacent molecules, or elements in the compound, because all nuclei in molecules are surrounded by electron clouds which change the encompassing magnetic field and thereby alter the absorption frequency.

Light-Scattering (LS) Detectors

When a source emits a parallel beam of light which strikes particles in solution, some light is reflected, absorbed, transmitted, or scattered. Two forms of LS detection may be used to measure the two latter occurrences:

❖ **Nephelometry**

This is defined as the measurement of light scattered by a particulate solution. This method enables the detection of the portion of light scattered at a multitude of angles. The sensitivity depends on the absence of background light or scatter since the detection occurs in a black or null background.

❖ **Turbidimetry**

This is defined as the measure of the reduction of light transmitted due to particles in solution. It measures the light scatter as a decrease in the light that is transmitted through the particulate solution. Therefore, it quantifies the residual light transmitted. The sensitivity of this method depends on the sensitivity of the machine employed, which can range from a simple spectrophotometer to a sophisticated discrete analyzer. Thus, the measurement of a decrease in transmitting light from a large signal of transmitted light is limited to the photometric accuracy and limitations of the instrument employed.

Near-Infrared Detectors

Operates by scanning compounds in a spectrum from 700 to 1100 nm. Stretching and bending vibrations of particular chemical bonds in each molecule are detected at certain wavelengths. This is a fast growing method which offers several advantages: speed (sometimes less than 1 second), simplicity of preparation of samples, multiple analyses from single spectrum, and non consumption of the sample.

Applications of HPLC

Pharmaceutical applications

❖ Tablet dissolution study of pharmaceutical dosage form.

❖ Shelf-life determinations of pharmaceutical products.

- Identification of active ingredients of dosage forms.
- Pharmaceutical quality control.
- Determination of partition coefficients and pka values of drugs and of drug protein binding.

Environmental applications

- Detection of phenolic compounds in Drinking Water.
- Identification of diphenhydramine in sediment samples.
- Bio-monitoring of pollutant.

Forensics

- Quantification of the drugs in biological samples.
- Identification of anabolic steroids in serum, urine, sweat, and hair.
- Forensic analysis of textile dyes.
- Determination of cocaine and metabolites in blood.

Clinical

- Quantification of ions in human urine.
- Analysis of antibiotics in blood plasma.
- Estimation of bilirubin and bilivirdin in blood plasma in case of hepatic disorders.
- Detection of endogenous neuropeptides in extracellular fluids of brain.

Food and Flavor

- Ensuring the quality of soft drink and drinking water.
- Analysis of beer.
- Sugar analysis in fruit juices.
- Analysis of polycyclic compounds in vegetables.
- Trace analysis of military high explosives in agricultural crops.

Limitations of HPLC

- There is still a requirement for reliable and inexpensive detectors which can monitor compounds that lack a chromophore.
- Drugs have to be extracted from their formulations prior to analysis.
- Large amounts of organic solvent waste are generated, which are expensive to dispose off.

1.2. The basic steps involved in method development [9]

1.2.1. Literature collection

Search for literature (USP, EP, JP, IP, Chromatography Journals, patents, internet, etc) for the same molecule or for similar molecules having similar structures.

Take the method from literature to check suitability to meet the requirements or modify the method to suit the requirements such are resolution of possible impurities as per the synthetic process.

1.2.2. Chemical structure

By observing the structure, based on the functional groups present in the molecule, it can be determined whether the compound is Basic, acidic and neutral. Based on the nature of the compound, the pH of the mobile phase can be selected.

If the compound is acidic, acidic mobile phase is preferable. For a basic compound, low pH and basic mobile phases are preferable. For a neutral compound neutral mobile phase is suitable. Elution of the compounds will follow on 'like solves like' concept.

1.2.3. Molecular weight

If the molecular weight is less than 500 and based on the solubility and nature (acid/base/ionic/non-ionic) of the molecule we can select the techniques given below.

Reversed phase chromatography (for acids, bases and non-ionic samples) Ion-pair chromatography (for ionic samples), Ion exchange chromatography (for ionic samples) Normal phase chromatography (for isomers, non-polar/non-ionic samples, chiral molecules) Size exclusion chromatography (for protein related samples)Capillary electrophoresis (for red-ox samples)

1.2.4. pH / pKa value of compounds

Based on pH / pKa values the nature of the compound (acidic, basic and neutral in nature) and polarity of the compound can be assumed.

1.2.5. Sample solubility

Check the solubility of all components in the solutions like organic mixtures, aqueous-organic mixtures and mixtures of mineral acids like perchloric acid, phosphoric acids, and buffer solution etc.

1.3. Optimization of chromatographic condition

Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that all the compounds are detected by more or less symmetrical peaks on the chromatogram. By a slight change of the mobile phase composition, the shifting of the peaks can be expected. From a few experimental measurements, the position of the peaks can be predicted within the range of investigating changes. An optimized chromatogram is the one in which all the peaks are symmetrical and are well separated in less run time. [10-12]

The peak resolution can be increased by using a more efficient column with higher theoretical plate number, N.

The parameters that are affected by the changes in chromatographic conditions are,

- Resolution (R_s),
- Capacity factor (k'),
- Selectivity (α),
- Column efficiency (N) and
- Peak asymmetry factor (As).

Retention time (t_R)

Retention time is the difference in time between the point of injection and appearance of peak maxima. Retention time is the time required for 50% of a component to be eluted from a column. Retention time is measured in minutes of seconds. Retention time is also proportional to the distance moved on a chart paper, which can be measured in cm or mm.

Retention volume (V_R)

Retention volume is the mobile required to elute 50% of the component from the column. It is the product of retention time and flow rate. Retention Volume = Retention time x flow rate

Resolution

Resolution is a measure of the separation of the two components and the baseline separation achieved. Resolution is generally defined as "the distance between the centers of two eluting peaks as measured by retention time or volume divided by the average width of the respective peaks "Resolution can be determined by using the following formula

$$Rs = \frac{2\,(t2 - t1)}{W2 + W1}$$

t_1 and t_2 – Retention times of two components.

w_2 and w_1 – Width of the two components at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

The parameters that contribute to a peak resolution column selectively, Column efficiency and the column retention factor.

$$Rs = \frac{1}{4} * \frac{(\alpha-1)}{\alpha} * \sqrt{n} * \frac{k\prime}{1+k}$$

Resolution Rs is a function of selectivity a, efficiency (Number of theoretical plates) and the average retention factor, K' for peaks 1 and 2.

Capacity factor

The capacity factor, K' is related to the retention time is a reflection of the proportion time of a particular solute residue in the stationary phase as opposed to the mobile phase. Long retention times results in large valves of K'. The capacity factor K', can be calculated for every peak defined in a chromatogram, using the following equations.

$$\text{Capacity factor} = \frac{\textit{moles of solute in stationary phase}}{\textit{moles of solute in mobile phase}}$$

$$K'' = \frac{Tr - To}{To} * \frac{Vr - Vo}{Vo}$$

Where T_R are the retention time and retention volume, respectively, of the solute and T_O and V_O the retention time and retention volume, respectively, of an un retarded solute.

Efficiency (N)

The number of theoretical plates expresses the efficiency of a packed column; N. 'N' is also called as number of theoretical plates count. It is a measure of the band spreading of a peak. Smaller the band spread, higher the theoretical plate indicates good column and system performance. A theoretical plate is an imaginary or hypothetical unit

of a column where an equilibrium has been established between stationary phase and mobile phase.

The column plate number N is calculated by

$$N = \frac{16\ Rt^2}{W^2}$$

Where

N = Number of theoretical plates

Rt = Retention time

W = peak width at base

Mutual measurement of the baseline bandwidth 'W' may be subject to error. Therefore, a more practical equation for N is

$$N = 5.54 \quad [t / W_{h/2}]^2$$

$W_{h/2}$ is the width of peak half height.

The number of theoretical plates is sometimes reported as plates per meter of column length (N/L). The height equivalent to theoretical plates, H, is given by

$$H = L/N$$

Where L is column length and N is the number of theoretical plates.

Asymmetry factor (or) tailing factor (T)

$$T = \frac{W0.05}{2f}$$

The assessment of peak shape is in terms of asymmetry factors.

1.4. Validation of analytical method [13]

Validation is the documented evidence, which provides a high degree of assurance for specific method. Validation is a systemic approach to identifying, measuring, evaluating, documenting, and evaluating all the critical steps responsible, before establishing the validity of the method. Validation of the analytical method is not only an integral part of quality control system, but cGMP also require assay validation.

FDA defines validation as "establish the documented evidence which provides a high degree of assurance that a specific process will consistently produce a product of predetermined specifications and quantity attributes".

Analytical method validation includes all the procedures and checks required to prove the reliability of a method for quantitative determination of concentration of an analyte or series of analytes in a given sample. For analytical method validation, US-FDA has given some guidelines in USP and are referred as "Eight steps of analytical method validation". These terms are also referred as "Analytical Performance Parameters".

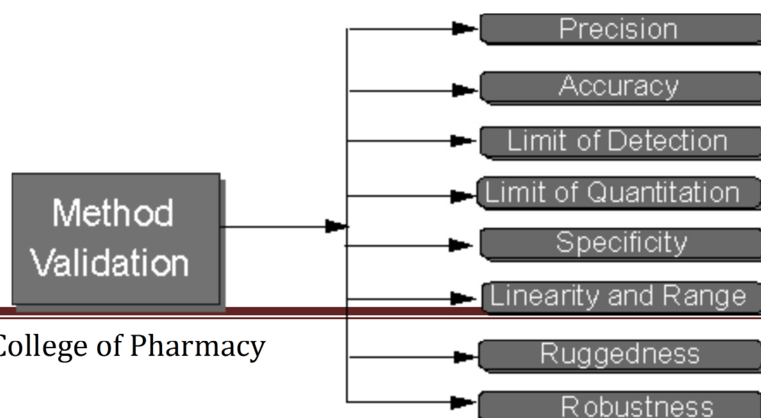


Figure - 7: Eight steps of method validation

Specificity

It is the ability of an analytical method to assess unequivocally the analyte of interest in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components. In the case of the assay, demonstration of specificity requires that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substances or product with appropriate levels of impurities or excipients, and demonstrating that the assay is unaffected by the presence of these extraneous materials. If the degradation product impurity standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure. These comparisons should include samples stored under relevant stress conditions (e.g. Light, heat, humidity, acid/base hydrolysis, oxidation, etc.).

Linearity and range

The linearity of an analytical method is its ability to obtain test results in direct or well-defined mathematical transformation proportional to the concentration of analyte in samples within a given range. It should be established across the range of analytical procedure. Linearity is generally represented as the correlation coefficient, the slope of the regression line, etc. The range is normally expressed in the same unit as test results (e.g. Percentage, ppm, ppb, etc.).

Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogenous sample. The precision of an analytical method is determined by assaying sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimates of standard deviation or related standard deviation.

Accuracy

It is defined as the closeness of agreement between the actual (true) value and mean analytical value obtained by applying a test method number of times. The accuracy of an analytical method is determined by systematic error involved. The accuracy is acceptable if the difference between the true value and mean measured value does not exceed the RSD values obtained for repeatability of the method. The parameter provides information about the recovery of the drug from a sample and effect of matrix, as recoveries are likely to be excessive as well as deficient.

Limit of detection (LOD)

It is the lowest amount of analyte in the sample that can be detected, but hasn't necessarily constituted under the stated experimental conditions. Thus, limit test merely substantiates that the amount of analyte is above or below a certain level. The detection limit is usually expressed as the concentration of analyte (e.g. Percentage, ppm, ppb, etc.) in the sample.

Based on the standard deviation of the response and the quantitation limit may expressed as

$$\text{LOD} = \frac{3.3\sigma}{S}$$

σ = The standard deviation of the response

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

Limit of quantitation (LOQ)

The limit of quantitation is the lowest amount of analyte in the sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. It is usually expressed as the concentration of analyte (e.g. Percentage, ppm, ppb, etc.) in the sample.

Based on the standard deviation of the response and the quantitation limit may expressed as

$$LOQ = \frac{10\sigma}{S}$$

Where

σ = The standard deviation of the response.

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

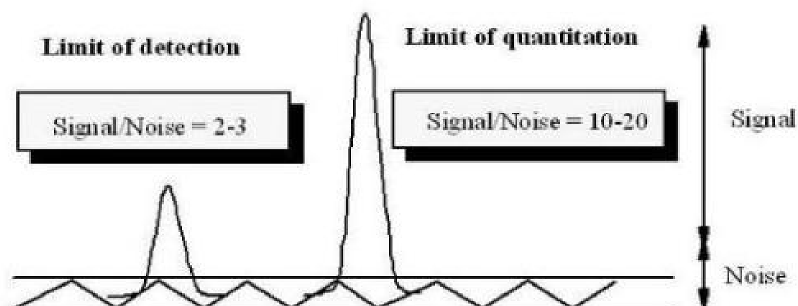


Figure – 8: Representative figure of limit of detection and limit of quantitation via signal to noise

Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days, etc.

Robustness

The robustness of analytical method 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.

Stability test

The term “Stability” can be defined as the product’s ability to maintain its various properties and characteristics within the prescribed limits, from the time of the products manufacture until its self life. One of the main reasons for conducting the stability test is product reflects its efficacy, quality and safety aspects evaluation of product stability gives a rough measure of such aspects.

