A NOVEL ANALYTICAL

Methods For Simultaneous Estimation Of Pharmaceutical Drugs

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Preface

The analytical methods assume a great importance in quality assurance of many pharmaceutical industries due to i) Development of new drugs ii) continuous changes in manufacturing processes for existing drugs and iii) setting up of threshold limits for individual and total impurities of drugs by regulatory authorities. This is accomplished by modern pharmaceutical analytical techniques which include the physico- chemical methods of analysis. Our objective in the present study is to develop new liquid chromatographic methods for the assay of some selected drugs. This book deals with the chemistry of chromogenic reagents, the reactions used in the present investigation. The general methodology for developing new chromatographic methods optimization of experimental conditions (effect of pH, reagent concentration and order of addition, keeping time and temperature during each addition, effect of solvent, color development and stability) optical characteristics. Selectivity, precision, standard deviation, percent range of error, testing of significance by F-test, accuracy(comparison of the proposed and reference methods of pharmaceutical formulation, testing of significance by t-test and recovery experiments. This research also includes the study of HPLC system components (solvent delivery systems, solvent degassing systems, gradient elution devices, sample introduction systems liquid chromatography detectors, column packing materials inclusive of bonded phase, derivatization, gradient elution), performance calculations (relative retention, theoretical plates, plates per meter, height equivalent to theoretical plate, capacity factor, resolution, peak asymmetry), linear fit properties of solvents used in chromatography and validation of analytical methods(recovery, response function, sensitivity, precision and accuracy). Keeping this in view, an attempt was made by the author in the present investigation to develop new analytical methods for some of the important drugs in pure and pharmaceuticals dosage forms. All the methods described in the book are simple, rapid, reliable and valid. The methods could be used not only for quality control but also for process development of bulk drugs. The work carried out in the present investigation was described in Six chapter

Acknowledgement

I great fully acknowledge my sincere thanks to my Research Supervisor Prof. Rambabu, Head Department of Chemistry, RVR & JC College of Engineering, Chowdavaram, Guntur, for encouraging me at every stage with exemplary patience and kindness.

We would also like to express our gratitude to the PACE Institute of technology and sciences (autonomous), ongole, for their support in every level int this book.

We would also like to express our gratitude who have shared their knowledge and experiences, both formally and informally.

We extend our thanks Empyreal Publishing House for publishing this edited book

Once again, thank you to all those who have played a part, big or small, in bringing this book to fruition. With heartfelt appreciation.

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Table of Contents

1

INTRODUCTION TO HPLC AND PROCEDURE FOR METHOD DEVELOPMENT

Isolating, recognizing, and determining the relative quantities of constituents **(1)** in a specimen are all part of pharmaceutical investigation, which is a subdivision of chemical analysis. It is crucial for quality assurance and control in pharmaceutical formulations (**2**). Pharmaceutical investigation entails both qualitative and quantitative characterisation of the sample. Quantitative investigation displays the relative amounts of elements in the sample in number terms, whereas qualitative analysis revealed the sample's chemical identity. The rapid growth of pharmaceutical companies and medication production in many regions of the world has resulted in a surge in demand for novel analytical techniques in the industry.

Chemical methods and instrumental methods are two types of analytical procedures. Chemical methods emphasised theoretical implementation with an efficient reaction in chemistry, as well as the precise proportion of chemical quantities required to complete the reaction or create the appropriate amount of yield formed in the reaction. Titrimetric, volumetric, and gravimetric approaches, for example.

Instrumental techniques are based on the proportion of some physical features of a material or compound, such as optical or electrical, and are also used to determine the concentration of a substance in a specimen.

Instrumental methods are now widely recognized due to their superior speed, selectivity, precision, and specificity of analysis when compared to traditional methods. They're really sensitive. As a result, they can provide exact and accurate information for even small samples. Depending on the nature and kind of material, a suitable analysis method (**3, 4**) is used, either alone or in fusion. Instrumental techniques such as Spectroscopy (UV- Visible Spectrophotometry, IR, Mass Spectroscopy, NMR, Nephelometry, Fluorimetry, and Turbidimetry), Chromatography (GC, TLC, and HPLC), and Chromatography (GC, TLC, and HPLC) enable accurate assay investigation of medications and formulations with negligible substance, reagents, and time consumption.Various chromatographic techniques are utilised in the

pharmaceutical sector for a wide range of samples. The HPLC technique is often utilised in those chromatographic procedures.

LCMS Introduction:

LC/MS is a hyphenated technology that merges the dividing capability of HPLC with mass spectrometry's detecting power.

Mass spectrometry is a broad systematic approach that involves the generation of charged species, followed by their separation and identification.

The abbreviation LCMS (Liquid Chromatography-Mass Spectrometry) [**Fig 1.01**] is used to refer to a wide range of applications. This lesson will look at the various instrument accession procedures and the types of data that can be generated by such apparatus.

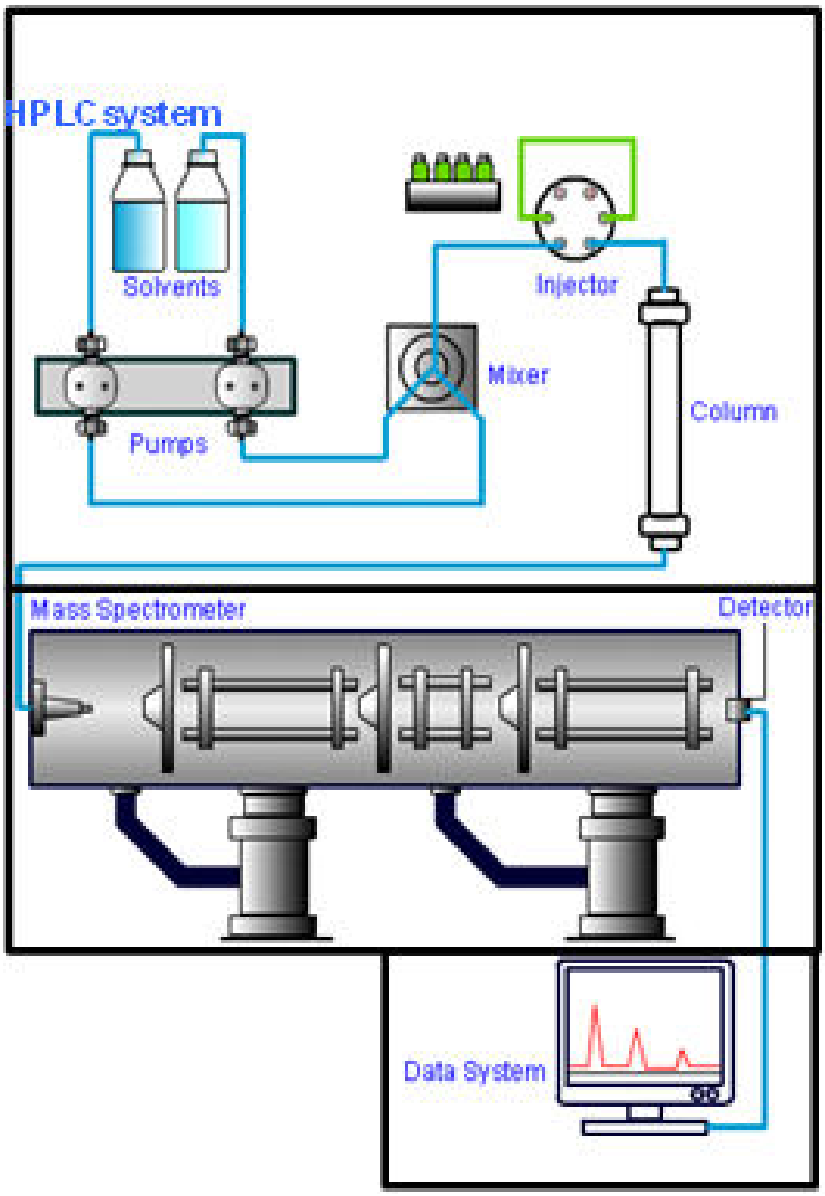


Figure 1.01: LCMS Diagram

1.1 Instrument Principles

The mass spectrometer is a device that separates ions in the gas phase based on their m/z (mass to charge ratio) value.

These parathion of charged species are created by a number of ionisation techniques in LC-MS and are used in mass spectrometry. Electro spray ionisation (ESI) and atmospheric pressure chemical ionisation are two examples.

Under atmospheric pressure, the charged species are formed as gas phase ions in all circumstances.

The gas phase ions are separated in the mass spectrometer using current field and/or field of magnet to discriminate them.

The mass spectrometer additionally has an air ionisation chamber, a vacuum system, and a detector in addition to the analyzer. **Figure 1.02** shows the main elements of LC/MS instrument.

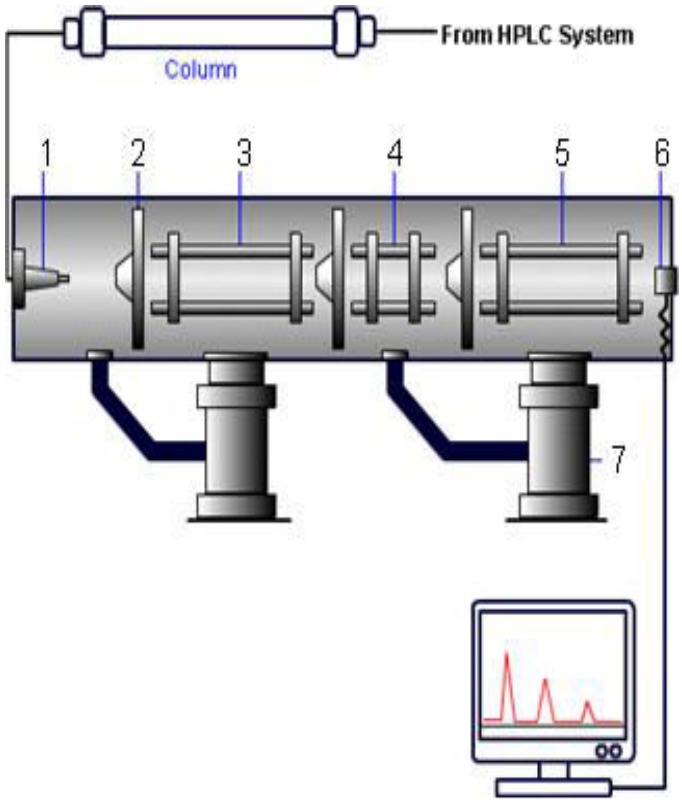


Figure 1.02: Main elements of LC/MS equipment

WHERE:

1. The HPLC (**5**) eluent is scattered into the atmospheric pressure zone as the ion source.
2. **Skimmer Cone:** A cone with a smaller sampling gap that preferentially samples gas phase ions and reduces the gas charge into the mass analyser device's vacuum system.
3. **Quadrupole:** A device that separates ions based on their m/z as they move through the center line of four side by side same distant rods using electric fields.
4. **Collision Cell:** Ions from the first mass investigator are quickened by a potential variation and strike with impartial gas molecules like H2, N2, or Ar, fragmenting the analyte.
5. **Detector:** Once the ions have been created and isolated, they must be detected and converted into an useable signal. Most modern mass spectrometer systems use reproduce, dynode, photodiode, and multi channel plate (MCP) ion detection methods.
6. **Vacuum system:** In order to use in a expected and well organized manner, mass analyzers require a high degree of vacuum. Most current LC-MS systems (**6**) use a vacuum system that consists of two or more distinctive pushed vacuum cells splitted by baffles or opening plates that vary in outline turn on the instrument seller.

Process

HPLC column partitioning, in which the reagents are partitioned differently between the movable and unmoving phases (covered onto a hold up matter and fill into the column). Some retention and partition procedures use Hydrophobic Interaction, Ion Exchange (**7**), Ion Pair, Surface Localization, or other chromatography techniques.

After the separated species have been sprayed into an API, the majority of the eluent is pushed to the trash.

Ions are sorted using a mass analyzer based on their mass to charge ratio. Quadrupole (seen flip side), the most popular is time of flight (**8**), ion trap (**9**), and magnetic zone. Ions with a given mass to charge ratio can be extracted from the mass analyzer, or they can be "scanned" through all of the ion m/z results.

The detector counts the ions that emerge from the mass analyzer and may additionally magnify the signal produced by each ion. Electron reproduce, dynode, photo diode, and several-channel plate are all common detector types.

All mass analysis and detection takes place at a high vacuum level, which is achieved by combining foreline (roughing) and turbo molecular pumps.

Detection of MS

- Contains compounds that can be identified (structural elucidation via spectral interpretation combined with elemental composition from accurate mass analysers is possible)
- Apt to be emotionally affected (fempto-gram amounts have been detected by certain mass analyzer types)
- Difficult to please" (certain analyzer and experiment combination scan lead to highly selective and sensitive analysis of a wide range of analytes).
- the process of ionization is known as ionization
- Electron- or atom-exchange processes, also called ionisation, manipulate the internal structure of atoms or molecules to form ions. In LC-MS, charged molecules like protons can interact with the molecule to which you are applying the charge.
- Strong electric fields are used in LC/MS systems to form these ions in the vapour or condensed phase. Ionization or dissolution at sub atmospheric pressure is known as Atmospheric Pressure Ionization (AP-I) (API).
- Electro spray Ionization (ESI) - condensed phase ionization
- APCI–gas phase ionization
- AERIAPAP (APPI)–gas-phase ionization

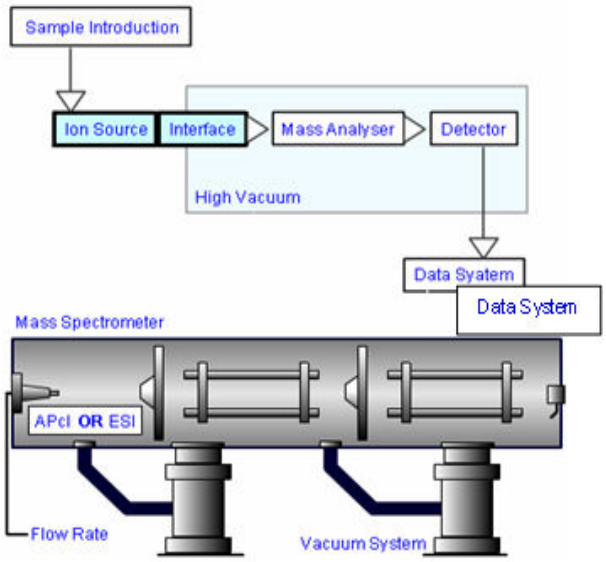


Figure 1.03: Diagram for Ionization

1.2 Ionization of the Atmospheric Pressure (API)

The dissolver expulsion and ionisation processes are integrated in Atmospheric Pressure Ionization (API), which takes place in the ion source.

All interface types have two main considerations in common:

1. Desolvation of the analyte molecule: To form gas phase analyte ions, the dissolver fragments must be eliminated from the eluent of HPLC.
2. Substance fragments Charging: Ions must be produced in order for the substance (or substance by-product) to be sent from the interface into the mass spectrometer, where they will be sieved from other heaps and then determined.

Electro spray ionisation (ESI) (**10**) and APCI are the two major forms of ionisation employed in API LC/MS (APCI). The primary processes employed in API ionisation techniques are depicted in the schematic image below [**Fig 1.04**].

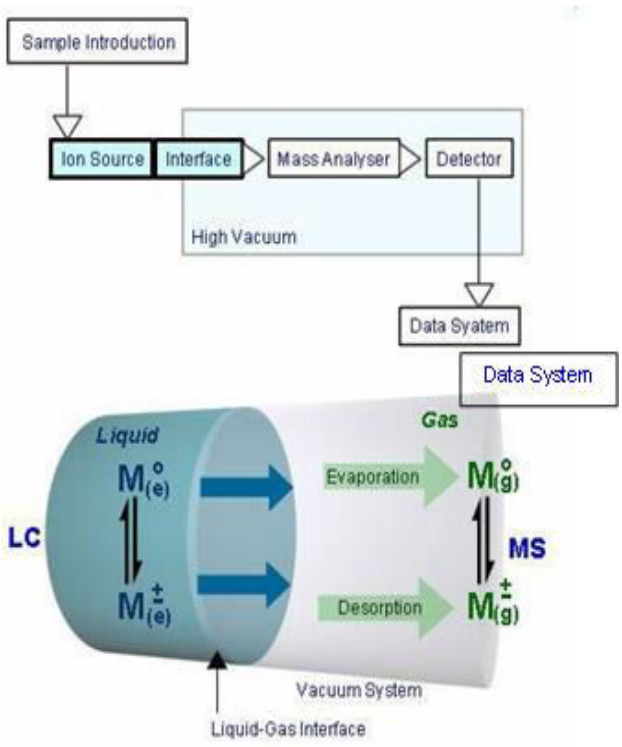


Figure 1.04: Ion production process

- In Electro spray ionization, substance ions are formed in the movable phase prior to approaching the API interface. In the API interface for APCI, ions are produced via charge transfer mechanisms in the gas phase.
- electro spray ionizations
- Electro spray ionization (ESI) [**Fig 1.05**] employs liquid phase (liquid) charge detachment and ion escape processes to produce vapour phase analyte ions. Before spraying the analyte molecules into the Electro spray interface, they must first be ionized. This also indicates that analytes are ionized in the HPLC column before or after partitioning in the column.
- Electro spray ionization uses three key processes to separate ions from the HPLC eluent into the gas phase of the mass spectrometer. To manufacture charged droplets, one of these

processes must be taking place at the tip of the capillary.

- the drops turning into a puddle
- Ion production in the gas phase from small/highly charged droplet

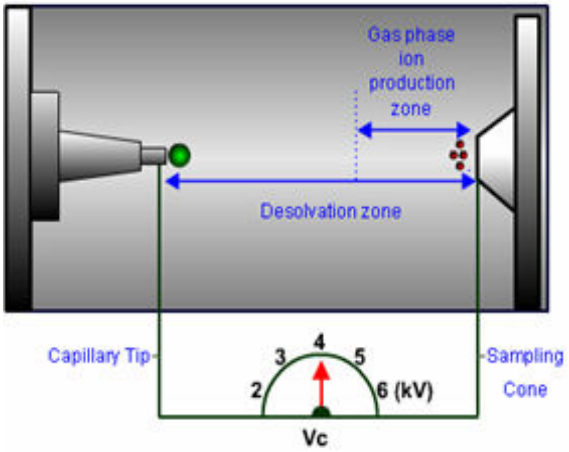


Figure 1.05: Diagram for Electro spray Ionizations

Within the contact, hot drying gas dissolved sprayed eluent droplets.

Small solvated droplet or gas phase analyte molecule ([M+H]+) with a more number of fundamental charges than the initial sprinkled bubbles.

Chemical Ionization of Atmospheric Pressure (APCI) [Fig 1.06]
To produce vapour phase analyte ions, APCI involves substance dissolvation and charge shift processes in the vapour phase.

The eluent is injected into the interface in APCI via a capillary that is comparable to the ESI source. However, instead of applying a potential to the capillary, the liquid exits from the capillary into a heated zone, surrounded by a flow of inert, nebulizing gas.

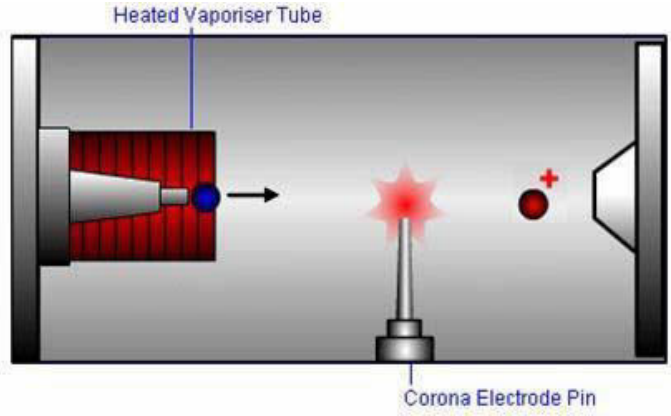


Figure 1.06: Diagram for APCI process

APPI (Atmospheric Pressure Photo Ionisation)
It is a complementary technique to ESI and APCI that was designed to expand the scope of ionizable substances at air pressure.