2. REVIEW OF LITERATURE

Maria-Cristina Ranetti et al., (2009) reported the development and validation of a simple HPLC method for the simultaneous determination of metformin (MTF) and gliclazide (GCZ) in the presence of glibenclamide, in human plasma, for the clinical monitoring of MTF and GCZ after oral administration or for bioequivalence studies. Ion-pair separation followed by UV detection performed on deproteinised plasma samples was chosen for the determination of metformin and gliclazide. The internal standard was glybenclamide. The HPLC method used a Zorbax Eclipse XDB-C18 150x4.6 mm i.d. (5μm) column and analytical guard column 12.5x4.6 mm (5μm), with a gradient elution (1 mL/min) at 40°C column temperature. The mobile phase was acetonitrile: methanol (1:1v/v) and sodium dodecylsulphate 5mM, pH=3.5 with H3PO4 85% and gradient elution. The eluent was monitored at 236 nm. The calibration curve was linear within the range of 0.05-5.00 μg/mL (r2=0.99, n=6). The lowest limit of quantification (LLOQ) was 50 ng/mL for metformin and 49 ng/mL for gliclazide. The proposed method was validated and proved to be adequate for metformin and gliclazide clinical monitoring, bioavailability and bioequivalence studies.[14]

Mubeen and Khalikha Noor et al., (2009) reported a simple and sensitive spectrophotometric method has been developed and validated for the estimation of metformin hydrochloride in bulk and in tablet formulation. The primary amino group of metformin hydrochloride reacts with ninhydrin in alkaline medium to form a violet colour chromogen, which is determined spectrophotometrically at 570 nm. It obeyed Beer's law in the range of 8-18 μg/ml. Percentage recovery of the drug for the proposed method ranged from 97-100% indicating no interference of the tablet excipients. The proposed method was found to be accurate and precise for routine estimation of metformin hydrochloride in bulk and from tablet dosage forms.[15]

Sujana et al., (2010) reported two new UV-spectrophotometric methods have been developed for simultaneous estimation of Pioglitazone hydrochloride and Metformin hydrochloride in tablets. The first method was based on application of vierodt's method which involves the formation and solving of simultaneous equations at

225nm and 237nm, as absorbance maxima of Pioglitazone hydrochloride and Metformin hydrochloride, respectively. The second method employed was absorption correction method which involves direct estimation of pioglitazone hydrochloride at 267 nm, as at this wavelength Metformin hydrochloride has zero absorbance and shows no interference. For estimation of Metformin hydrochloride, corrected absorbance was calculated at 237.0 nm due to the interference of Pioglitazone hydrochloride at this wavelength. Calibration curves were linear with correlation coefficient between 0.999 over the concentration range of 6-14 µg/mL and 1-5 µg/mL for both the drugs. The mean percent recovery was found in the range of 99.41-99.80 and 100.36-100.52 for vierodt's method and 99.18-99.80 and 99.64-99.92 for absorption correction method, for pioglitazone hydrochloride and Metformin hydrochloride, respectively. The results of analysis were validated statistically. The proposed methods are simple, rapid, accurate, precise and economical and can be used successfully in the quality control of pharmaceutical formulations and routine laboratory analysis.[16]

Prachi Saxena et al., (2010) reported a sensitive and accurate UV spectrophotometric method for the determination of metformin hydrochloride in tablet dosage form has been evolved. The various parameters, such as linearity, precision, accuracy, limit of detection and limit of quantitation were studied according to International Conference on Harmonization guidelines (ICH).The method is accurate, precise (% CV=0.763) and linear within the range 1-16 µg/mL with coefficient of correlation, R^2 = 0.9995. The limit of detection and limit of quantitation were found to be 22.34 and 67.69 ng/mL respectively. The proposed method was successfully employed for the quantitative determination of metformin tablet dosage form with no interference from any other excipients and diluents.[17]

Dhabale et al., (2010) reported two simple, accurate, precise, reproducible and economical procedures for simultaneous estimation of Gliclazide and Metformine hydrochloride in tablet dosage form have been developed. First method based on solving of simultaneous equation using 228 nm (λmax of GLZ) and 234 nm (λmax of MET) as two analytical wavelengths for both drugs in mixture of Water and Methanol (60:40) solvent. Second method based on an equation of area calculation of curve at two

wavelength resion (233 to 223nm and 239 to 229 nm).Linearity was observed in the concentration range of 2-24 µg/ml for GLZ and 2-14 µg/ml for MET. The result of analysis have been validated satistically and by recovery study.[18]

Madhukar et al., (2011) reported a simple, specific, accurate and isocratic reversed phase - HPLC method was developed and subsequently validated for the determination of Metformin Hydrochloride. Separation was achieved with an Inertsil - Extend - C18 HPLC column 250mm in length and having an internal diameter of 4.6mm. A mobile phase comprising 10m.mol 1-Octane sulfonic acid: Acetonitrile in the volume ratio of (80:20) was developed. The detection was carried out using a PDA detector set at a wavelength of 232nm. Validation experiments were performed to demonstrate System suitability, specificity, precision, linearity and Range, Accuracy study, stability of analytical solution and robustness. The method was linear over the concentration range of 1-250µg/ml and get the correlation Regration (R^2) 0.9995, showed good recoveries (100.25 - 101.13%), the relative standard deviations of intra and inter-day assay were 99.4% and 99.94% respectively. The method can be used for quality control assay of Metformin Hydrochloride.[19]

Havele and Dhaneshwar S,et al., (2011) reported a simple, rapid, and precise reversed-phase high-performance liquid chromatographic method for simultaneous analysis of metformin hydrochloride, gliclazide, and pioglitazone hydrochloride in a tablet dosage form has been developed and validated. Chromatography was performed on a 25 cm × 4.6 mm i.d., 5µm particle, C18 column with 85:15 (v/v) methanol: 20 mM potassium dihydrogen phosphate buffer as mobile phase at a flow rate of 1.2 ml/min. UV detection at 227 nm; metformin hydrochloride, gliclazide, and pioglitazone hydrochloride were eluted with retention times of 2.15, 3.787, and 4.57 min, respectively. The method was validated in accordance with ICH guidelines. Validation revealed the method is specific, rapid, accurate, precise, reliable, and reproducible. Calibration plots were linear over the concentration ranges 50– 250 mg/ml for metformin hydrochloride, 3.0 –15.0 mg/ml for gliclazide, and 2–10 mg/ml for pioglitazone hydrochloride. Limits of detection were 0.20, 0.04, and 0.10 mg/ml and limits of quantification were 0.75, 0.18, and 0.30 mg/ml for metformin hydrochloride, gliclazide, and pioglitazone hydrochloride,

respectively. The high recovery and low coefficients of variation confirm the suitability of the method for simultaneous analysis of the three drugs in tablets. Statistical analysis proves that the method is suitable for the analysis of metformin hydrochloride, gliclazide, and pioglitazone hydrochloride as a bulk drug and in pharmaceutical formulation without any interference from the excipients. It may be extended to study the degradation kinetics of three drugs and also for its estimation in plasma and other biological fluids.[20]

Ramzia I. El-Bagary et al., (2012), In this work, a reversed-phase liquid chromatographic (RP-LC) method has been developed for the determination of alogliptin (ALG) based on isocratic elution using a mobile phase consisting of potassium dihydrogen phosphate buffer pH (4.6) - acetonitrile (20:80, *v/v*) at a flow rate of 1 mL min^{-1} with UV detection at 215 nm. Chromatographic separation was achieved on a Symmetry® cyanide column (150 mm × 4.6 mm, 5 μm). Linearity, accuracy and precision were found to be acceptable over the concentration range of 5-160 μg mL^{-1} for ALG in bulk. The optimized method was validated and proved to be specific, robust and accurate for the quality control of ALG in pharmaceutical preparations.[21]

Amruta B. Loni ea al., (2012) reported two simple, precise and economical UV methods have been developed for the simultaneous estimation of Sitagliptin phosphate and Metformin hydrochloride in bulk and pharmaceutical dosage form. Method A is Absorbance maxima method, which is based on measurement of absorption at maximum wavelength of 266 nm and 232 nm for Sitagliptin phosphate and Metformin hydrochloride respectively. Method B is area under curve (AUC), in the wavelength range of 244-279 nm for Sitagliptin phosphate and 222-240 nm for Metformin hydrochloride. Linearity for detector response was observed in the concentration range of 25-225μg/ml for Sitagliptin phosphate and 2-12 μg/ml for Metformin hydrochloride. The accuracy of the methods was assessed by recovery studies and was found to be 99.64 % and 98.98% for Sitagliptin phosphate and Metformin hydrochloride. The developed method was validated with respect to linearity, accuracy (recovery), precision and specificity. The results were validated statistically as per ICH Q2 R1 guideline and were found to be satisfactory. The proposed methods were successfully applied for the

determination of for Sitagliptin phosphate and Metformin hydrochloride in commercial pharmaceutical dosage form.[22]

Joshi et al., (2012) reported a simple, rapid, precise, and accurate, stability-indicating reversed phasehigh performance liquid chromatographic method was developed and validated for si-multaneous determination of metformin HCl and repaglinide. The chromatographicseparation was achieved on YMC Pack AM ODS (5μm, 250 mm length × 4.6 mm i.d.)column at a detector wavelength of 210 nm, using an isocratic mobile phase consisting ofmethanol and 10 mM potassium dihydrogen phosphate buffer (pH 2.5) in a ratio of 70:30v/vat a flow rate of 1 mL min^{-1}. The retention times for metformin and repaglinide werefound to be 2.6 and 11.3 min, respectively. The drugs were exposed to thermal, photo-lytic, hydrolytic, and oxidative stress conditions, and the stressed samples were ana-lyzed by the proposed method. Validation of the method was carried out as per Interna-tional Conference on Harmonization (ICH) guidelines. Linearity was established formetformin and repaglinide in the range of 5–200μg $mL^{-1}$and 1–200μg mL^{-1}, respec-tively. The limits of detection were 0.3μg mL−1and 0.13μg mL−1for metformin and re-paglinide, respectively. The method was found to be specific and stability-indicating asno interfering peaks of degradants and excipients were observed. The proposed methodis hence suitable for application in quality-control laboratories for quantitative analysisof both the drugs individually and in combination, since it is simple and rapid with good accuracy and precision.[23]

Usharani Gundala et al., (2013) reported a simple, accurate, precise and reproducible method has been developed for the simultaneous estimation of Vildagliptin and Metformin hydrochloride in combined tablet dosage forms. As there are no reported UV methods for the simultaneous estimation of Vildagliptin and Metformin hydrochloride in their combined dosage form, a need was felt to develop new methods to analyze the drugs simultaneously. The estimation was done by multi-wavelength technique, at wavelengths of 217 nm and 234 nm over the concentration ranges of 0.7μg/ml and 7 μg/ml with mean recovery 100% for both drugs Vildagliptin and Metformin hydrochloride respectively. The results of the analysis were validated

statistically and recovery studies were carried out as per ICH guide lines. Thus the proposed method can be successfully applied for the simultaneous estimation of Vildagliptin and Metformin hydrochloride in routine analysis work.[24]

Sujani et al., (2013) reported a simple, sensitive, rapid, economic, UV Spectroscopic method was developed for the estimation of Sitagliptin-Metformin in Pure and Tablet dosage forms. The linearity for Metformin was found between 200-400 μ/ml and between 20-45 μg/ml for Sitagliptin. Metformin showed the maximum absorbance at 232nm & Sitagliptin at 266nm and Validation parameters like Precision, Accuracy, and System suitability parameters were determined and examined by applying validated parameters. [25]

Himal Paudel Chhetri et al., (2013) reported the optimization of a simple HPLC-UV method for the determination of metformin in human plasma. Ion pair separation followed by UV detection was performed on deproteinized human plasma samples. The separation was carried out on a Discovery Reversed Phase C-18 column (250 X 4.6 mm, 5μm) with UV detection at 233 nm. The mobile phase contained 34% acetonitrile and 66% aqueous phase. Aqueous phase contained 10 mM KH_2PO_4 and 10 mM sodium lauryl sulfate. Aqueous phase pH was adjusted to 5.2. The mobile phase was run isocraticcally. The flow rate of the mobile phase was maintained at 1.3 ml/min. The linearity of the calibration curve was obtained in the concentration range of 0.125–2.5 μg/ml and coefficient of determination (R^2) was found to be 0.9951. The lowest limit of quantification and detection was 125 and 62 ng/ml respectively. No endogenous substances were found to interfere with the peaks of drug and internal standard. The intra-day and inter-day coefficient of variations was 6.97% or less for all the selected concentrations. The relative errors at all the studied concentrations were 5.60% or less. This method is time efficient and samples are easy to prepare with minimum dilution. So, it can be applied for monitoring metformin in human plasma. [26]

3. RESEARCH ENVISAGED

The number of drugs and drug formulations are introduced into the market has been increasing at an alarming rate. These drugs or formulations may be either new entities in the market or partial structural modifications of the existing drug or novel dosage forms.

Standard analytical procedures for the estimation of these drugs are most necessary for the development of newer analytical methods which are more accurate, precise, specific, linear, simple and rapid. The modern method of choice of analysis is HPLC, which requires highly sophisticated equipment, trained personnel, high purity chemicals and proper maintenance. Pharmaceutical industry's main role in quantitative chemical analysis to ensure that the raw materials used and the final product obtained meets the required specifications or not.

3.1. Aim

The aim of the present work was to develop and validate a new simple, precise, accurate, and reliable RP-HPLC method for the simultaneous estimation of Alogliptin and Metformin HCl bulk and Pharmaceutical dosage form.

3.2. Objective

The literature survey reveals that there are several methods reported for the estimation of Alogliptin and Metformin HCl alone or in combination with other drugs in their pharmaceutical dosage forms but none of the method available for the estimation of these drugs in the selected pharmaceutical dosage form.

In the analysis of formulations containing two or more drugs, one drug can interfere in the estimation of another drug. To avoid this, separation of component mixture by extraction is usually carried out which make the procedure time consuming and complicated and often lacks accuracy. Therefore, it was thought worthwhile to

develop such a method of analysis, which can estimate both the drugs in combination without prior separation.

Hence on the basis of the literature survey, it was thought to develop a precise, accurate, simple and reliable method for estimation of drug in tablets using the following technique of RP HPLC method for Alogliptin and Metformin HCl

3.3. Plan of work

- Literature collection.
- Study of physicochemical properties of drug (pH, pKa and solubility).
- Procurement of chemicals and API.
- Selection of suitable solvent.
- Selection of suitable wavelength.
- Development of RP-HPLC method for the quantification of Alogliptin benzoate and Metformin HCl in bulk and pharmaceutical dosage form.
- Validation of proposed method as per ICH Q2 (R1) guidelines.
- Estimate the amount of Alogliptin benzoate and Metformin HCl in bulk and pharmaceutical dosage form.

4. DRUG PROFILE

4.1. Alogliptin benzoate [38-43]

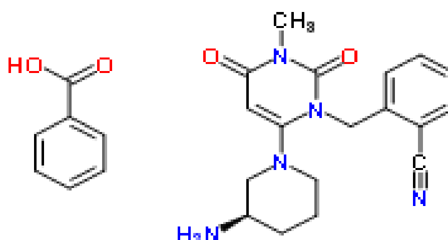
Synonyms:

2-[[6-[(3R) -3-Amino-1-piperidinyl] -3,4-dihydro-3-methyl-2,4-dioxo-1 (2H) -pyrimidinyl] methyl] benzonitrile.

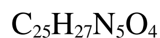
IUPAC Name:

2-({6-[(3R) -3-aminopiperidin-1-yl] -3-methyl-2,4-dioxo-3,4- dihydropyrimidin-1 (2H) - yl} methyl) benzonitrile.

Structural Formula:



Chemical Formula:


$C_{25}H_{27}N_5O_4$

Molecular Mass:

461.513

Description:

Alogliptin benzoate is a white to off-white, crystalline powder containing one asymmetric carbon in the aminopiperidine moiety.

Solubility:

It is soluble in dimethylsulfoxide, sparingly soluble in water and methanol, slightly soluble in ethanol, and very slightly soluble in octanol and isopropyl acetate.

Melting point:

127-129°C

Category:

Dipeptidyl peptidase 4 (DPP-4) Inhibitor

Storage:

Store it at room temperature between (20°C to 25°C).

Mechanism of Action:

Alogliptin inhibits dipeptidyl peptidase 4 (DPP-4), which normally degrades the incretins glucose-dependent insulinotropic polypeptide (GIP) and glucagon like peptide 1 (GLP-1). The inhibition of DPP-4 increases the amount of active plasma increments, which helps with glycemic control. GIP and GLP-1 stimulate glucose dependent secretion of insulin in pancreatic beta cells. GLP-1 has the additional effects of suppressing glucose dependent glucagon secretion, inducing satiety, reducing food intake, and reducing gastric emptying.

Side Effects:

Alogliptin is an anti-diabetic drug used as an adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes mellitus. It should not be used in patients with type 1 diabetes mellitus or for the treatment of diabetic ketoacidosis. Common side effects include sore throat, sinus infection, or headache.

Use:

Oral antihyperglycemic drugs *used* to treat type 2 diabetes.

Dose:

12.5 mg.

Brand name:

Kazano.

4.2. Metformin HCl [27-37]

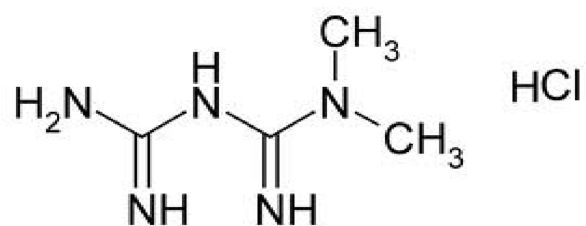
Synonyms:

1,1-Dimethylbiguanide hydrochloride, N,N-Dimethylimidodicarbonimidic Diamide Hydrochloride; Diabetosan; Diabex; Glucophage; Metiguanide; Glucoform; Glucomet; Glucomin; Glucomine; Orabet; Siamformet; Walaphage.