Using APPI, it is possible to ionize difficult-to-ionize mixtures, including low- and non polar mixtures (APPI has been used in the investigation of polycyclic aromatic hydrocarbons).

The ionization process in APPI is done by subjecting a droplet aerosol to light irradiation. When a molecule absorbs a photon, it produces a molecular radical ion. Only when their radiating Photon (of energy) exceeds the molecule's ionization potential (IP) is this process conceivable.

According to APPI [**Fig 1.07**], species that have an electrical charge may develop in either an ionic or non-ionic mode.

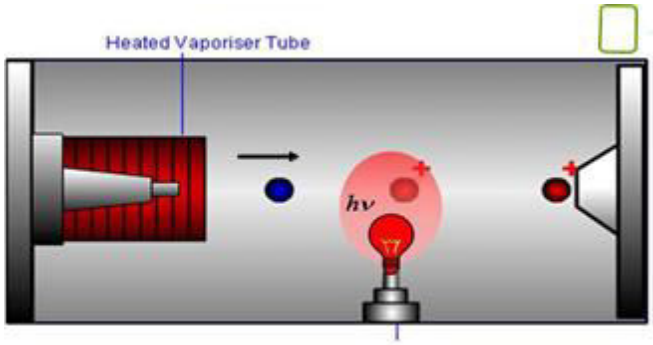


Figure 1.07: Diagram for APPI process

Mass Analyzers

It is a type of mass analyzer [**Fig 1.08**].

The most basic kind of LC/MS mass analysis is performed using aparathion or filtration of analyte ions or fragments of analyte ions in the API interface or in the regions between the API interface and the high vacuum region of the mass analyzer (products of collision-produced separation etc.).

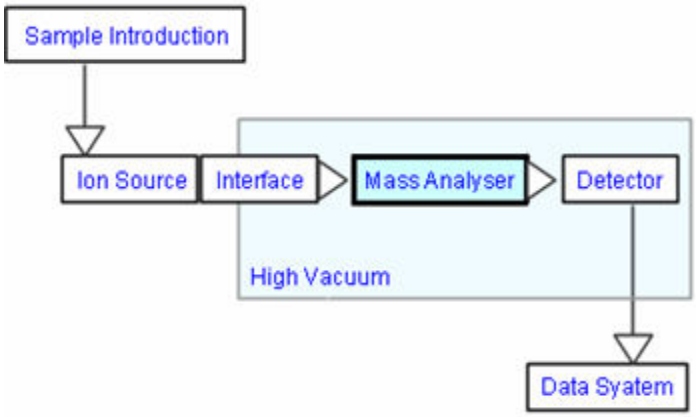


Figure 1.08: Mass analyzer

There are numerous main categories of mass analyzers used in regular liquid chromatography mass spectrometric investigation, and they all vary in how they differentiate species on a mass-to-charge basis:

Ions are separated using electrostatic potentials that are provided to the elements of mass, which are used to “select” ions based on their m/z.

Mass spectrometers that use Time of Flight separate ions by applying a wide range of flying times to quicken ions as they fly along a long path.

The magnetic zone mass analyzers use magnetic fields to guide the stream of ions towards the detector, thus selecting ions of interest.

The relative abundance of each mass is graphed in terms of m/z ratio. This illustrates the results of the mass spectrum [**Fig 1.09**] of the analyte.

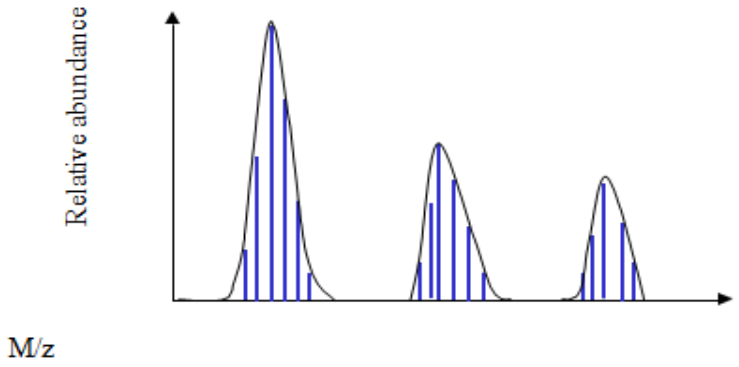


Figure 1.09: Diagram for Mass Spectrum

QUADRUPOLE

It is a four-pole structure.

Electric fields are used in quadruple mass analyzing devices to distinguish ions according on their m/z ratio as they pass through a lode (or pillar). To create an electrostatic zone interior of the instrument, ion detachment is accomplished by applying regulated voltages to the mass investigator rods.

As long as x and y, which control the point of an ion from the middle of the rods, remain constant, the ion will be able to pass through the quadrupole [**Fig 1.10**] without hitting the rods, remain < r0.

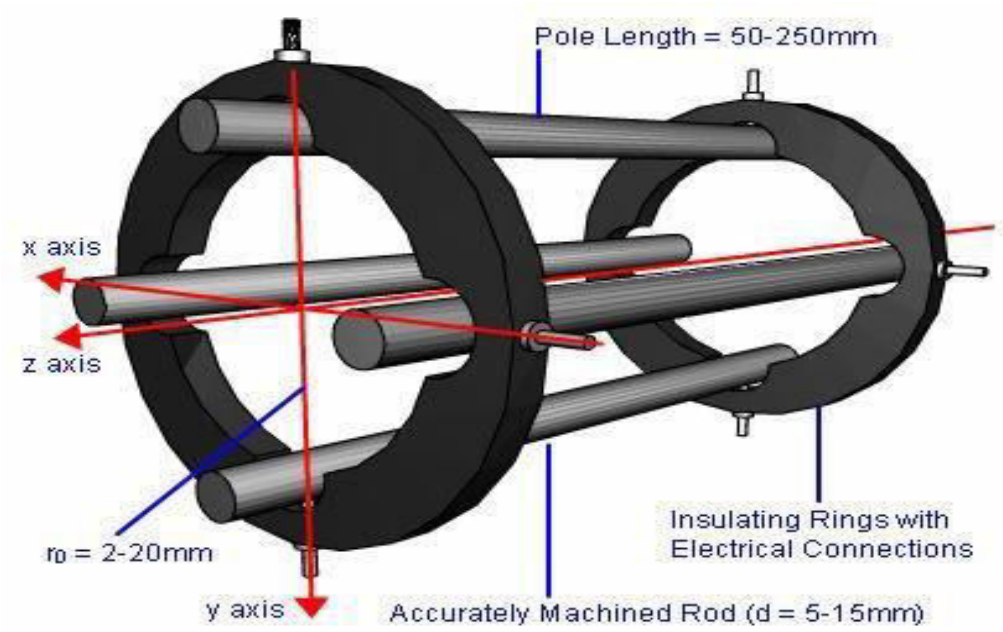


Figure 1.10: Diagram for Mass Spectrum Quadrupole

When an ion is made to vibrate with a path whose magnitude exceeds r0, it collides with a rod and is emitted, then pushed to waste. An unstable or collisional trajectory is what this is called. **Figure 1.11** shows the collisional energy diagram.

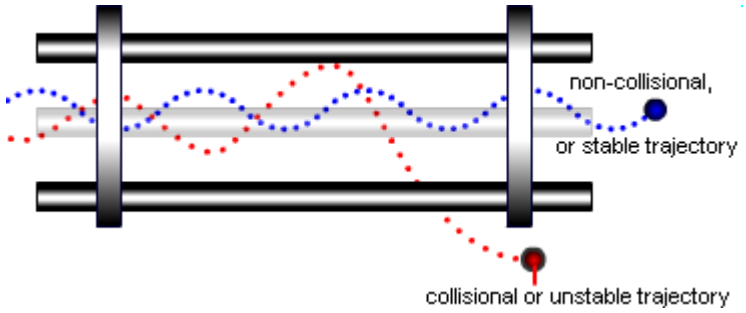


Figure 1.11: Collisional energy diagram

Quadrupole mass analyzer

Advantages	Disadvantages
• Reproducibility • Low cost	• Low resolution • Mass discrimination. Peak height vs. mass response must be 'tuned'

ETA (Estimated Time of Arrival) (TOF) (11)

In comparison to innumerable other common mass examined appliance, the basic concepts of mass study employing time-of-flight mass investigator [**Fig 1.12**] are rather simple.

In the ion source, ions are removed (or created) in small cracks or packets and then accelerated. The ions then „drift' or „fly' down a predetermined length („d') of evacuated tube. Once the ions are outside of the accelerating voltage area, the fast at which they move through the tube is determined by their mass (m) and charge (c) (z). This mass spectrometer is handy since it detects all ions (nearly) simultaneously. Because studying the mass period of total ions is done quickly, the instrument's inherent sensitivity is boosted.

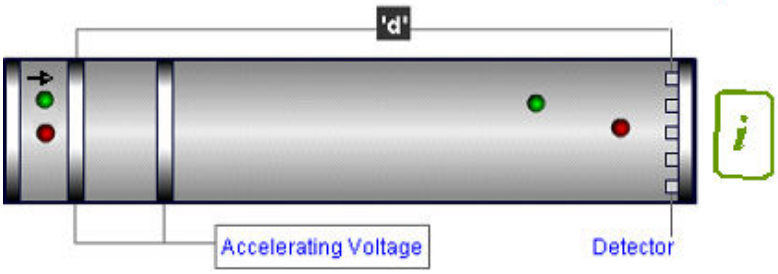


Figure 1.12: Diagram for Time-of-flight

Time-of-flight (TOF)

Advantages	Disadvantages
• High ion transmission • Highest practical mass range of all M S analyzers • Detection limit	• Fast digitizers used in TOF can have limited dynamic range

Mass Analyzer with Ion Trap [Fig 1.13]

Ion trap mass spectrometers work by trapping ions and modulating them with applied DC and RF fields. The electrostatic field gives non-selected ions path, causing them to exit the trap. It is feasible to fragment selected ions by stuffing the trap with an inert gas. When structural information is necessary, this is useful.

The system offers numerous unique features, such as the ability to do numerous production scans with excellent sensitivity (MSn). Due to the differing crash administration in the systems (collision energy/gas), the spectra obtained with an ion trap mass investigator may differ dramatically from those obtained with a triple quadrupole system.

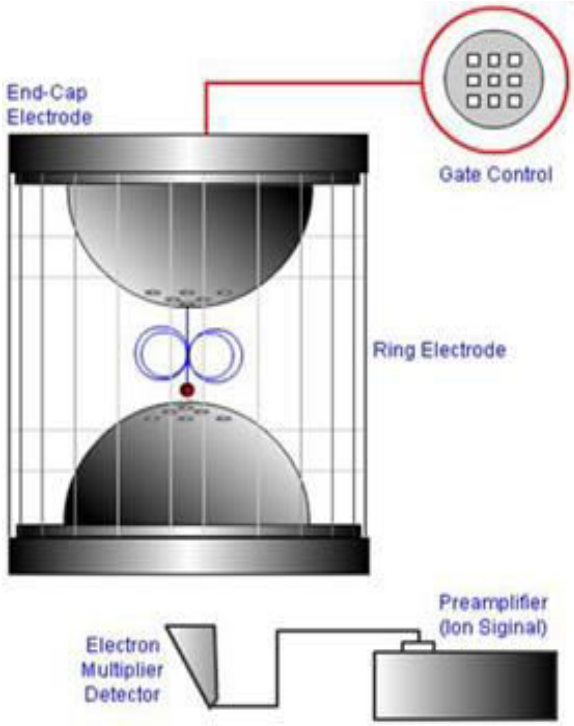


Figure 1.13: Diagram for Time-of-flight Ion Trap Mass Analyser Table 1.01 gives the advantages and disadvantages of ion trap mass analyzer.

Table 1.01: Ion Trap Mass Analyser

Advantages	**Disadvantages**
• High sensitivity • Multiple Product Ion scan capability$(MS)^n$ • High resolution • Good for DDA analyses	• Produces very unusual spectra if the ions are stored in the trap too long. • Easily saturated • Poor for low mass work (below100Da) • Poor dynamic range (except the most modern devices) and hence may have limited quantitative use

Tandem Mass Spectrometry (MS/MS) [Fig 1.14]

To put it another way, MS/MS can be described as a combination of two or more individual MS studies. Our goal is to either grow more structurally solid as a result of breaking apart the ions that were initially isolated through the first investigation, or to acquire better discernment and sensitivity in quantitative reasoning by employing both the first and second investigators.

Multiple analysers (of the same or different kinds) can be linked together to do MS/MS analysis, or an ion trap can be used to do consecutive fragmentations of trappedions.

Multiple ion generation and filtration in a single instrument is referred to as MS^n (should read MS to then). Instruments are typically made up of multiple quadrupoles combined with a collision cell. Fragmenting the emergents from the first analyser before secondary mass filtering is a typical design. Other massanalysing device combinations are feasible, such as quadrupoles with time off light, or quadrupoles with magnetic sector instruments.

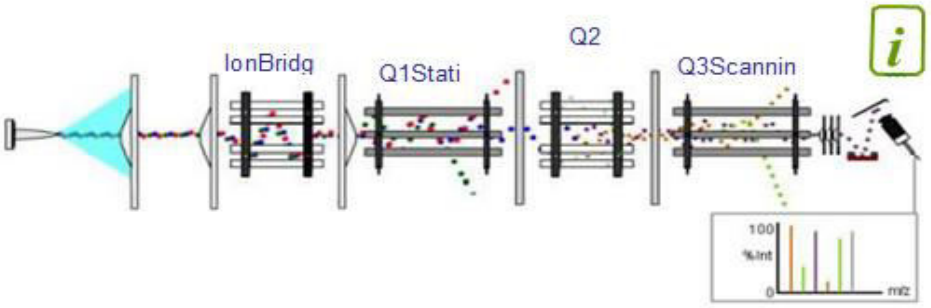


Figure 1.14: Diagram for Tandem Mass Spectrometry

Detectors [Fig 1.15]

After passing through the mass analyser, the ions must be identified and converted into a useful signal. A mass spectrometer's detector is essential to obtaining signals from either secondary electrons that are amplified or a current (generated by moving charges). An ion detector system can be divided into 2 types: passive and active.

Beer-sniffing tubes: Ions do not exist in one particular location and thus affect detector locations in a spectrometer over time, creating point detectors.

Array detectors: Ions are spatially determined, and total ions come at the same time (or almost at the same time) and are rerecorded along a plane with a bank of detectors.

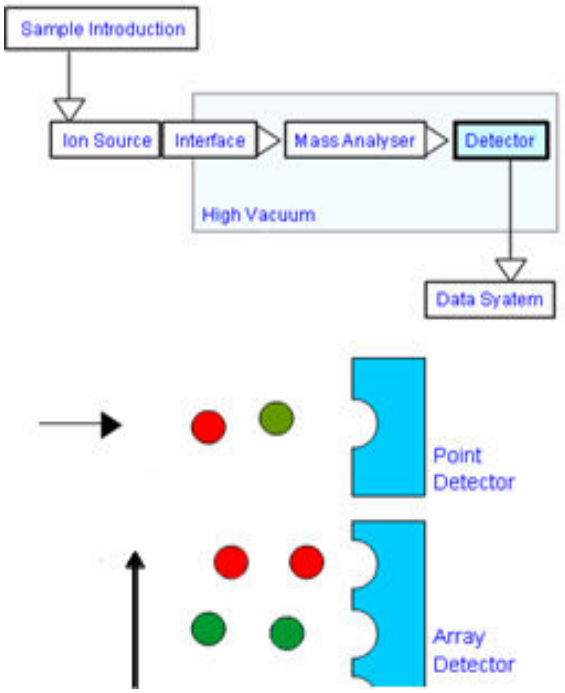


Figure 1.15: Diagram for Detectors

1.3 High pressure/performance liquid chromatography (HPLC)

From the last twenty five years, HPLC has been the most important component in pharmaceutical and biomedical research methodologies. HPLC is the most efficient systematic technique for API (**12**) or sample testing during the research, Evolution and synthesizing processes for drug allowance, evaluation, and recognisation. HPLC is a kind of column chromatography in which a fluid (referred to as the mobile phase) is

injected via the column and a test mixture or substance (referred to as the stationary phase) is transported through the column with chromatographic stuffed substance (called as the stationary phase) at a stable high compulsion. **Figure 1.16** depicts a systematic diagram of an HPLC.

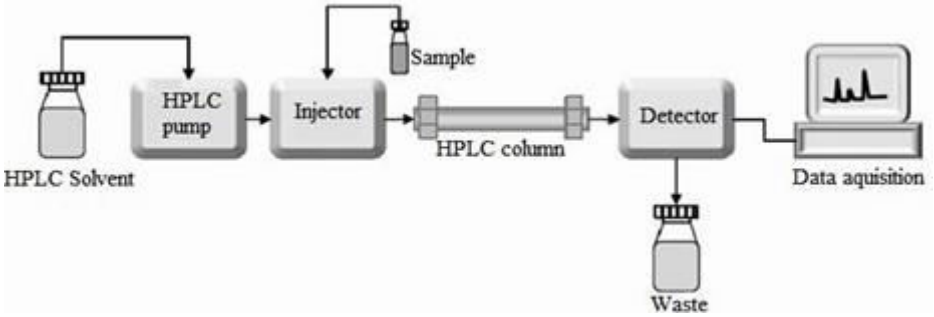


Figure 1.16: HPLC systematic diagram

The following are the main components of an HPLC apparatus:

Data Handling Device and Microprocessor Control for HPLC Solvent push dose Column Detector Data administration appliance and logic circuit Control for HPLC Solvent Pump Injector Column Detector

1.3.1 Solvent for HPLC

The solvent reservoir is another name for this section. This is where the movable phase is kept. Highly purified solvents such as HPLC grade solvents and water with a resistivity of 18.2Mcm at room temperature (25°C) are used to prepare the mobile phase.

1.3.2 Pump

Because it affects reproducibility and retention time, the pump is an important component of HPLC apparatus. With a constant flow rate, the HPLC pump delivers mobile phase to the column, which is pressurised by the solvent reservoirs (5000 pounds per square inch or more). Removing other gases and diffused air from the dissolver is imperative to the success of the pump. While it is important to have a good pump, the following characteristics should be avoided in order to avoid problems when switching solvents: a pump that has regular stream delivery with no build-up of end pressure, the ability to change worn components easily, solvent suitability, rust resistance, and low dead volume.

1.3.3 Injector

Injection ports are used to inject samples into HPLC. A testing loop and an injection pipe may be found on an injection port. After being decomposed into the moving action, or with a selected diluent, the instance was injected into the testing loop via an injection pipe. In order to introduce the specimen into the mobile process flow, the pipe rotor is utilized to open the curve and then close the pipe. The loop volume ranges from 10 litres to over 500 litres. The samples were immediately fed into a sophisticated HPLC apparatus.

1.3.4 Column

Critical components in HPLC are one of the most preferred columns because the sample mixture is separated through the column. Since of its porosity structure, exterior characteristics, and particle form, silica gel is widely utilized to heap columns. Because of its usually predictable and reproducible chromatographic action, silica is used to separate a variety of chemical substances. Silica also has a higher surface activity that is quickly altered by water and other solvents. There are two types of HPLC columns. A shield column is the first, while an actual column is the second. Analytical columns are typically 5cm, 10cm, 15cm, and 25cm in length and packed with particles with a diameter of 3, 5, or 10m. The interior diameter of a column is usually 4.6mm. The guard column is a disposable top of the primary analytical column that

protects the analytical column. It extends the analytical column's life and protects it from impurities and particulates in solvents. The most widely utilised material is octadecyl-silica, which is a reverse phase C18 column. HPLC is offered, as well as C8, C6, C8, C8, C4, cyano, and amino columns.