IUPAC Name:

1-carbamimidamido- N, N-dimethyl methanimidamide

Structural Formula:



Chemical Formula:

C4H11N5•HCl

Molecular Mass:

165.63.

Description:

Metformin hydrochloride is a white to off-white crystalline powder.

Solubility:

Metformin hydrochloride is freely soluble in water and is practically insoluble in acetone, ether, and chloroform.

Melting point:

222 °C to 226 °C.

Category:

Non-insulin-dependent diabetes mellitus (NIDDM)

Storage:

2-8°C

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 signaling, 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.

Side Effects:

Headache, dizziness, nervousness, depression, drowsiness, insomnia, vertigo and ringing in the ear, Chest pain, high blood pressure and fluid retention

Use:

Oral antihyperglycemic drugs used to treat type 2 diabetes.

Dose:

500mg.

Brand name:

Kazano.

5. MATERIALS AND METHODS

5.1. Equipments used:

Table - 3: Equipment

Name	Make/Model
Analytical Balance	Elico.
P^H meter	Metler Toledo.
Ultra sonicator	Bandelin.
HPLC instrument	Shimadzu, LC-10AT-vp Solvent delivery system (pump),
Detector	Shimadzu, SPDM – 10AVP photodiode array detector.
Column Phenomenex C 18 column (150x4.6mm, 5µm).	
UV-VIS Spectrophotometer	Schimadzu, SPD-2350A; LC solution.

5.2. Drugs and chemicals used:

5.2.1. Drug sample:

Table - 4: Drug samples

S.No	Drug Samples	Manufacturer/Supplier
1	ALogliptin Benzoate Jinlan Pharm-Drugs Technology co.,	

	Ltd.,	
2	ALogliptin	AN therapeutics
3	Kazano	Purchased from the Market

5.2.2. Reagents and chemicals:

Table - 5: Reagents and Chemicals

S. No	NAME	MAKE/GRADE
1	Methanol	Merck (AR-Grade)
2	Acetonitrile	Merck (AR-Grade)
3	Water	Milli – Q -Water
4	Di – Sodium Hydrogen ortho Phosphate (AR Grade)	S.D fine chemical Pvt Ltd.,
5	Ortho phosphoric acid (AR Grade)	S.D fine chemical Pvt Ltd.,

5.3. Methodology

5.3.1. Method development

5.3.1.1. Selection of wavelength (λ_{max})

The sensitivity of the HPLC method that uses PDA detection depends upon the proper selection of the wavelength. An ideal wavelength is one that gives good response for the drugs to be detected.

ALogliptin and Metformin HCL in Methanol, were scanned in the UV-Visible range of 200 to 400 nm against blank separately (Figure 9). Then suitable wavelength for the detection of ALogliptin and Metformin HCl was selected at 249nm by overlapping the spectrum of both the drugs.

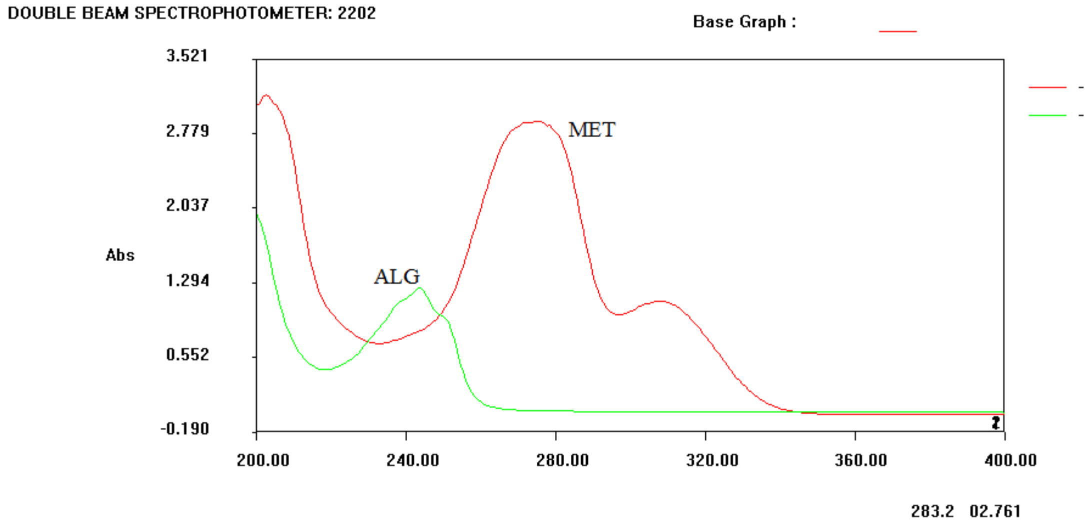


Figure - 9: UV spectrum of Alogliptin Benzoate and Metformin HCl

5.3.1.2. Trials for RP- HPLC method development

Trial – 1

Chromatographic conditions

HPLC	:	Shimadzu,LC-10AT-vp Solvent delivery system.
Column	:	Phenomene x C 18 column (150x4.6mm, 5µm).
Flow Rate	:	1.0 ml/min
PDA Detection	:	249 nm
Injection Volume	:	20 µL
Temperature	:	25°C
Run Time	:	20 min.
Mobile phase	:	Methanol: Water (50:50)
Diluent	:	Mobile phase.

Result:

More noise peaks are observed and drug is not eluted **(Chromatogram no. 2)**.

Trial – 2

Chromatographic conditions

HPLC	:	Shimadzu, LC-10AT-vp Solvent delivery system.
Column	:	Phenomene x C 18 column (150x4.6mm, 5µm).
Flow Rate	:	1.0 ml/min.
PDA Detection	:	249 nm.
Injection Volume	:	20 µL
Temperature	:	25°C.
Run Time	:	20 min.
Mobile phase	:	Methanol: Water (60:40).
Diluent	:	Mobile phase.

Result:

More noise peaks are observed and eluted peaks are broad in shape (Chromatogram no. 2).

Trial – 3

Chromatographic conditions

HPLC	:	Shimadzu, LC-10AT-vp Solvent delivery system.
Column	:	Phenomene x C 18 column (150x4.6mm, 5µm).
Flow Rate	:	1.0 ml/min.
PDA Detection	:	249 nm.
Injection Volume	:	20 µL
Temperature	:	25°C.
Run Time	:	20 min.
Mobile phase	:	Buffer: Methanol (50:50); pH 4.5.
Diluent	:	Mobile phase.

Result:

ALogliptin and Metformin HCL peaks are elated, but peak shape is not good, and failed in theoretical plates, Tailing factors and also Retention time of both peaks are more than 7 minutes (**Chromatogram no. 3**).

Trial – 4

Chromatographic conditions

HPLC	:	Shimadzu, LC-10AT-vp Solvent delivery system.
Column	:	Phenomene x C 18 column (150x4.6mm, 5µm).
Flow Rate	:	1.0 ml/min.
PDA Detection	:	249 nm.
Injection Volume	:	20 µL.
Temperature	:	25°C.
Run Time	:	20 min.
Mobile phase	:	Buffer : methanol (40:60); pH- 4.
Diluent	:	Mobile phase.

Result:

ALogliptin and Metformin HCL peaks are elated, but failed in tailing factor and also retention time both peaks are being more (Chromatogram no. 4).

Trial – 5

Chromatographic conditions

HPLC	:	Shimadzu, LC-10AT-vp Solvent delivery system.
Column	:	Phenomene x C 18 column (150x4.6mm, 5µm).
Flow Rate	:	1.0 ml/min.
PDA Detection	:	249 nm.
Injection Volume	:	20 µL.
Temperature	:	25ºC.
Run Time	:	10 min.
Mobile phase	:	Buffer : Methanol and (35:65); pH 3.5.
Diluent	:	Mobile phase.

Result:

Peaks are elated nicely and passed in all the efficiency parameters, but sharp peaks will not be appearing (Chromatogram no. 5).

Trial – 6

Chromatographic conditions

HPLC	:	Shimadzu, LC-10AT-vp Solvent delivery system.
Column	:	Phenomene x C 18 column (150x4.6mm, 5µm).
Flow Rate	:	1.2 ml/min.
PDA Detection	:	249 nm.
Injection Volume	:	20 µL.
Temperature	:	25ºC.
Run Time	:	4 min.
Mobile phase	:	Buffer: ACN:Methanol (30:5:650), pH- 3.5.
Diluent	:	Mobile phase.

Result:

Metformin and ALogliptin are eluted at 2.078 and 3.238 respectively, efficiency parameters are indicate the good separation, asymmetric. So this method was selected for further analysis (Chromatogram no. 6).

5.4. Validation of developed method:

The developed method was validated according to ICH [11-16] guidelines.

5.5. Preparation of solutions

5.5.1. Preparation of phosphate buffer solution

4.2568 gm of di-sodium hydrogen orthophosphate was weighed and sufficient water (HPLC grade) was added to dissolve it. Then sonicate for 10 min. Then 1ml of tri ethanol amine was added, the final volume was made up to 1000ml with water and adjusted the pH to 3.5 with ortho phosphoric acid.

5.5.2. Preparation of mobile phase:

Methanol, Buffer and Acetonitrile were mixed in the ratio of 65:30:5 and sonicated for 20minutes, Filtered with 0.45 µ membrane filter.

5.5.3. Preparations of working standard solution:

500mg of Metformin HCL and 12.5 mg of Alogliptin were accurately weighed and transferred in to a separate 50 ml volumetric flask and sufficient mobile phase was added to dissolve the drug. The final volume was made up to 50 ml with mobile phase

(primary stock solution). Pipette out 2ml from the above stock solution into a 50ml volumetric flask and the final volume was made up to the mark with the mobile phase.

5.5.4. Preparation of Sample solution

20 tablets were weighed and powdered, tablets powder equivalent to 500mg of Metformin HCL and 12.5mg of ALogliptin was transferred in to a 50 ml volumetric flask, sufficient amount of mobile phase was added and dissolved by 20 minutes ultrasonication. Then made the volume up to the mark with the mobile phase and filtered with 0.45 µ filter paper. Pipette out 2 ml from the above solution and diluted to 50ml with the mobile phase.

The amount of Metformin HCl and Alogliptin present in each tablet were calculated by using the following formula:

$$\text{Amount present} = \frac{\text{Sample Area}}{\text{Standard Area}} \text{ X } \frac{\text{Standard weight}}{\text{Standard Dilution}} \text{ X } \frac{\text{Sample Dil}}{\text{Sample weight}} \text{ X Average weight}$$

$$\text{\% Amount present} = \frac{\text{Amount present}}{\text{Label Claim}} \text{ X } 100$$

6. Results and Discussion

6.1. Validation of developed method:

The developed method was validated according to ICH [13] guidelines. The method was validated in terms of specificity, system suitability, linearity, precision, accuracy, robustness, LOD and LOQ.

6.1.2. Specificity:

The specificity of the method was established by injecting the blank and standard solution. It is observed that there is no interference of the blank (Chromatogram no. 7) with principal peaks (Chromatogram no. 8), hence the method was specifically for these two drugs.

6.1.2. System suitability:

The working standard solution was injected 5 times into the HPLC, chromatograms were recorded and measured the responses for the major peaks. System suitability parameters such as retention time, theoretical plates and asymmetric factor. Then the percentage of RSD of all the parameters were calculated and presented in the table 6 the percentage RSD.

Table – 6: System suitability parameters

	MET Area	ALG Area	MET Theoretical plates	ALG Theoretical plates	MET Tailing factor	ALG Tailing factor
	4208745	400587	3568.305	4836.127	1.276	1.105
	4208746	396574	3586.231	4836.241	1.285	1.118
	4198754	398567	3528.97	4863.727	1.256	1.126
	4184764	397854	3594.212	4758.963	1.274	1.113
	4207841	399852	3567.422	4698.521	1.293	1.133
AVG	4201770	398686.8	3569.028	4798.716	1.2768	1.119
SD	10397.99	1592.19	25.1889	68.2888	0.01388	0.0109
% RSD	0.247467	0.3993	0.7057	1.4230	1.0872	0.9769

6.1.3. Linearity:

Appropriate volume from the stock solution was diluted to get the final concentration of 200, 300, 400, 500, 600 µg/mL for Metformin HCl and 5, 7.5, 10, 12.5, 15 µg/mL for Alogliptin. Then the chromatogram was recorded (Chromatogram no. 9-13) for each concentration, plot the graph concentration versus area. The calibration graph was shown in figure -10 and 11.

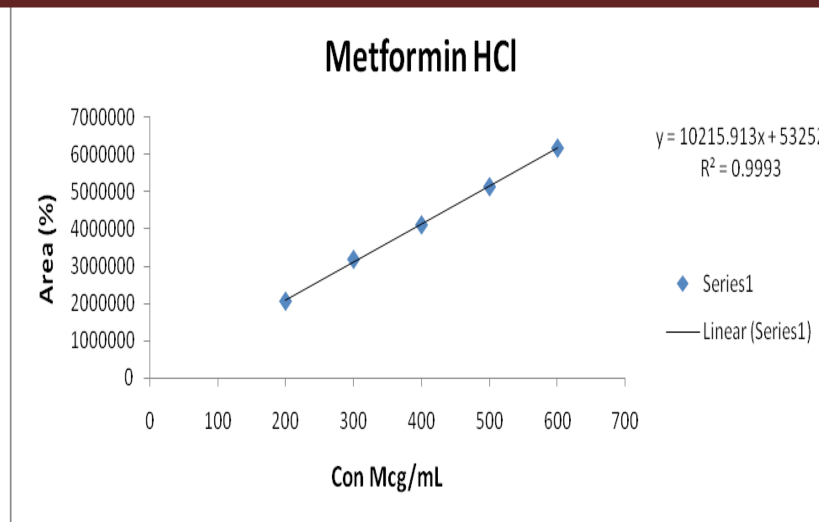


Figure - 10: Calibration graph of Metformin HCl

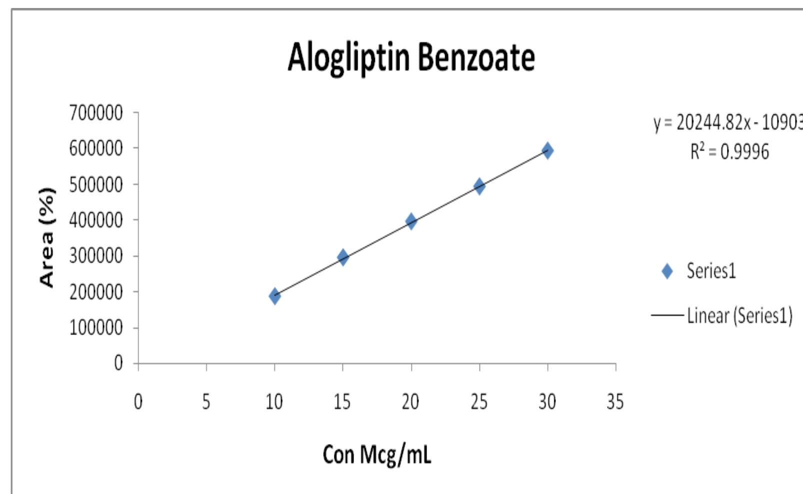


Figure- 11: Calibration graph of Alogliptin

From the linearity study, Metformin HCl and ALogliptin were found to be linear in the concentration range from 200 - 600 µg/ml and 5 - 15 µg/ml. Correlation coefficient values (R^2) were found to be 0.9993 and 0.9996 for Metformin HCl and Alogliptin respectively. Results revealed that good correlation exists between the concentration of the sample and their area.

6.1.4. Precision:

Precision study was carried out by injecting a sample solution in to HPLC without changing the assay procedure and the results are shown the % RSD is less than 2 % for metformin HCl and Alogliptin. The low RSD value indicated that the method was precise.

6.1.4.1. Repeatability:

This study was performed with a minimum of three replicate measurements of sample solution at 0 hrs, 8 hrs and 16 hrs in a same day.

6.1.4.2. Intermediate precision:

Intermediate precision was performed by injecting the sample solution in to HPLC at three different days, different analysts and in different instruments.

6.1.4.3. Reproducibility:

Reproducibility studies were done in two laboratories and the results were compared.

All the precision study results presented in the table 7 and chromatogram of this study show chromatogram no 14 - 21.

Table - 7: Precision results for MET and ALG

Parameters	Sampling time	MET			ALG		
		Amount present (mg)	Amount present (%)	RSD (%)	Amount present (mg)	Amount present (%)	RSD %
Repeatability	0 hrs	495.11	99.02	0.0920	12.62	100.97	1.4542
	8th hrs	499.69	99.93	0.9449	12.37	100.62	0.5498
	16th hrs	503.98	100.79	0.3633	12.60	100.83	0.7566
Intermediate precision	I st Day	504.63	100.92	0.4993	12.55	100.42	0.7712
	2nd day	503.59	100.71	0.3197	12.63	101.06	0.6141
	3rd day	497.53	99.50	0.1257	12.70	101.64	0.1250
	Analyst -1	502.26	100.45	0.1907	12.63	101.07	0.8081
	Analyst -2	504.35	100.87	0.1197	12.61	100.94	0.6498
	Instrument -1	501.00	100.20	0.7276	12.66	101.30	0.1559
	Instrument -2	504.86	100.97	0.1219	12.61	100.94	0.4287

6.1.5. Accuracy:

This study was performed using a minimum of 3 concentration levels, each in triplicate determinations (Chromatogram no. 22-25).

75, 100 and 125% from the label claim of metformin HCl and Alogliptin benzoate was taken in to a 50 ml volumetric flask and sufficient mobile phase was added, sonicated 20 min for dissolving the drugs, final volume was adjusted up to the mark with the mobile phase. Pipette out 2 ml from the above solution into a 100 ml volumetric flask and final volume was adjusted up to the mark with the mobile phase. Then chromatogram (Chromatogram no. 14) was recoded in triplicate and the results are shown in table – 8.

Table - 8: Recovery results for MET and ALG

Concentration (%)	Added AMT amount (mg)		Amt recovered (mg)		Amt recovered (%)	
MET / ALG	MET	ALG	MET	ALG	MET	ALG
75	375	9.375	374.65	9.394	99.90	100.20
100	500	12.5	495.17	12.649	99.03	101.19
125	625	15.625	621.83	15.487	99.49	99.11

The percentage recoveries of the three concentrations (75 %, 100% and 125%) were found to be close to 100%, indicative of high accuracy of this method.

6.1.6. Robustness

Robustness of the method were determined by changing the method parameters (wavelength ± 1nm from 1nm, Flow rate ± 0.1 (Chromatogram no. 26,27), pH ± 0.05 and the mobile phase ratio ± 2 %), the results were presented in Table 11. Based on the results of these studies show, the small changes made to the method procedure, but it will not affect the method results so this method is robust.

Table - 11: Results observed by changing the wavelength ± 1nm

		MET			ALG		
Parameters		Amount present (mg)	Amount present (%)	RSD %	Amount present (mg)	Amount Present (%)	RSD %
Wavelength (nm)	248	493.04	98.60	0.1139	12.64	101.16	0.0549
	250	505.57	101.11	0.1237	12.63	101.11	0.0504
Flow Rate (mL/min)	1.3	502.87	100.57	0.3725	12.61	100.94	0.4278
	1.1	502.90	100.58	0.7906	12.65	101.23	0.0153
Mobile phase (% of (Methanol)	67	502.99	100.59	0.3907	12.65	101.27	0.1750
	63	504.86	100.97	0.09942	12.58	100.66	0.3853
pH	3.55	498.76	99.75	1.1828	12.64	101.18	0.0634
	3.45	500.30	100.06	1.3808	12.63	101.08	0.0801

6.1.7. Limit of Detection (LOD) and Limit of Quantification (LOQ):

The LOD and LOQ of the developed method were determined by analyzing progressively low concentration of the standard solution using the developed methods. The LOD is the concentration of the analyte that gives a measurable response (signal to noise ratio 3.3). The LOQ is the lowest concentration of the analyte, which gives a response that can be accurately quantified (signal to noise ratio of 10). LOD and LOQ of Metformin HCl and Alogliptin was found to be 359.561 ng/mL, 84.037 ng/mL and 1.08958 ng/ml, 254.658 ng/m (Table 12).

Table – 12: LOD and LOQ

	MET	ALG
	2056745	188634
	2057246	187858
	2058874	187658
SD	1113.106	515.5502
Slope	10215.91	20244.82
LOD (μg/mL)	0.359561	0.084037
LOQ (μg/mL)	1.08958	0.254658

6.1.8. Stability Studies:

Stability of drug solution was determined by keeping the drug solution for 3 days at room temperature. The chromatogram was recorded by injecting the sample solution at once per day and calculated the amount of drug present. There is no significant degradation was observed. The stability test results (Table 13) are indicating the drug solutions are stable up to 3 days at room temperature.

Table – 13: Stability studies

	MET		**ALG**	
Parameters	**Amount present (mg)**	**Amount present (%)**	**Amount present (mg)**	**Amount Present (%)**

Day 1	504	98.60	12.65	101.25
Day 2	504.38	100.87	12.64	101.17
Day 3	503.88	100.77	12.64	101.16

6.1.9. Apply the developed and validated method for the estimation of Metformin HCl and Alogliptin in marketed tablet dosage form:

Sample solution was prepared according to the above described procedure. Then the solution was injected in to the HPLC and calculated the amount of Metformin HCl, Alogliptin present in the each tablet by using the above mentioned formula, results are present in the table – 14, 15 and Chromatogram no 28 - 30.

Table – 14: Assay results for MET

SAM Area	STD Area	Amt present	% Amt present
4257964	4201770	501.7721	100.3544
4287561	4201770	505.2599	101.0517
4287956	4201770	505.3064	101.0612

4281863	4201770	504.5884	100.9176
4178293	4201770	492.3834	98.4766
4187956	4201770	493.5221	98.7044
	AVG	500.4721	100.0944108
	SD	5.977577	1.195515343
	% RSD	1.194388	1.194387712

Table – 14: Assay results for ALG

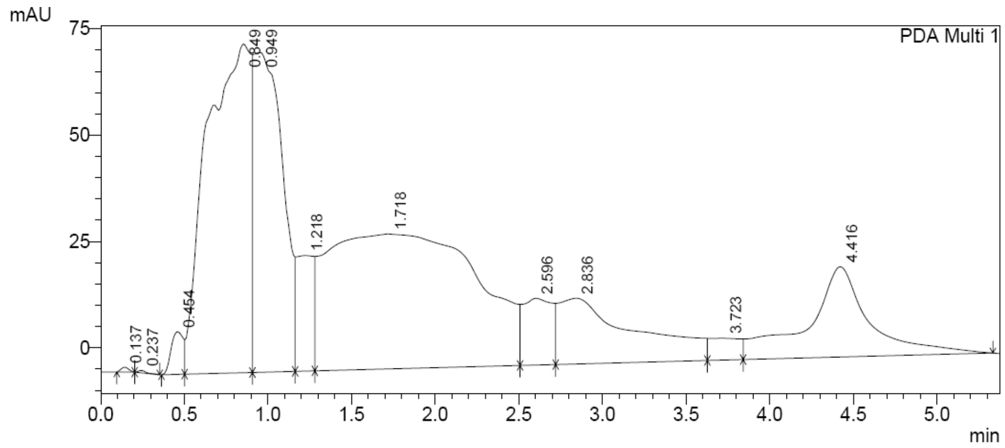
SAM Area	STD Area	Amt present	% Amt present
403156	398687	12.54026	100.3220
406321	398687	12.63871	101.1096
403652	398687	12.55569	100.4454
406328	398687	12.63893	101.1114

401357	398687	12.4843	99.8744
398741	398687	12.40293	99.2234
	AVG	12.54347	100.3477463
	SD	0.091207	0.729659142
	% RSD	0.727131	0.727130573

The developed RP-HPLC method for simultaneous estimation of Metformin Hcl and Alogliptin benzoate in combined dosage from through the C18 column and Methanol: 30 mM of Di – sodium hydrogen ortho phosphate as mobile phase. Detection of eluent carried out using a UV detector at 249 nm. The run time per sample is just 4 min. The excipients in the formulation did not interfere in the accurate estimation of metformin HCL and ALogliptin benzoate. The method was validated as per ICH guidelines in terms of linearity, accuracy, specificity, precision, and robustness. Since this developed method can be used for routinely for the analysis of these drugs.

7. CHROMATOGRAMS

CHROMATOGRAM NO 1: TRAIL -1



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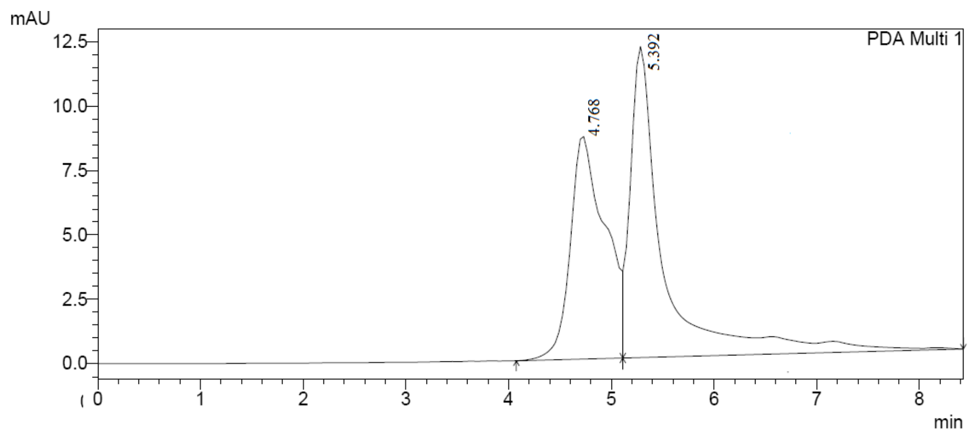

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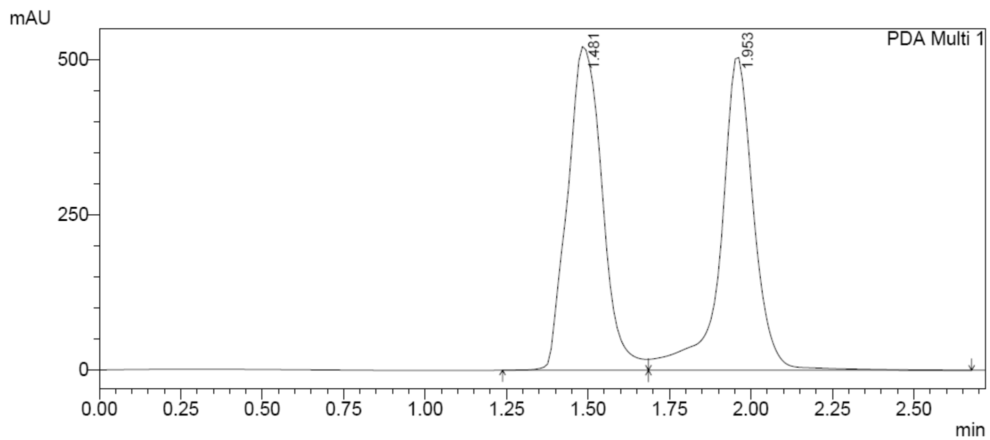

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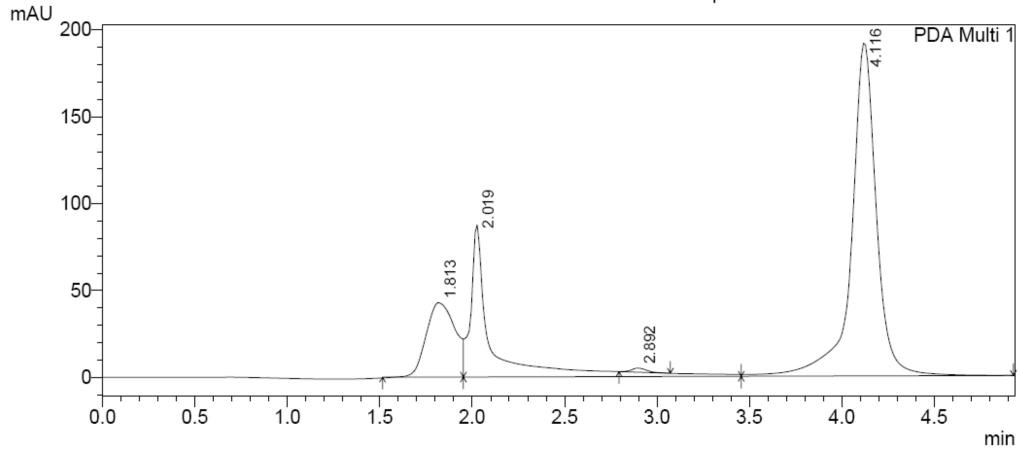

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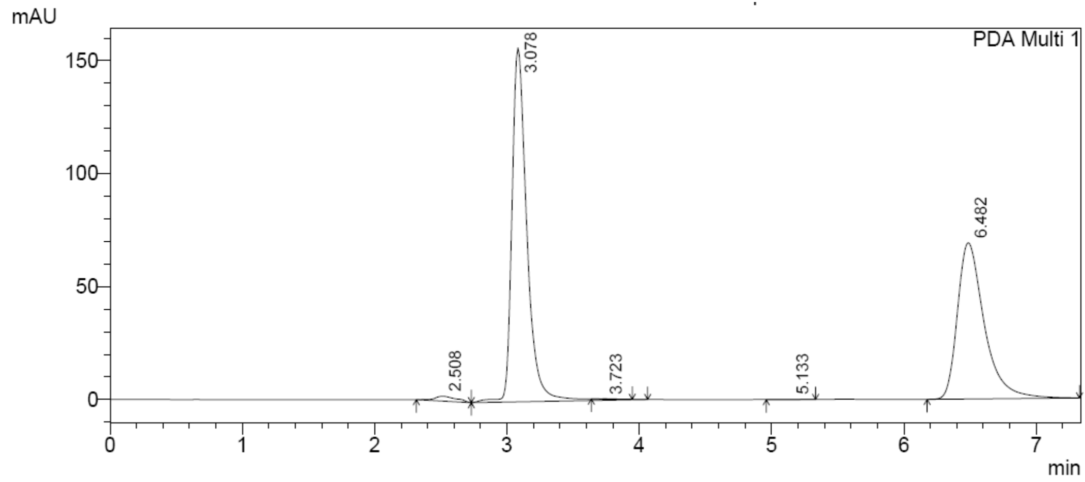