1.3.5 Detectors

Without a detector, it's nearly impossible to carry out HPLC analysis. One of the HPLC ingredients that releases a reaction and then indicates a peak on the chromatogram is used to elute the compound. To identify the chemicals that are eluted from the column, it is located behind the static phase. Coarse and fine-tuning settings were utilised to determine the overall band width and height of the peaks. The fine- tuning and coarse settings can be adjusted to change the detection and sensitivity characteristics in various situations. In HPLC detection, the benefits are that they can differentiate between all of the chemical components in a mixture. Non-disastrous operational simplicity and accuracy; and non-disastrous. Some of the most often used HPLC detectors are as follows:

1.3.5.1 Refractive index detectors

The refractive index detector measures the index of refraction difference in the middle of the eluent that passes all over the stream cell and the uncontaminated movable phase. It's referred to as a universal detector. It functions on an isocratic basis. As a result, it is especially sharp to replace in pressure, stream, and normal temperature, and it cannot be utilized for gradient extraction. This detector comes in handy when it comes to identifying nonionic substances. UV and fluorescence detectors are unable to detect these substances because they do not absorb ultraviolet light or emit fluorescence.

1.3.5.2 UV (ultraviolet) detectors

The UV-visible absorbance detector is the most frequent type of detector utilized in HPLC nowadays. UV detectors are utilized in HPLC to notice and recognize mixtures by displaying a spectrum in the visible or UV range (between 190 and 600 nm). A emit lamp with a wavelength of 190-380nm is utilized in UV detectors to use deuterium as a light source. When components are identified at wavelengths greater than 380 NM, a tungsten light is utilised in addition. The light from the lamp scatters according to its wavelength when it is focused on the grating. The diffraction grating's angle is tuned to test the desired wavelength. The brightness then goes via the reflector, dividing into two beams, one of which travels along the flow cell and the other via the reference-side light-receiving section. The difference in light intensity in the middle of the brightness from the standard cell and brightness from the stream cell can be quantified. As a result of the procedure, the absorbance was obtained. The UV detector detects all of the components with excellent sensitivity. **Figure 1.17** shows a schematic of the UV detector.

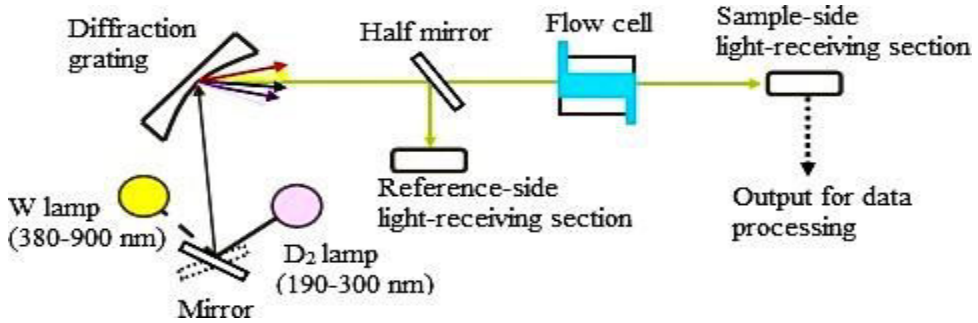


Figure 1.17: shows a diagrammatic representation of a UV-VIS detector optical apparatus

Fixed wavelength: only detects one wavelength, which is commonly 254nm.

Wave length variation: The UV detector is capable of detecting a wide variety of wavelengths, but one at a time.

Diode array: The brightness from the origin is sucked by the stream cell and then scattered by a diffraction grating into different wavelengths. The dispersed light is detected using a PDA (Photodiode Array). The CPU scans each photodiode, which gathers a different narrow wavelength band. The readings are displayed on the monitor.

Figure 1.18 shows a schematic diagram of a diode array detector.

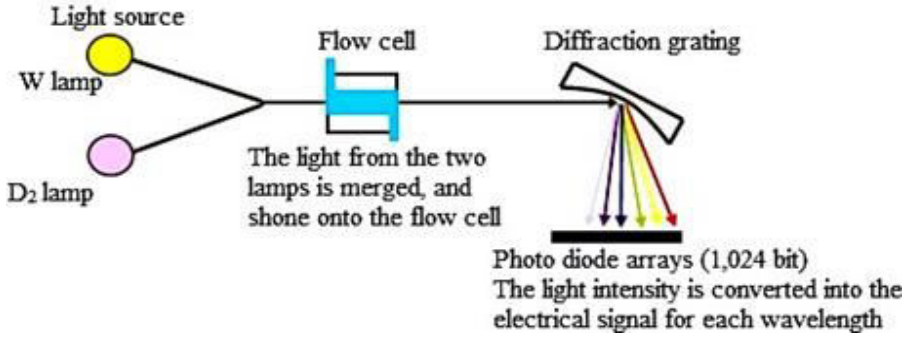


Figure 1.18: PDA optical system is depicted in diagrammatic form.

1.3.5.3 Fluorescence Detectors

A fluorescence detector is utilized to examine mixtures with fluorescence characteristics. The usage of this detector is limited due to the fact that only a few substances have fluorescence qualities. It is the most sensitive detector of the HPLC

detectors now available. This detector can identify a single analyte in a flow cell. For heavy UV absorbing materials, illumination perceptivity is 10 to 1000 times larger than UV detector perceptivity. Due to its spectroscopic and chemical particularity, it is particularly selective for analyzing chemicals from complicated matrix. It's most commonly used to measure samples that contain certain fluorescent species.

1.3.5.4 Electrochemistry-based detectors

Electrochemical detectors detect molecules that undergo oxidation or reduction processes. As the sample passes between the electrodes, they calculate the difference in electrical potential.

1.3.5.5 Evaporative Light Scattering Detector (ELSD)

The components that do not absorb UV rays are analysed using ELSD detectors. The mobile phase solvent is pushed through a nebulizer from an HPLC. The mobile phase evaporates and small particles remain after passing through a heated funnel. These particles scatter light, which is detected by a photomultiplier, when they travel across a narrow light beam. The detector's response is determined by particle size and number. It gives a flat baseline even with gradient elution.

1.3.6 Microprocessor control and data handling device

Microcomputers are used to combine the signals after they have been seen. The integrator reports the retention time, peak area, and peak height, making qualitative and quantitative analysis easier. Using various chromatography software, the obtained data is evaluated and transformed to a presentable format.

1.4 HPLC applications

1. The pharmaceutical industry

Prepare massive amounts of pure materials. Complex molecule separation

Quantitative and qualitative research

To look for trace contaminants in the purified chemicals Pharmaceutical product self-life determinations

2. The environment

It's used to monitor air quality and test drinking water.

To detect trace amounts of pollutants in pesticides and waste oil, such as PCBs.

3. Disease and disorder clinical diagnosis

4. Forensic examination

5. The food processing industry

It is used in the food sector to detect, separate, and analyse additives, preservatives, proteins, vitamins, and amino acids for quality checks and analysis.

6. Steroid determination in urine, sweat, and blood

It's used in bioinformatics and DNA fingerprinting, for example.

1.5 Approaches to the development of analytical method

The reverse phase chromatographic separation technique is the most widely used analytical technology in the pharmaceutical (**13)** business. Reverse phase chromatography is widely utilised for medicinal substance assay and impurity profiling. In a Quality by Design (QbD) (**14**) setting, the importance of technologies such as HPLC and UPLC is expanding. The method's requirements are usually determined by the drug's stage of development. The emphasis in the early stages is on quick turnaround and high throughput, whereas in the latter stages, the emphasis is on high throughput and low turnaround time. Pharmaceutical manufacture must be a simple procedure that is also sturdy, tough, and technically simple. The following parameters must be closely assessed while developing simple, robust, and tough analytical procedures.

- Literature gathering
- Chemical design pH and pKa values of substance
- Diluent choice/Sample dispersible
- Column choice
- Detector choice
- Mobile phase choice

1.6.1 Gathering of literature

Before beginning the technique creation process, the same or similar types of pharmaceuticals can be found in pharmacopoeias such as IP, USP, JP, EP, and chromatographic publications and copyright, among others. If the needed technique is

useable, assess its applicability to satisfy or adjust needs, such as solve probable adulterants during the preparation phase, one of which is adulterations, as well as process adultarations and deterioration. Gather all contaminants, normal, and test mixtures, as well as data on physico-chemical effects, at each step.

1.6.2 Chemical make-up

Beginning substances, byproducts, middle products, adultarants, and deterioration results, also their polarity, are compared to the structure of the medicinal ingredient (whether they are polarised more or < the compound of interest). It is feasible to determine if a particle is neither acidic nor basic, acidic, or alkali depend on the functional group of the molecule. The pH value of the movable phase can be elected depend on the compound's nature. If the chemical is acidic,

acidic mobile phases are preferred. A low pH and a basic mobile step are desirable if the chemical is basic. A neutral mobile step is ideal for neutral substances. The elution of a substance is determined by its polarity. Under reverse phase circumstances, a molecule with a higher hydrophobicity will be preserved for a longer period of time. There is a chance that non-aqueous conditions will be required. If a substance is ionic, it is hydrophilic (less hydrophobic) in nature and hence has a shorter retention time. For the separation of these types of chemicals, HELLIC columns are used.

1.6.3 Compound pH and pKa values

The nature and polarity of a substance can be determined using pH and pKa values. When the movable phase pH is equal to the pKa of the substance, the chemical is 50% ionised. The transfer in degree of ionization is approximately 90percent if pH is one part away from the substance's pKa result; it is approximately 99% if pH is two parts away from the substance's pKa value; and it is approximately 99.9% if pH is 3 parts away from the analyte's pKa value. The rule of two pHs, which is used to estimate the degree of ionisation, was thus devised. Most pH-related alterations occur within 2 units of the pKa value. During this range, the compound either has an ionic or non-ionic structure, and the chemical retention duration remains constant regardless of pH.

1.6.4 Sample solubility/diluent choice

In movable phase results, movable phase-organic combination, and water- organic combination, the solubility of all components in pharmacological compounds is investigated. The movable phase is a secure alternative for test diluents since it detaches baseline noise, negative peaks, and ghost peaks. All of the components must be totally soluble to obtain a simple solution.

1.6.5 Selecting Columns

A number of different column-styles are offered in the business market place. To guarantee consistency, we must look at lot to lot and batch to batch reproducibility before choosing a column. All internal diameter, surface region, particle size, and carbon load must be tested in order to ensure system compatibility. The non-polar column and the polar period of mobility were utilized in the RP-chromatographic analysis. Columns C18, C8, C4, cyano, phenyl, and amino can be used to counteract a more polar mobile phase, i.e. C18, C8, C4, cyano, phenyl, and amino. Mobile phase polarity is lower in an NP-chromatographic analysis. Normal phase chromatography uses normal phase, cyano, phenyl, silica, and chiral columns.