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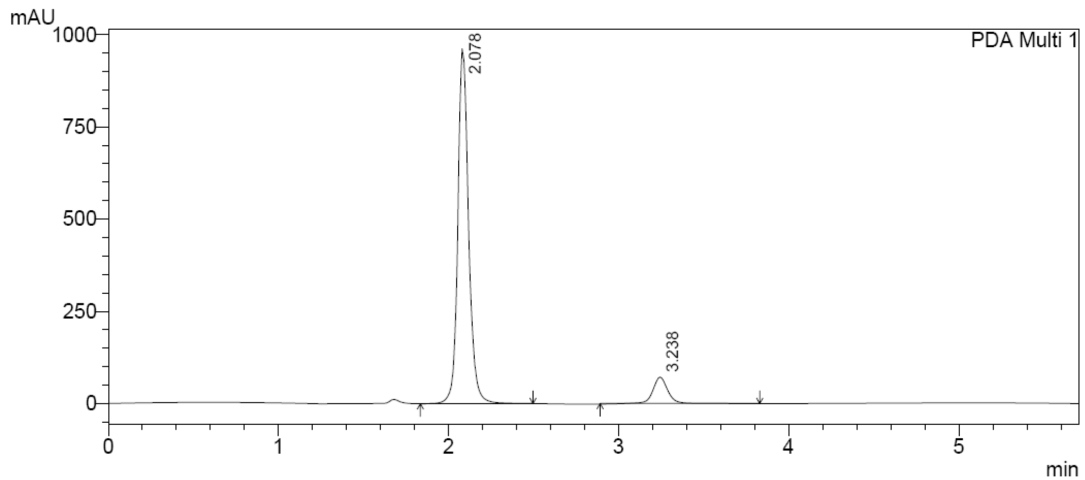

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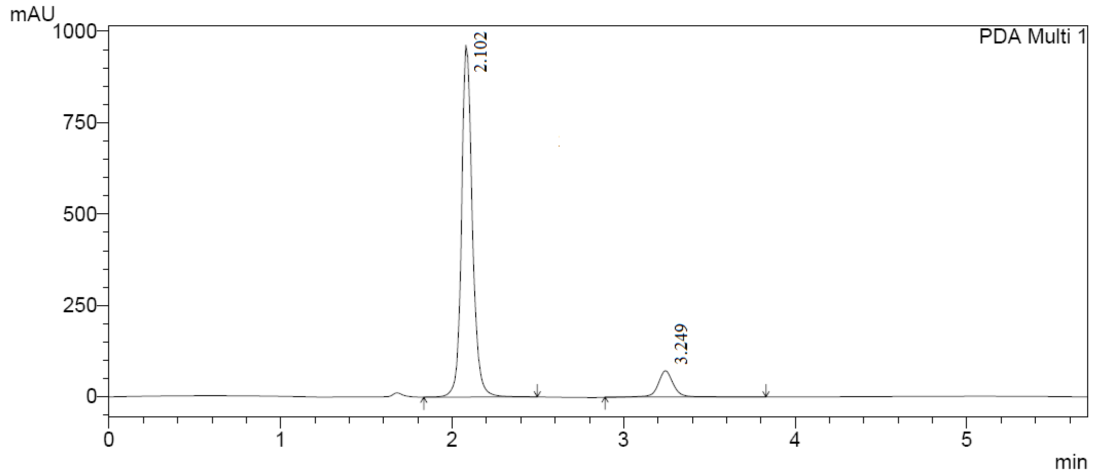

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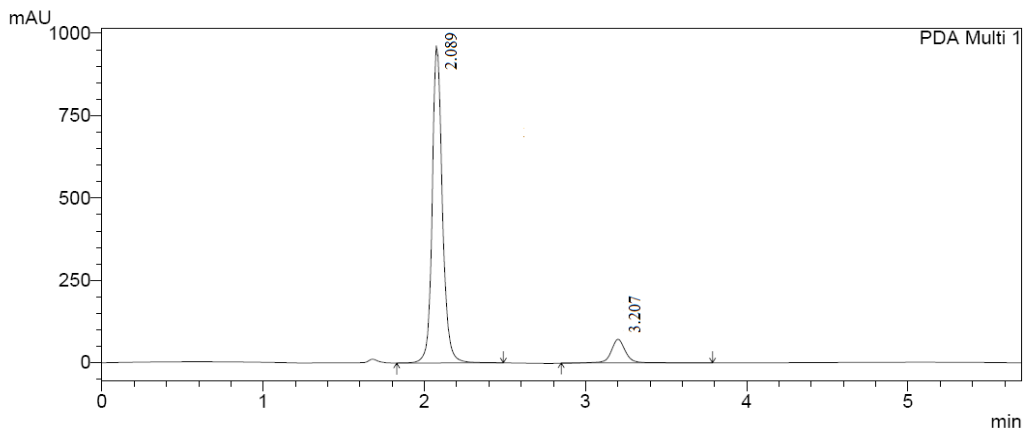

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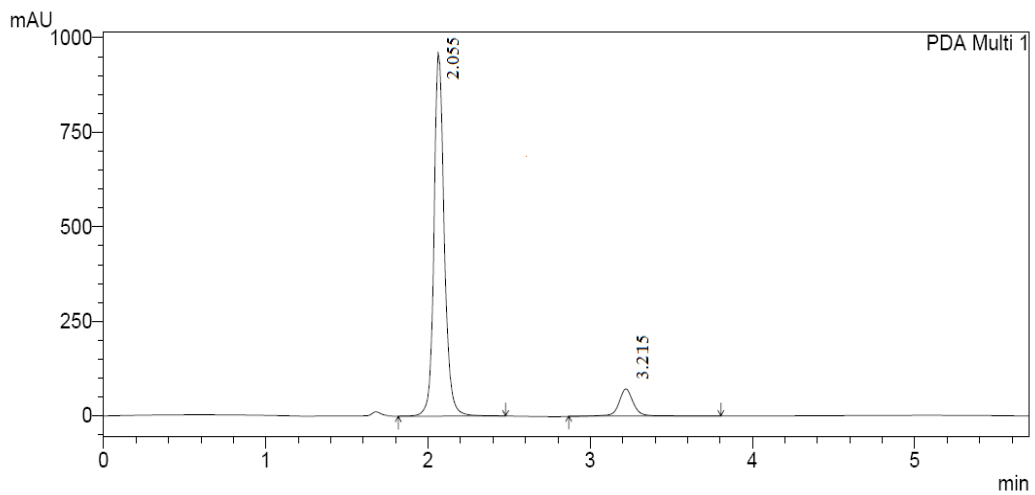

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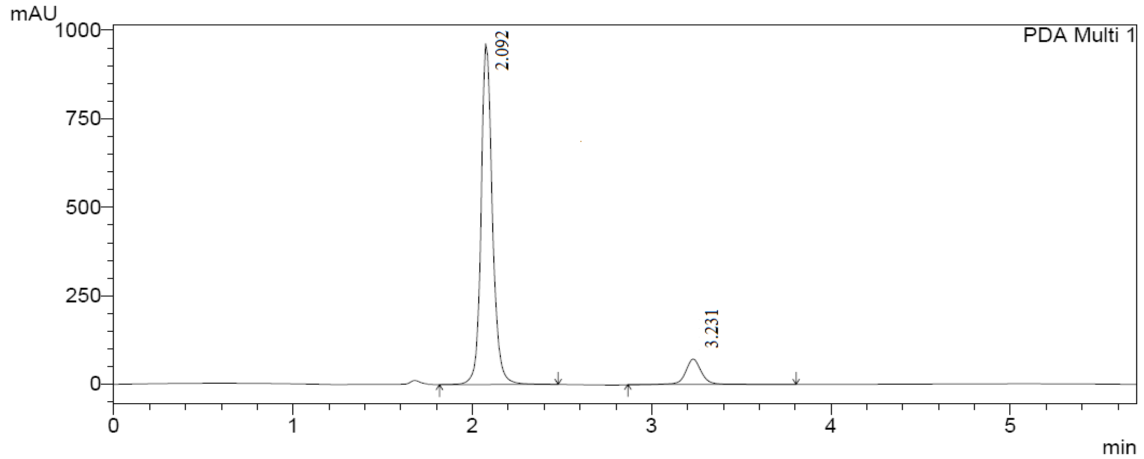

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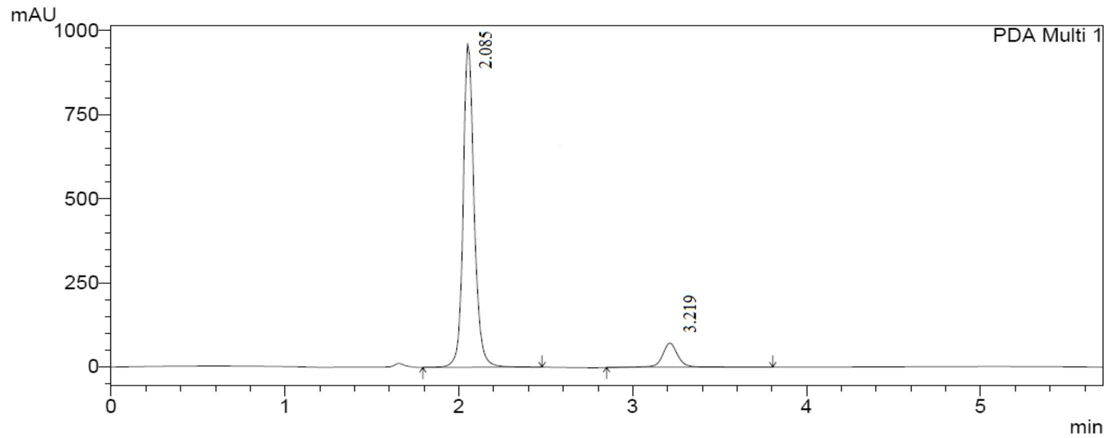
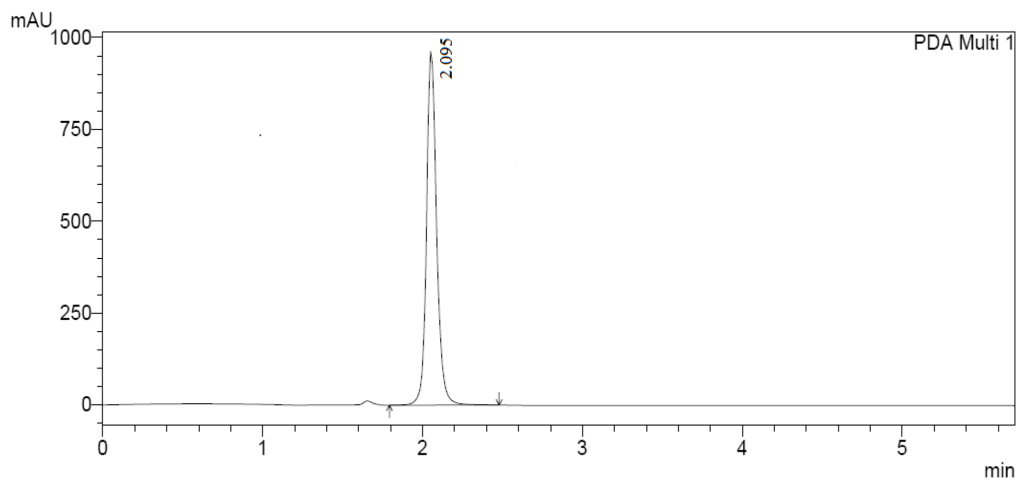


CHROMATOGRAM NO 12: METFORMIN HCl



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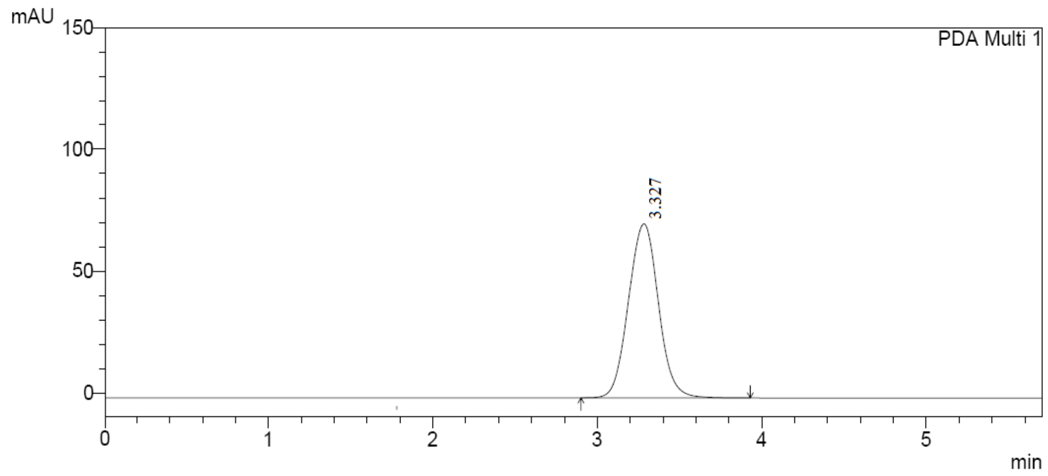
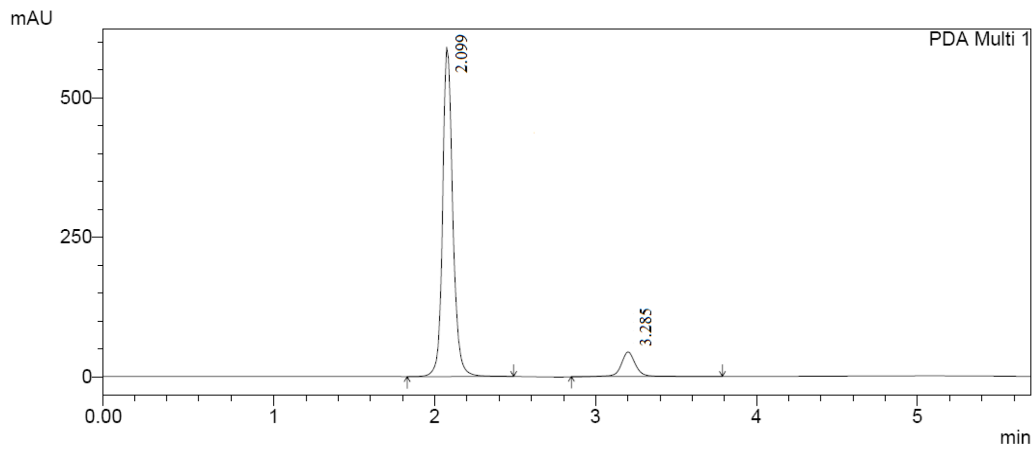
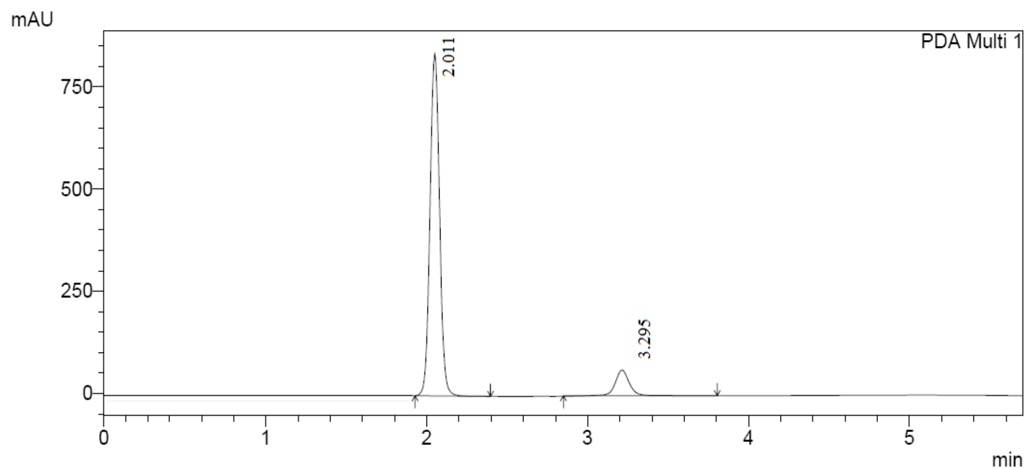