For very good resolution and retention, start with C8 or C18 columns for reverse phase chromatography. The ideal type of phases for development of early quick methods are C8 and C18, which are both C8/C18 phase complexes. This figure shows the polarity and hydrophobicity of columns, as shown in **Figure 1.19**.

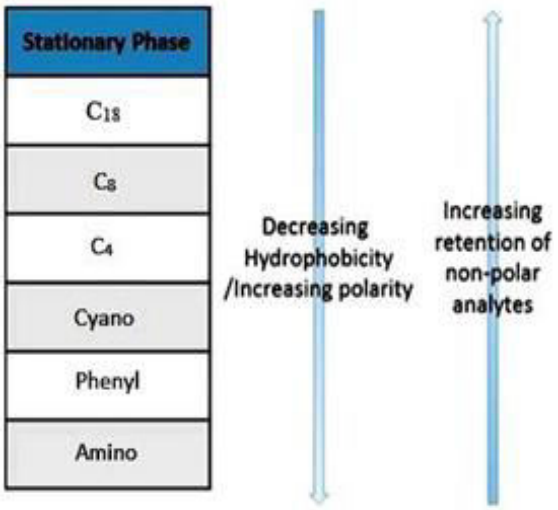


Figure 1.19: Shows the polarity order of different static phases.

1.6.6 Selecting a Detector

In liquid chromatography, the detector is a crucial component. For precise determination and selective separation, it should be carefully chosen. The most important component is determined by the progress of a feature separation that may be inspected and registered automatically. For solutes of different orders of magnitude, a satisfactory detector response is linear. It should be very reproducible, dependable, and stable. It should have a low dead volume to reduce extra-column band broadening. The suitable detection technology must be chosen depends on the type of the molecule and its makeup [**Table 1.02**].

Table 1.02: Detector selection depending on chemical application

Detection technique	Molecule structure and nature
UV	Compounds containing chromophores are referred to as chromophores.
Fluorescence	A characteristic of certain substances is used for fluorescence.
Electrochemistry-based detectors	When it comes to chemicals that are easily oxidised
RI detectors	It's a detector that you can take with you everywhere you go. It's employed for chemicals that don't need a lot of sensitivity and don't have a chromophore. For gradient elution, however, it is unsuccessful.
Evaporative Light Scattering Detectors (ELSD)	These are superior to the RI detectors used in gradient elution because they have a higher sensitivity. A universal detector is another name for it.

When a compound lacks chromophores, alternative detectors such as RI, GC, or ELSD are used. Record the spectra of all the molecules in a component whether the detecting technology is a UV-Visible spectrophotometer or a photodiode array (PDA). Select the product's absorption limit by considering all of the product's elements. Other issues, such as component intermediates, degradations, and process-related contaminants, must be addressed. supplies of raw materials If any component acts differently, conduct a dual wavelength analysis and compare data from different scanning ranges. Peak purity with all elements must be ensured, and the purity threshold must be greater than the purity angle.

1.7 VALIDATION OF ANALYTICAL METHOD

Validation is a critical component of any material presented to international regulatory agencies. Unless using methods published in any recognised standard references or the applicable pharmacopoeia, procedures must be validated. All testing analytical methods must be evaluated for system suitability in actual use conditions and must be accurately recorded. All analytical techniques must be validated, according to ICH recommendations for addressing Q2 (R1), which represents analytical processes for validation. Excluded methods should be validated. The main goal of the International Conference on Harmonization is to obtain agreement between the European Community, the United States of America (EC) and Japan, the three biggest geographic markets, on the technical requirements for product registration. According to the United States Pharmacopoeia (USP) and the United States Food and Drug Administration (US FDA), the current method validation guidance documents used by the United States Pharmacopoeia (USP) and the United States Food and Drug Administration (US FDA) both refer to the International Conference on Harmonisation (ICH) guidelines. Linearity, specificity, reliability, detection limit, range, quantitation limit, exactness, and resilience are some of the most commonly used typical validation characteristics for many types of tests. Analytical method validation also considers the applicability of the system and the stability of analytical solutions.

1.7.1.1 Selectivity

Both terms selectivity and precision are utilized interchangeably, as in "selectivity is precision with selection." Selectivity is a term that refers to a process that produces a response for a wide range of possible chemical entities, whereas specificity is a process that produces a response for a single analyte. the answer stands out from the rest Selectivity is a better term to use, because few analytical procedures respond to a single analyte. Specificity is defined as the capability to measure the analyte of interest with complete certainty while simultaneously excluding all other potentially interfering substances from the sample matrix. The measure of how intense other interferences are is called specificity. The peak response is a result of a single analyte, which means there are no other components present. Tailing factor, resolution, and plate count are used to calculate it. The resulting spectra are also compared to determine peak homogeneity using a contemporary PDA device, which calculates peak homogeneity by measuring the spectra obtained over a peak. To accurately measure the purity of a sample, make sure that the concentration of the sample is in the acceptable range.

1.7.2 Precision

It refers to the precision of the analytical process, as well as the consistency of the expected point of reference and the result calculated. The proportion of component recovered by test or spiking in a blind sample is the metric. components or contaminants. For the test of a drug product, It is calculated by using known concentrations of analytes in combination with predetermined formulations. A drug sample is tested using a comparison method or a method which is highly similar to the second. Quantification of impurities is measured using impurity-spiked samples.

1.7.3 Accuracy

Under normal operational conditions, it defines how reproducible an analytical methodology is. Precision is commonly stated as a percent RSD when a statistically significant number of samples are considered. In accordance with ICH standards, precision is carried out in three stages. They are described as having intermediate accuracy, reproducibility, and repeatability. The repeatability of an analytical method performed under the same conditions within a short time period is referred to as an analytical method's effect. To have precision, at least 9 determinations (concentrations 3 times) are performed using the analytical technique's defined range, or at least 6 determinations (concentrations of 100 percent) are performed using the target

concentration. Reproducibility refers to the level of uniformity among laboratories. The outcomes found in random events, such as within-lab variation, facilities, various days and so on, reflect intermediate precision. Confidence intervals, coefficient of variance, relative standard deviation, and standard deviation should all be included in the documentation research.

1.7.4 LOD (detection limit)

An amount of analyte which can be detected but not quantified in a specimen that has been quantified properly is the smallest that can be detected. A determination of whether an analyte is above or below a certain threshold level is a type of limit test. If you have a S/N ratio of 3 or 2:1, you'll use the LOD calculation.

1.7.5 Limit of Quantification (LOQ)

Under the given conditions of the technology, the minimal concentration of an analyte in a specimen that can be detected with sufficient exactness and reliability is found. The LOQ, which is calculated using the S/N ratio of 10:1, is widely employed. It is calculated by calculating the lowest level of reliability and exactness that can be achieved for the analyte, and measuring known concentrations of the analyte. When you calculate LOD and LOQ values, you can use the linear regression method to obtain estimates for them. The device response, y, is assumed to be linearly related to the nominal concentration, x, over a certain concentration range. It's possible to display it in a variety of ways.

The letter „a' represents an interception in this example.

When b is the slope of the line, and x is the explanatory variable,

y is the dependent variable.

This model can estimate LOD, as well as LOQ.

Where b denotes the steepness of the curve.

The standard deviation of the response is denoted by Sa.

Use this approach in any context. When research is conducted in a setting where there is no background noise, it is most productive.

1.7.6 Range/Linearity

The term linearity refers to the test results being directly proportional to the concentration of the analyte in the specimen. Most commonly, linearity is defined as the slope of the regression line's regression equation's standard deviation. The range of analytical methods are defined as the distance between the lowest and highest amounts (concentration) of the analyte for which the exactness, reliability, and linearity of the method have been exhibited. It is possible for ranges to be given in the same units as the procedure results. There are five concentration levels recommended by the International Conference on Harmonization, as well as minimum ranges (ICH). When screening tests have a minimum specific range of 80 to 120 percent of the target concentration, this is known as a minimum specific range screening test. When screening tests have a minimum specific range of 80 to 120 percent of the target concentration, this is known as a minimum specific range screening test. To ensure the correct reading, a range needs to be specified that reflects the total number of harmful or powerful contaminants that need to be tracked.

1.7.7 Ruggedness

Ruggedness is defined as the degree of reproducibility of results obtained under various settings, as measured by percent RSD, according to the United States Pharmacopeia (percentage of the relative standard deviation). Instruments, analyzers, laboratories, experimental periods, and chemical reagents are among the circumstances listed above. The ICH guideline on definitions

and nomenclature included no mention of toughness. Instead of covering the toughness topic, an ICH prefers to discuss reproducibility (precision).

1.7.8 Robustness

It's described as the ability to remain unaffected by tiny changes in the method's analytical parameters in the face of adversity. Process variables like pH, percent organic solvent, temperature, or ionic strength affect the results, and those variables can be adjusted to manipulate the results. Designers should keep in mind the principles of the International Conference on Harmonization, and they should ensure that their systems are as robust as possible early in the design process (ICH). It is important to evaluate various equipment, such as analyzers, as well as the stability of analytical solutions before making any decision. if the results of a process or further measurements are sensitive to changes in analytical technique parameters, the procedure or measurements should be followed under acceptable analytical technique specification changes.

1.8 Assay method indicating stability

Patients who use a pharmaceutical medicine for a specific ailment expect it to be effective and safe. The product must retain its potency, purity, identity, and quality for the duration of its market availability, as required by international pharmaceutical regulatory organisations. Due to the use of the marketing application, agencies want to see data on product stability in order to help support the product's suggested expiration date. So the agency needs to conduct stability studies in order to do so. The ICH requires medicinal compound stability studies to be conducted using hydrolytic, oxidative, thermal, and photolytic stress tests.

1.8.1 Degradation under duress

The forced degradation research is a crucial component of method development; these investigations are carried out to define intrinsic stability features. Deliverables for stress degradation must be focused on development activities rather than degradant isolation and identification. It's a process that involves degrading pharmaceuticals and other compounds under more severe settings than accelerated settings. As a result, degradation products are produced in order to measure the analyte's stability. Forced degradation investigations are carried out if a stability indicating approach is required. Degradation studies must be done whenever methodologies, formulations, or processes are changed. Hydrolytic (acidic and basic degradation), thermal, oxidative, and photolytic degradation were applied to the drug product or drug material in order to establish a stability indicating approach. As far as stress studies for all of the pharmaceuticals being looked at are concerned, they are all done in the same conditions, but only with time and temperature varying from drug to drug.