CHROMATOGRAM NO 14: LENIEARITY -1



CHROMATOGRAM NO 15: LENIEARITY -2



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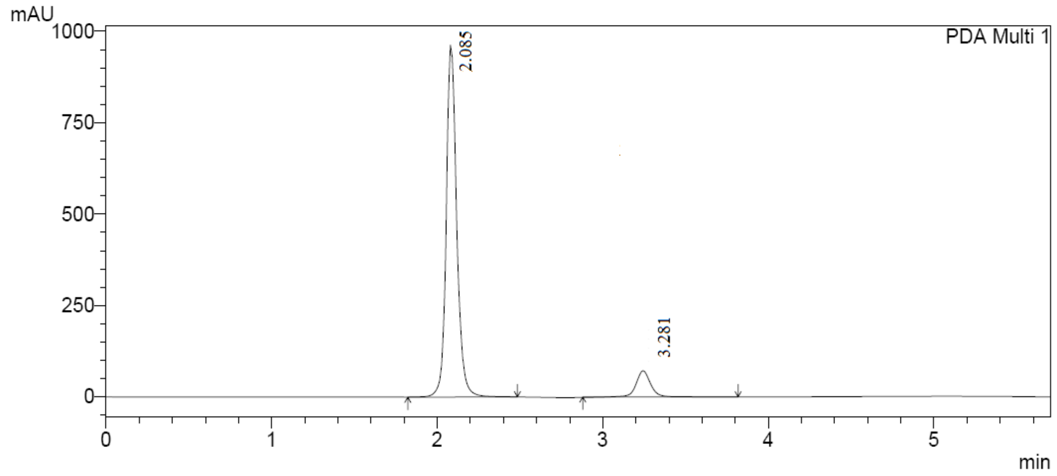

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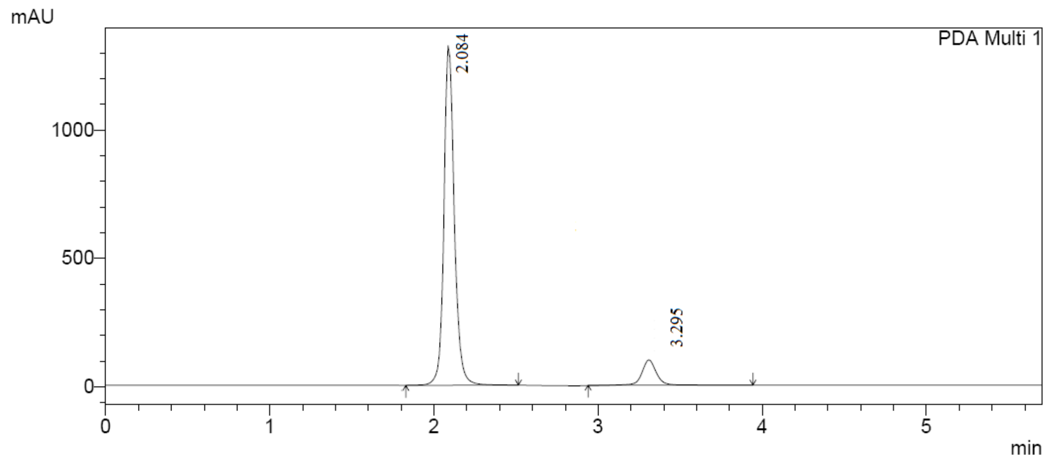

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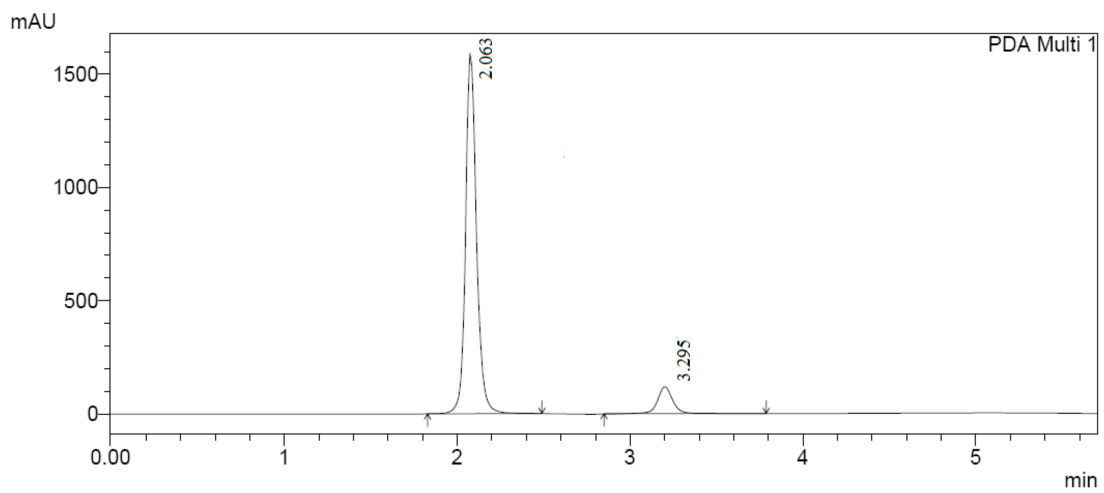
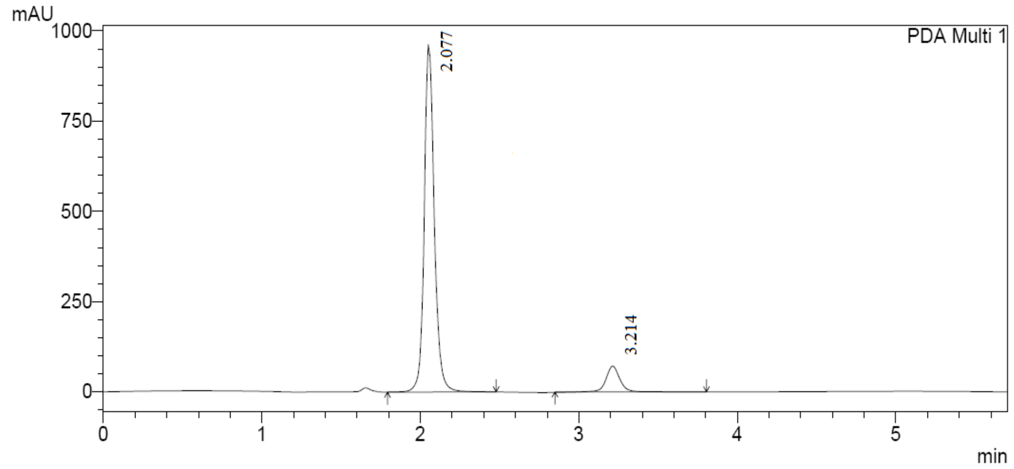


CHROMATOGRAM NO 19: PRECISION 0HRS



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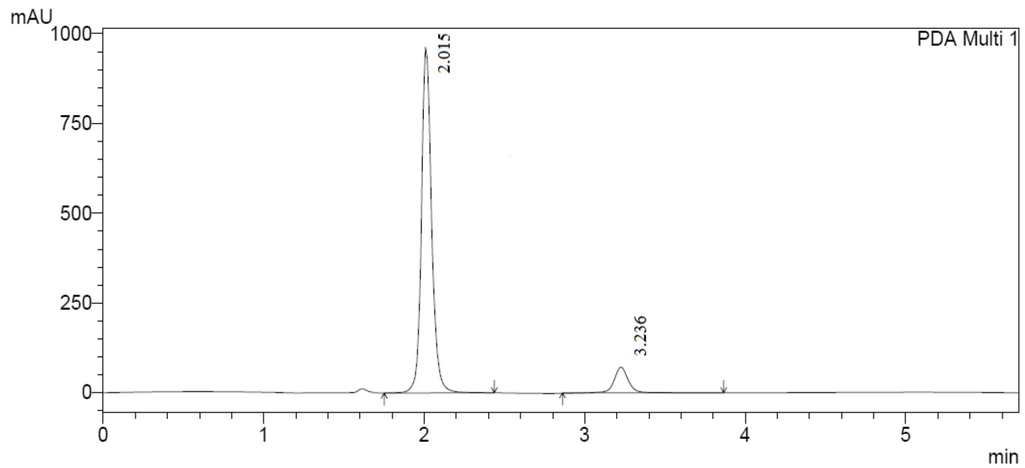

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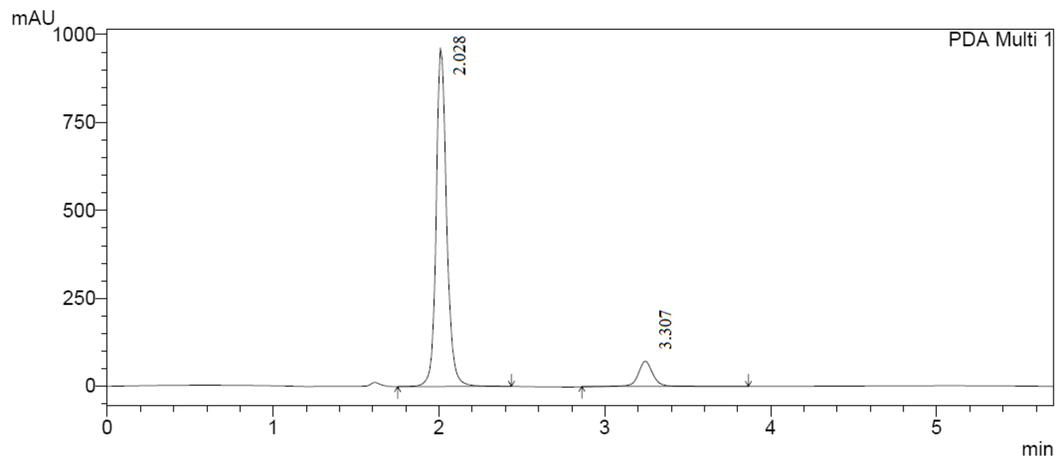
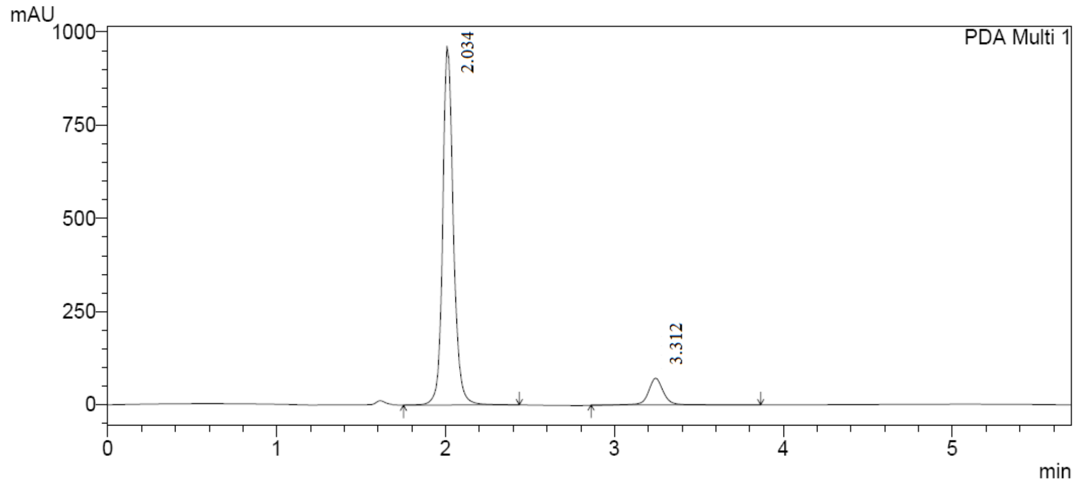
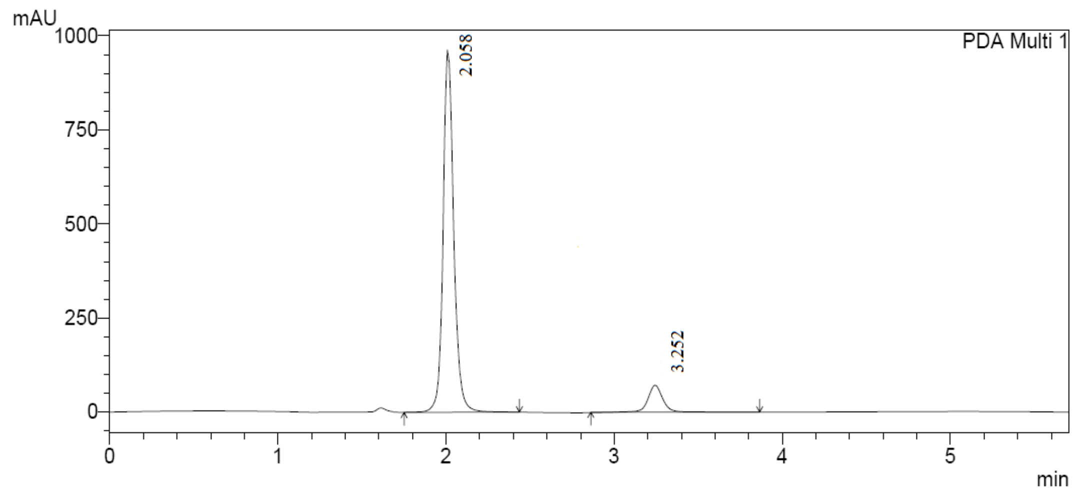


CHROMATOGRAM NO 22: PRECISION DAY -1-1



CHROMATOGRAM NO 23: PRECISION DAY -1-2



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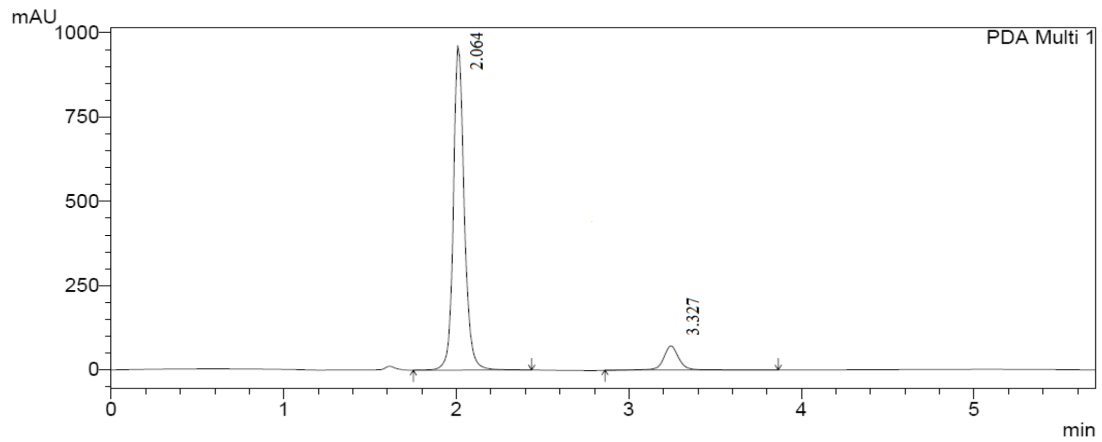

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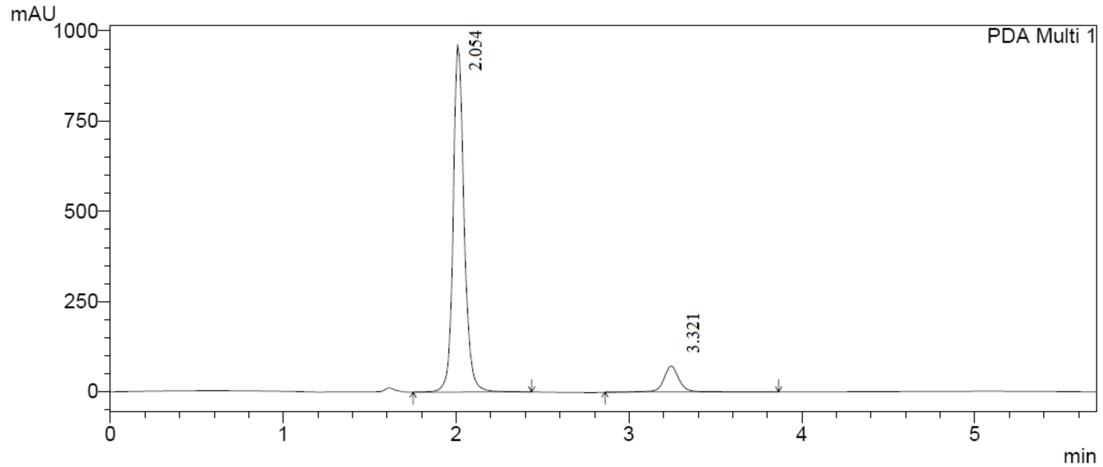

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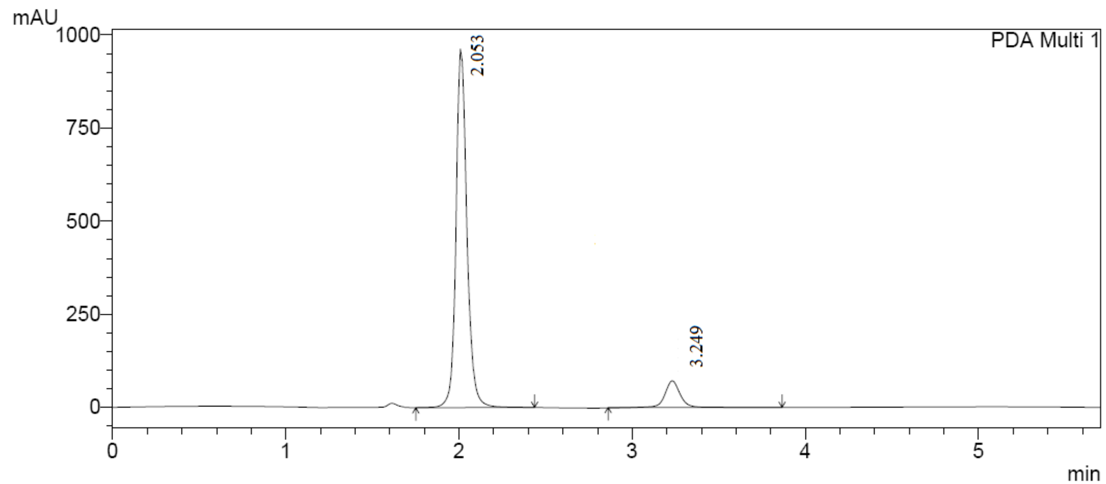

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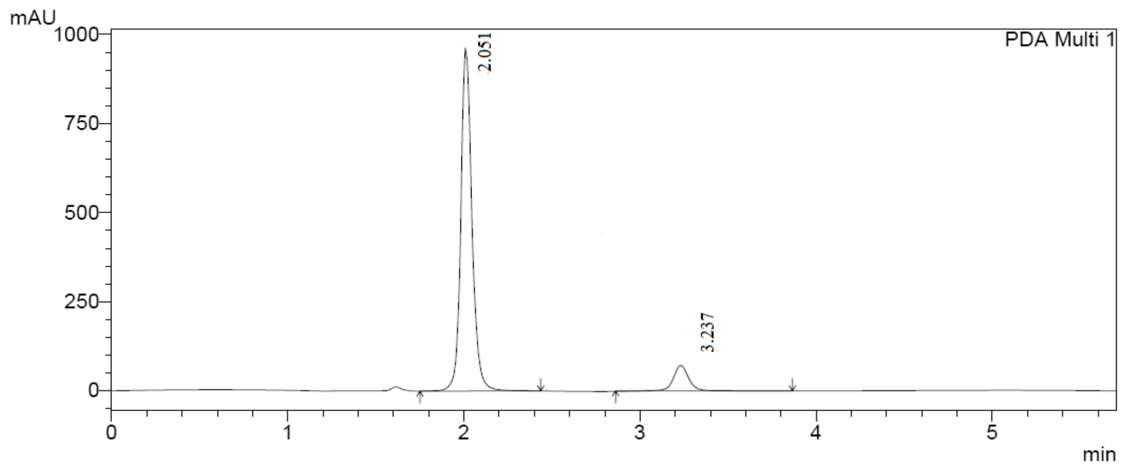
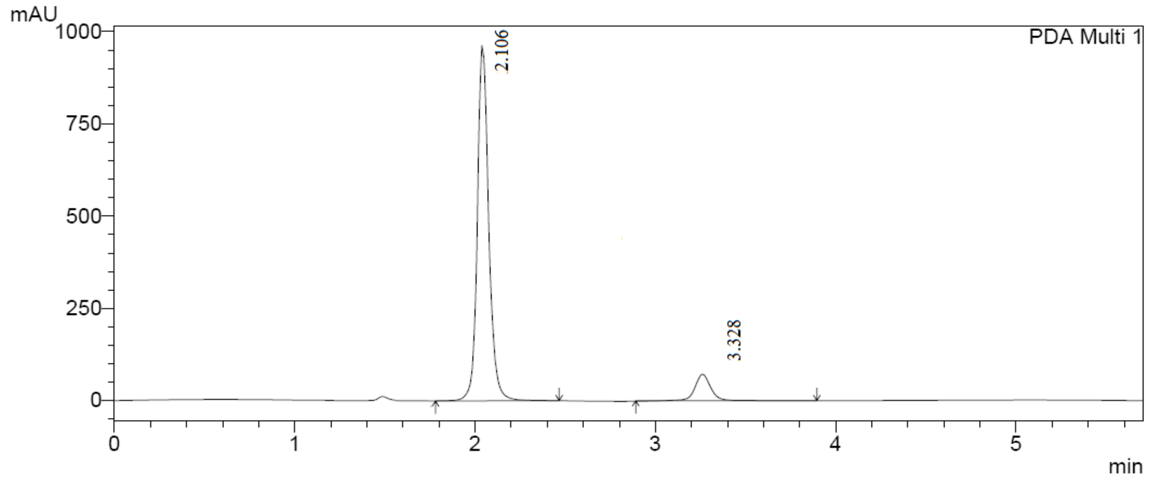


CHROMATOGRAM NO 28: PRECISION DAY -3-1



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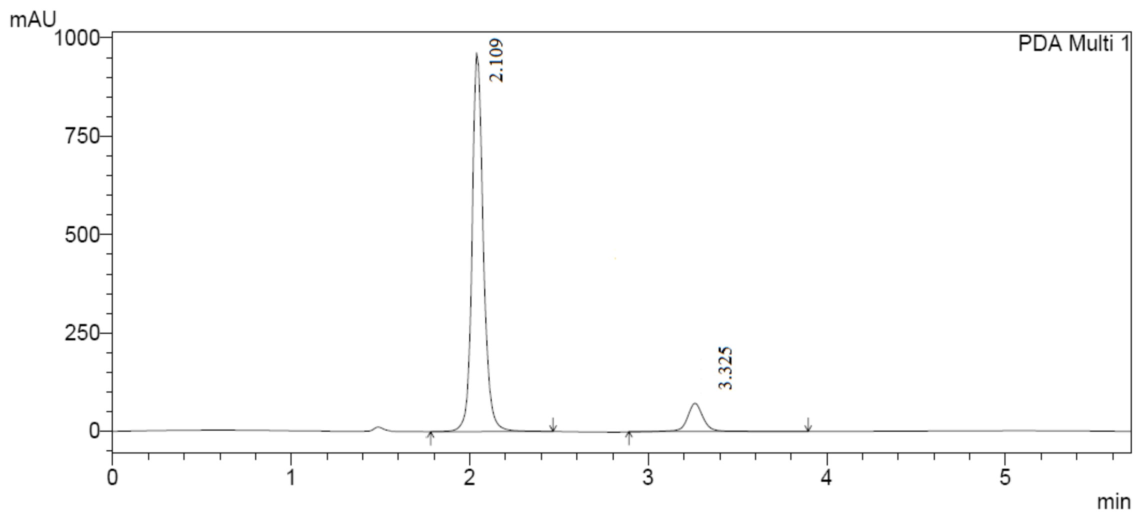

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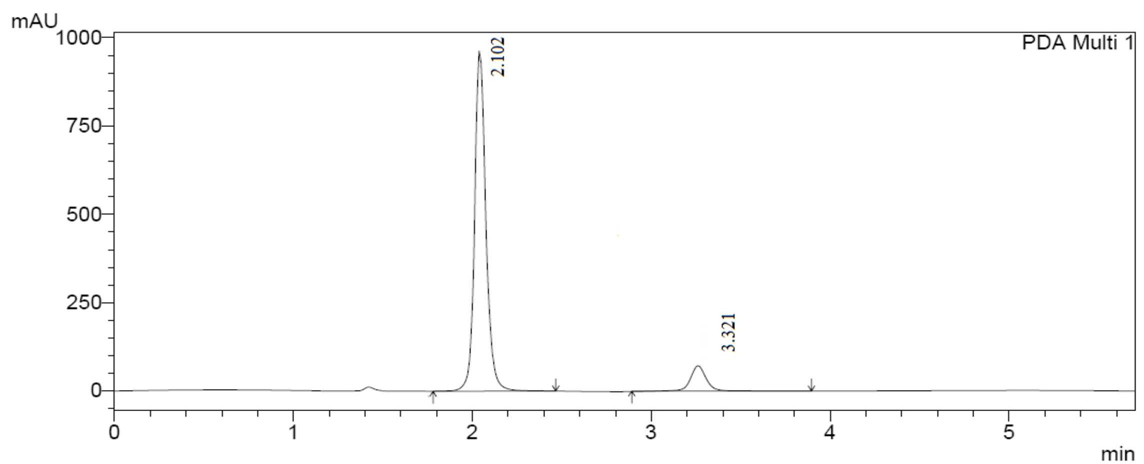
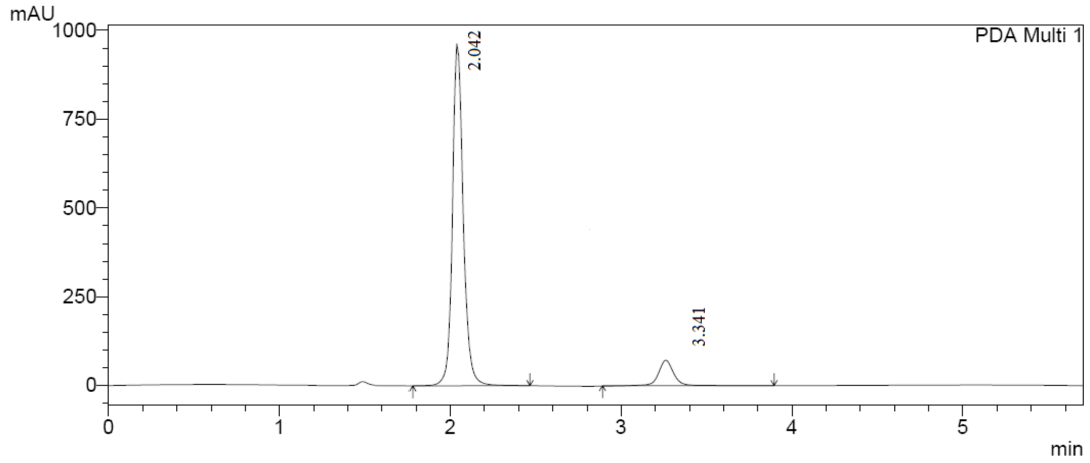


CHROMATOGRAM NO 31: PRECISION DAY –INSTRUMENT –I



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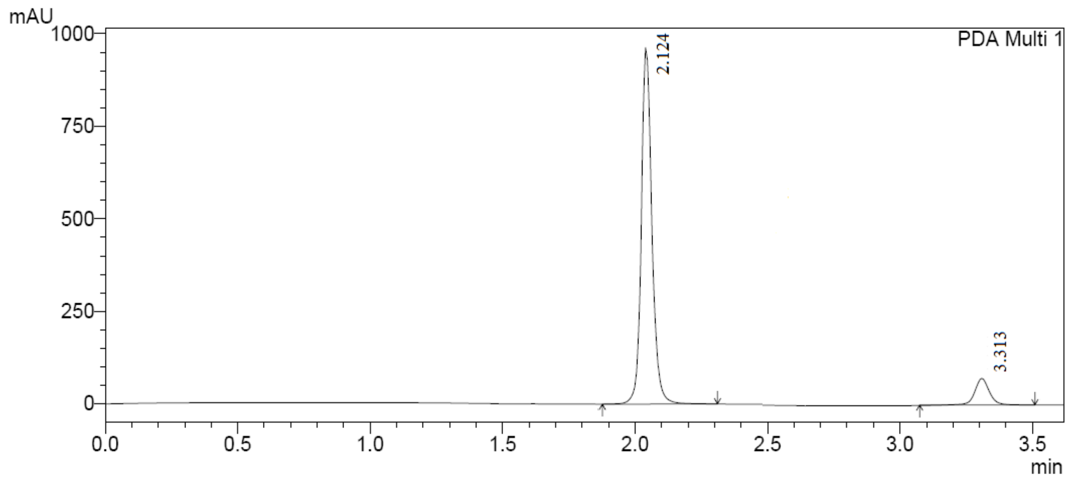

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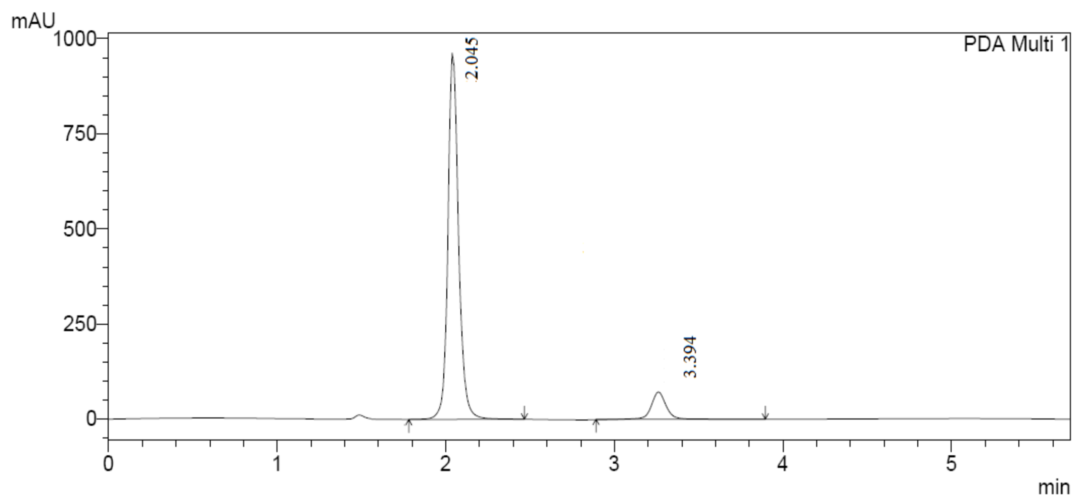