- Thermolytic deterioration,
- Hydrolytic deterioration,
- Oxidative deterioration and
- Photolytic deterioration

Are the most well-known investigations of forced deterioration.

1.8.1.1 Thermolytic degradation

This test was carried out by melting a substance to determine its melting point. If this is the case, the sample will be strained at 70 °C or 40 °C below its melting point at a temperature of 150 °C. This component undergoes strain at a temperature of 105°C, having a melting point greater than 150°C. To achieve the desired deterioration, heat the sample to a high temperature (10 percent to 20 percent).

1.8.1.2 Degradation by hydrolysis

The breakdown produced by hydrolysis is called hydrolytic degradation. A simple and acidic hydrolysis process is one type of hydrolysis process, while an acidic and simple hydrolysis process is the other. Acidic and basic conditions as well as water should be present for more hydrolytic reactions to occur. Be sure to test the drug molecule of interest for its ability to dissolve in water, as hydrolysis is done in water.

Molecules that are hydrophobic won't dissolve in water, so you need to dissolve them with a co-solvent. Co-solvents like acetonitrile and methanol are frequently used. For acidic degradation, the samples were treated with a suitable concentration of hydrochloric acid and refluxed for 12 hours, or until a degradation of 1-20% was achieved. It was neutralised before injecting the stress solution. The sample was treated with an appropriate strength sodium hydroxide and refluxed for 12 hours, or until a degradation of about 1-20% was achieved. It was neutralised before injecting the stressed solution.

1.8.1.3 Degradation due to oxidation

Treated the sample with hydrogen peroxide (H2O2) or a mixture of KMnO4 and K2Cr2O7 at room temperature, and then analysed the solution.

1.8.1.4 Photolysis induces chemical degradation

A UV light illuminates the sample with an average of 1.2 million Lux hours and 200 Watt Hrs/Sq. Meter for an exposure time of one hour. The sample is then collected and analysed at regular intervals**.**

1.9 Study of system suitability

The analytical validation of the method and evidence of correct analytical system function must be provided prior to beginning laboratory studies. The system's suitability is determined by a replication assessment of the standard or reference solution. The system precision value defines the upper limits for each of the tailing factor, theoretical plates, RSD, and resolution parameters, but if these limits are not exceeded, the system is suitable.

1.9.1 Resolution [Fig 1.20]

Chromatography peak distance calculation uses Resolution. In quantitative estimations, resolving the finest peaks is the main objective. Affinity for the stationary phase governs the separation between closely spaced peaks. Increasing the length of the column, changing the static phase particle size, and changing the polarity of the mobile phase is all effective at co-eluting the co-eluting chemicals. It is deemed complete if the resolution is equal to or greater than 1.5.

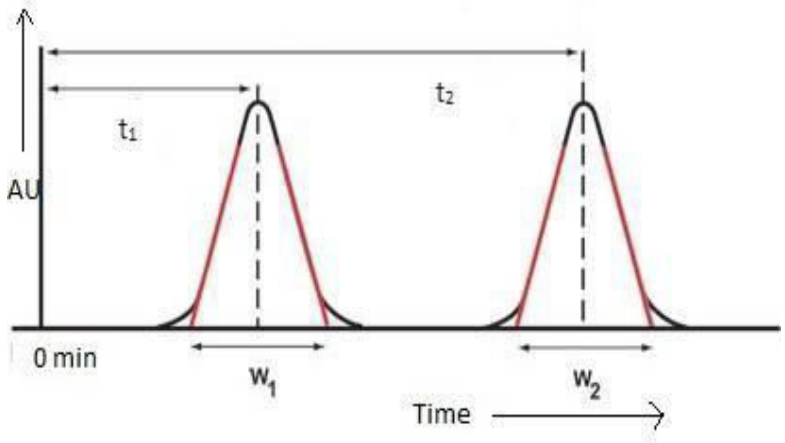
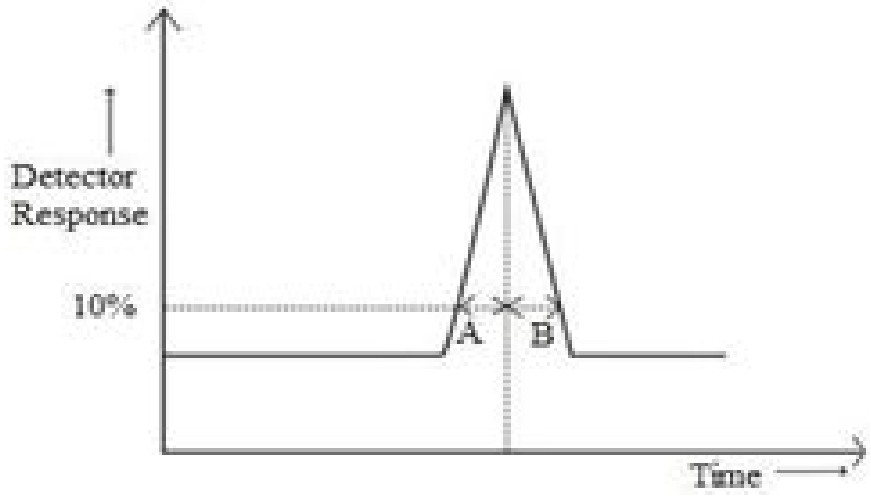


Figure 1.20: Resolution

The peak widths W1 and W2 are obtained from the tangent points where the baseline intersects, and the retention times for peak2 and peak1 are also noted.

1.9.2 Asymmetry or Tailing Factor (AS) [Fig 1.21]

There are numerous factors which influence the shape of the chromatographic peak, which results in an asymmetrical shape. Peak deformation causes peak tailing or peak fronting. When a component has more than one retention mechanism, peak tailing occurs. When the stationary phase has been capped, or the pH of the movable phase has been adjusted, the number of discrete components can be decreased.



Asymmetry factor

Figure 1.21: Asymmetry or Tailing factor (A_S)

Peak widths A and B represent ten percent of the peak height of the tallest peak Since Ideal peak, AS =1. 0, AS (or equivalent amount) must be between 0.90 and 1.10.

When AS is greater than or equal to 1.2, trailing is apparent

Tailing is calculated by applying the USP formula that includes widths A and B, which are each at 5% of peak height, as shown.

For the specification of tailing, T ≤2.00 is acceptable.

1.9.3 Precision

Replica injections of compound preparation are compared to verify the precision requirements. There are a total of six copies. If the required number is larger than 2, compound data injections are utilized to determine the percent RSD. As long as the required value is less than 2.0, five replicate injections of data are used to determine the percentage of random variation.

1.9.4 Theoretical plates

This assumes that chromatography columns have many independent layers, known as theoretical plates. These imagined layers have movable and stationary phases that balance each other out. This molecule moves the equilibration movable phase from one plate to the next in the column by moving the equilibration mobile phase. Plate count is a measure of how effective the column is (N). Plates should not be fewer than 2000 in total.

1.9.5 Retention factor (k) [Fig 1.22]

One-and-a-half the ratio of time spent in the mobile phase to time spent in the stationary phase is known as the retention factor.

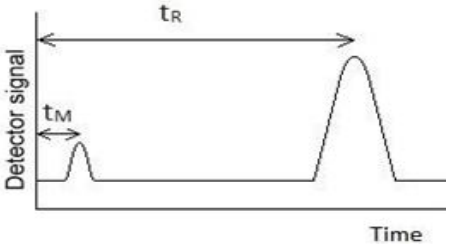


Figure 1.22: Retention factor (k′)

Ideally, k' is greater than or equal to 2.0.

When the k' value is high, the ketonate is higher in the compound holding the column.

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2.

STABILITY INDICATING VALIDATED HPLC METHOD FOR THE DETERMINATION OF ACECLOFENAC AND MISOPROSTOL IN BULKAND PHARMACEUTICAL FORMULATION

2.1 ACECLOFENAC

2.1.1 Drug profile of Aceclofenac:

Aceclofenac is a nonsteroidal anti-inflammatory drug (**1**) (NSAID) analog of diclofenac. It is used for the relief of pain and inflammation (**2**) in rheumatoid arthritis (**3**), osteoarthritis (**4**) and ankylosing spondylitis (**5**). Aceclofenac should not be given to people with porphyria (**6,7**) or breast-feeding (**8**) mothers, and is not recommended for children. It should be avoided near term in a pregnant woman because of the risk of having a premature closure of ductus arteriosus (**9**) leading to fetal hydrops (**10**) in the neonate.

Structure of Aceclofenac [Fig. 2.01]:

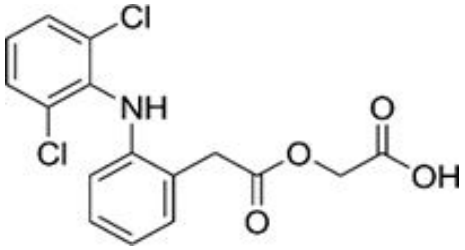

Fig 2.01: Aceclofenac's chemical structure

2.1.2 Name of the IUPAC: 2-[2-[2-(2,6-dichloroanilino)phenyl]acetyl]oxyacetic acid

2.1.3 Molecularformula: C16H13Cl2NO4

2.1.4 Molecular weight: 354.18 $g \cdot mol^{-1}$

2.1.5 Category:

Aceclofenac is classified as a BCS Class II, poorly soluble and highly permeable medication, and is used to treat pain. Prostaglandins (PG) are responsible for pain, edoema, inflammation, and fever. Aceclofenac acts by blocking the enzyme cyclooxygenase (COX).

2.1.6 Mechanism of action:

Aceclofenac reduces the generation of inflammatory mediators such as prostaglandin E2 (PGE2), IL-1, and TNF from the arachidonic acid (AA) route by inhibiting COX-2, a key enzyme in this pathway. Diclofenac, which is converted from aceclofenac 6, is hypothesised to have a role in the inhibition of IL-6. Reactive oxygen species generation is decreased when inflammatory cytokines are suppressed. This drug has been found to reduce the formation of nitrogen oxide in human joints by aceclofenac. aceclofenac also inhibits neutrophil adherence to endothelium by reducing the expression of L-selectin (CD62L), a cell adhesion protein produced on lymphocytes. Glycosaminoglycan synthesis in human osteoarthritic cartilage may be facilitated by aceclofenac's ability to decrease IL-1 production and activity.