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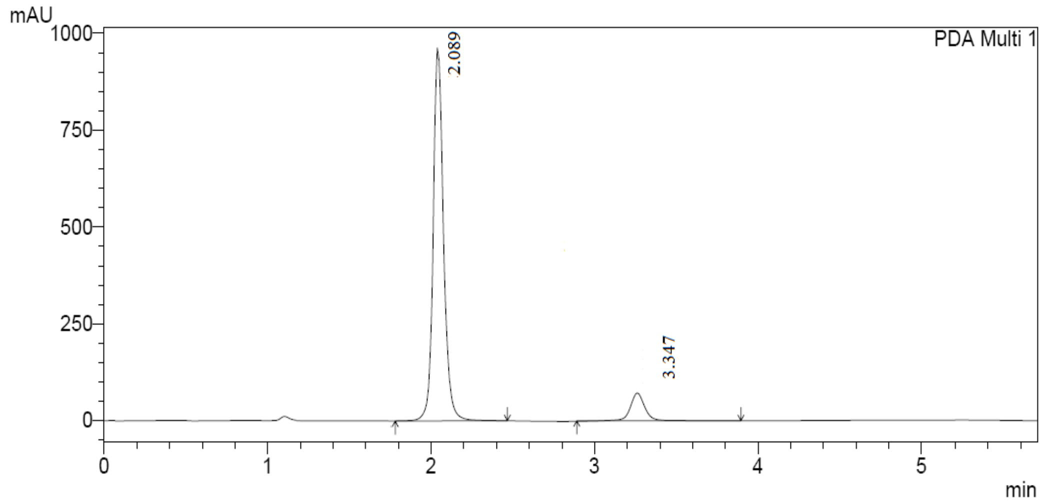
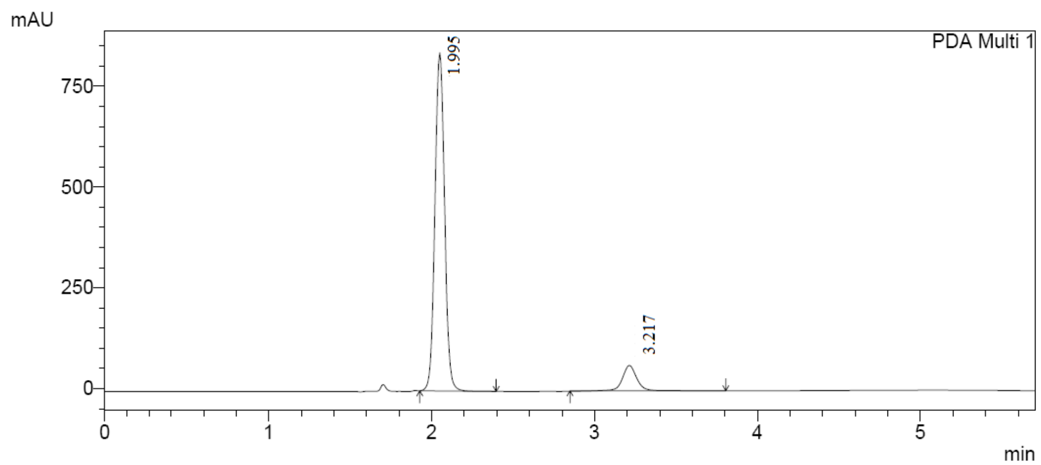
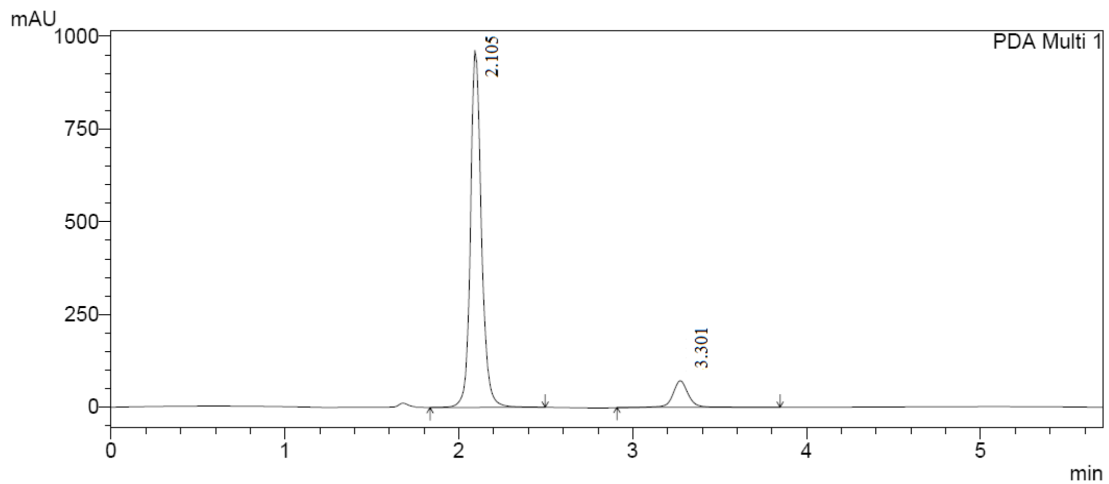


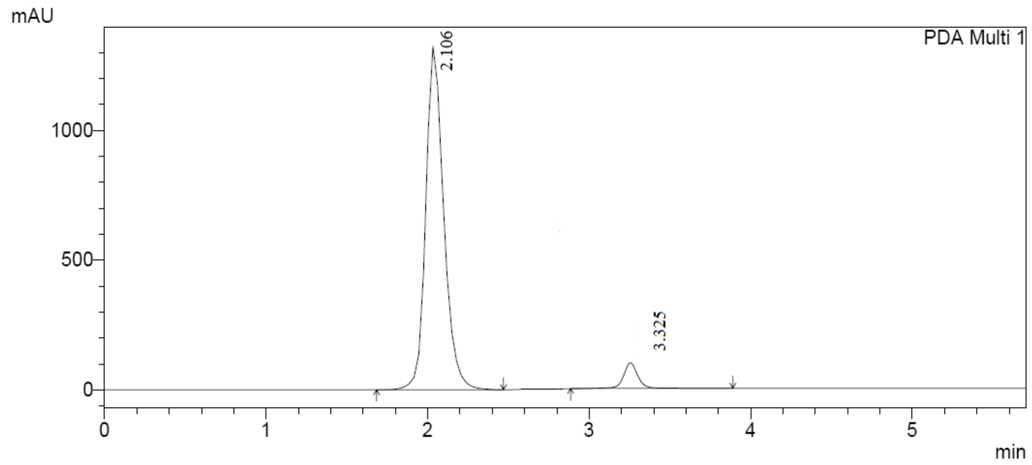
CHROMATOGRAM NO 34: ACCURACY - 75%



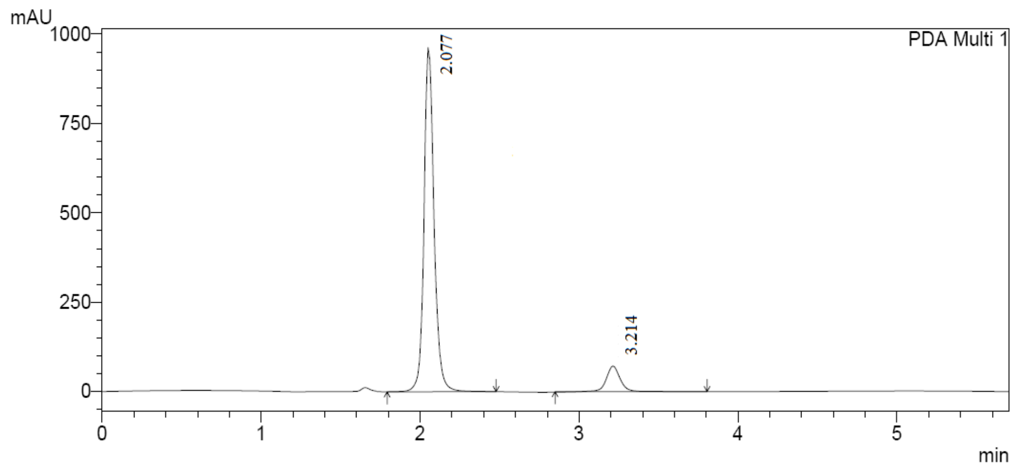
CHROMATOGRAM NO 35: ACCURACY - 100%



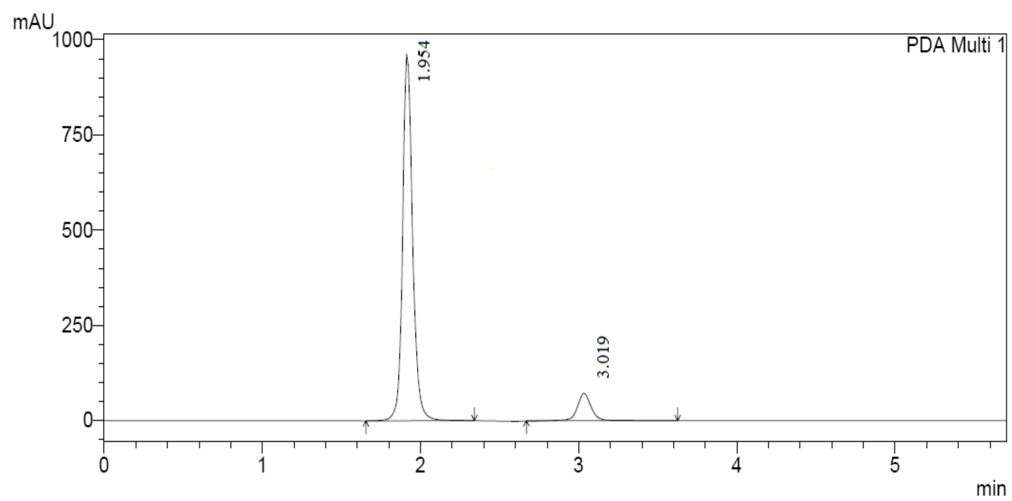
CHROMATOGRAM NO 36: ACCURACY - 125%



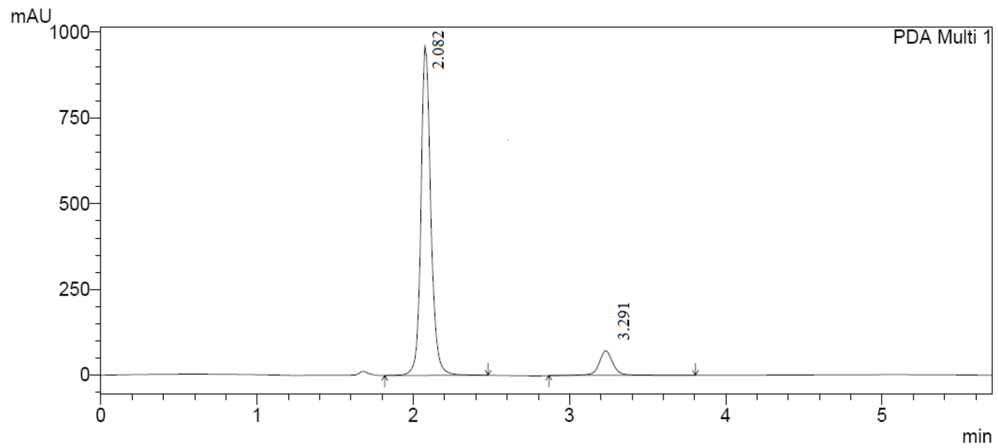
CHROMATOGRAM NO 37: ROBUSTNESS – FLOW RARE – 0.1 mL/min



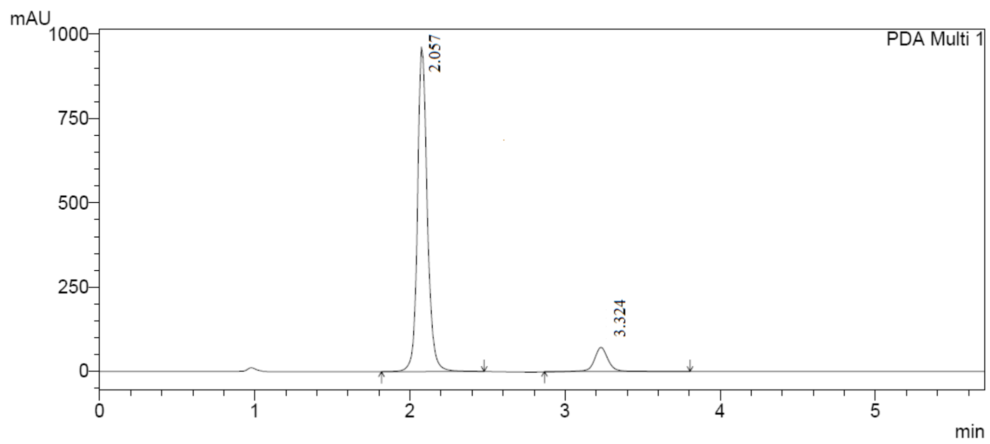
CHROMATOGRAM NO 38: ROBUSTNESS – FLOW RARE + 0.1 mL/min



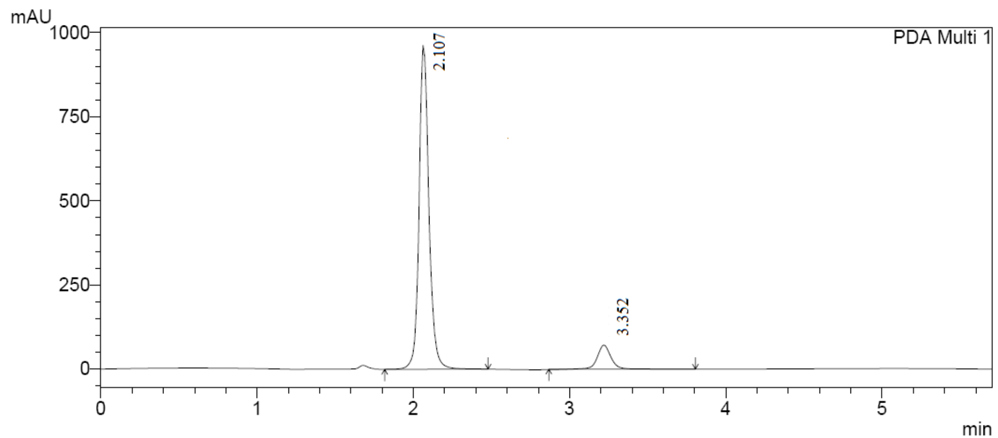
CHROMATOGRAM NO 39 : ROBUSTNESS – MOBILE PHASE – 2 %



CHROMATOGRAM NO 40: ROBUSTNESS – MOBILE PHASE + 2 %



CHROMATOGRAM NO 41: ASSAY



8. Conclusion

The developed RP-HPLC method was specific, simple, sensitive, precise, accurate and robust, for the detection of Metformin HCL and Alogliptin benzoate in pure and pharmaceutical dosage form. The proposed method is quite simple and do not require any pretreatment of the drug and tedious extraction procedure. The method has a wider linear dynamic range with good accuracy and precision. So the proposed method is used for the routine analysis of Metformin HCL and Alogliptin benzoate in pharmaceutical quality control.

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