By suppressing the IL-1-induced synthesis of promatrix metalloproteinases-1 and- 3, 4'-hydroxyaceclofenac provides chrondroprotection by inhibiting the release of proteoglycan from chrondrocytes.

2.1.7 Side effects of Aceclofenac:

- Abdominal pain.
- Constipation.

- Diarrhoea.
- Nausea.
- Vomiting.
- Skin rash.
- Dizziness.
- Visual Disturbance.

2.1.8 Contraindications:

- Ischaemic heart disease.
- Peripheral arterial disease.
- Cerebrovascular disease.
- Congestive heart failure (New York Heart Association, NYHA, classification II-IV)

2.1.9 Absorption:

Aceclofenac is promptly and fully absorbed as an unmodified medication after oral administration. 1.25 to 3.00 hours after intake, peak plasma concentrations are obtained in the blood. There are roughly 57 percent more concentrations of aceclofenac in synovial fluid than there are in blood plasma.

2.1.10 Uses:

To alleviate discomfort, aceclofenac is prescribed. One of the most often prescribed medications for rheumatoid arthritis, ankylosing spondylitis, and osteoarthritis. Aceclofenac is a non-steroidal anti-inflammatory medication (NSAIDS).

2.2 MISOPROSTOL

2.2.1 Drug profile of Misoprostol:

Misoprostol is a synthetic prostaglandin (**11**) medication used to prevent and treat stomach ulcers, start labor, cause an abortion, and treat postpartum bleeding due to poor contraction of the uterus. Misoprostol is taken by mouth when used to prevent gastric ulcers in persons taking NSAIDs. For abortions it is used by itself and with mifepristone or methotrexate (**12**). By itself, effectiveness for abortion is between 66% and 90% (**13,14**). For labor induction or abortion, it is taken by mouth, dissolved in the mouth, or placed in the vagina (**15**). For postpartum bleeding it may also be used rectally (**16**). Common side effects include diarrhea and abdominal pain. It is pregnancy category X meaning that it is known to result in negative outcomes for the fetus if taken during pregnancy. In rare cases, uterine rupture (**17**) may occur. It is a prostaglandin analogue (**18**) specifically, a synthetic prostaglandin E_1 (PGE_1).

Structure of Misoprostol [Fig 2.02]:

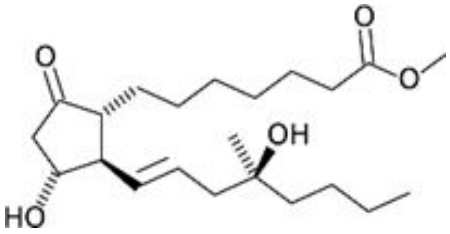

Fig 2.02: Misoprostol chemical structure

2.2.2 Name of the IUPAC: methyl 7-[(1R,2R,3R)-3-hydroxy-2-[(E)-4-hydroxy-4-methyloct-1-enyl]-5-oxocyclopentyl]heptanoate

2.2.3 Formula molecular : $C_{22}H_{38}O_5$

2.2.4 Molecular weight: 382.541 g/mol^{-1}

2.2.5 Category: Gastrointestinal Agents, Other; Prostaglandins, Endocrine include Cytotec in their family of medications.

2.2.6 Mechanism of action:

Synthetic prostaglandin E1 analogue misoprostol inhibits gastric acid output by stimulating prostaglandin E1 receptors on stomach parietal cells. 3 Mucosal bilayer thickening and increased mucus and bicarbonate secretion allow the mucosa to produce more new cells. 3 By binding to uterine smooth muscle cells, misoprostol increases contraction force and frequency while also degrading collagen and lowering cervical tone. 3

2.2.7 Side effects of Misoprostol :

- Diarrhea.
- Abdominal pain.
- Headache.
- Severe allergic reaction (anaphylaxis)
- Anemia.
- Abnormal heart beat.
- Chest pain.
- Gas (flatulence)

2.2.8 Contraindications:

Anticoagulant treatment, bleeding disorders, and ectopic or molar pregnancies are all examples of contraindications for a misoprostol-induced abortion procedure.

2.2.9 Absorption:

Misoprostol is frequently undetectable in plasma because of its fast de- esterification prior to or during absorption. The active metabolite of misoprostol, misoprostol acid, has a clearance rate of 0.286 litres per kilogramme per minute.

2.2.10 Uses:

Preventing ulcers in patients who use aspirin and other arthritis or pain medications may be done with the aid of misoprostol. In addition, it reduces the amount of stomach acid produced.

2.2.11 Adult dose:

- 100mcg
- 200mcg

2.3 Literature resurvey

B. Jagadeesh Naidu, K.E. Pravallika, Ravi Parimi, Validation of a new stability- indicating RP-HPLC technique for simultaneous measurement of aceclofenac in bulk and misoprostol in their combination dose form: Using reverse phase technology, we developed and validated a technique for simultaneous determination of the bulk and combination tablet formulation of Aceclofenac and Misoprostol, as well as stability tests for both formulations. Aqueous 0.01M triethylamine buffer (pH 2.5) and acetonitrile: aqueous 0.01M triethylamine buffer (pH 2.5)

were used as the mobile phase and the flow rate was set at 1 ml/minute. The eluent was monitored by UV detector wavelength at 227 nm in high-performance liquid chromatography (HPLC) with isocratic elution. There were 2.541 and 3.831 minutes of retention for Aceclofenac and Misoprostol, respectively; the LOQ was 0.250 and 0.255 nm; the correlation coefficient was 0.98 and 0.999; and the LOD was 0.125 and 0.127 nm. Aceclofenac and Misoprostol were intended to have a linearity range of 0.5-7.52 g/ mL and 0.51-7.56 g/ mL, respectively. Relative standard deviation (percent RSD) values of 0.21 and 0.28 for Aceclofenac and Misoprostol in accuracy studies indicated percentage recovery rates of 100.1% to 100.8 percent and 100.0% to 100.4 percent. According to ICH guidelines, the new approach was verified and confirmed to be optimum and optimal for routine laboratory analysis.

Kanhaiyalal Patidar, Jigar Mehta, Vipul Patel , Nayan Kshatri and Niranjan Vyas, **An in vitro dissolving technique for misoprostol with HPLC analysis was developed and validated:** A reverse-phase liquid chromatographic technique will be used to create and verify a dissolving test for misoprostol tablets comprising 200 g of

misoprostol [1 percent in HPMC]. It was found that using a 500-ml deaerated water dissolving medium and swirling it at a speed of 50 revolutions per minute (rpm), together with sink conditions and dissolution mediums of various densities, provided optimal circumstances for the stability of the medication. Specificity, precision, linearity, and accuracy were all evaluated as part of the method's validation in preparation for submission to international regulatory agencies for approval. Over the course of the trial, more than 85% of the label quantity was released in the medium. Using the dissolvability test devised, the dosage form of misoprostol may be tested for quality control.

D.Vijaya Bharathi, B. Jagadeesh Kishore Kumar, Hotha Uday Patil, Indu Bhushan, Method development and validation for the detection of misoprostol free acid in human plasma by liquid chromatography–electrospray ionisation tandem mass spectrometry, with application to a clinical pharmacokinetic investigation: For the determination of misoprostol free acid in human plasma utilising misoprostol acid-d5 as an internal standard, a highly sensitive, selective, and evaporation-free SPE extraction, ESI-LC–MS/MS approach was established (IS). Using isocratic mobile phase on a reverse phase column and the corresponding [MH] anions with a mass range of 367–249 m/z for misoprostol acid and 372–249 m/z for IS, the analyte was separated and analysed by MS/MS in the multiple reaction monitoring mode. Misoprostol acid and misoprostol acid-d5 (IS) were eluted after 3.6 minutes of running time. A quantitative limit of 2.5 pg/mL was established for the newly proposed approach in human plasma. Misoprostol acid in human plasma exhibited a linear response function ($r > 0.998$) for the concentration range of 2.5–1200 pg/mL. It was determined that the FDA-accepted precision values for misoprostol acid were acceptable on an intra- and inter-day basis. Benchtop, auto-sampler, and freeze/thaw cycles all found misoprostol acid to be stable. An oral pharmacokinetic investigation in humans using the newly designed test technique.

HuiyingSongGetuKahsay, FranEerdekens, YaxinTie, DannyHendriks, AnnV anSchepdaelet al, Misoprostol-related compounds and diastereoisomers are being separated using LC techniques that have been developed and validated: In addition to being used to treat and prevent stomach ulcers, misoprostol may be utilised to induce labour during an abortion owing to its labor-inducing properties. Many contaminants are present because of the drug's instability at higher temperatures and moisture levels. Misoprostol occurs as a combination of diastereoisomers (1:1). Reversed phase liquid chromatography (RPLC) and normal phase liquid chromatography (NPLC) methods are given for the separation of the related compounds and misoprostol diastereoisomers, respectively. An Ascentis Express C18 (150 mm x 4.6 mm, 5 m) column was used for the RPLC technique. At a flow rate of 1.5 mL/min, the mobile phase was a gradient mixture of 28:69:3 v/v/v ACN–H2O–MeOH and 47:50:3 v/v/v ACN–H2O–MeOH.

Detection was made at a wavelength of 200 nm. At 35 °C, an XBridge bare silica column was used to perform the NPC technique. 1-propanol–heptane–TFA (4:96:0.1% v/v/v) was injected into the mobile phase at 0.5 mL/min. At 205 nm, UV detection was carried out. The two diastereoisomers (Rs > 2) may be separated in less than 20 minutes using this LC approach. The ICH criteria were followed in the validation of both approaches. As a result, these novel LC techniques have been used effectively to monitor the purity and diastereoisomers ratio of misoprostol bulk medication, tablets, and dispersion.

2.4 Experimental

2.4.1 Chemical and reagents:

Merck India Ltd. in Mumbai, India provided the acetonitrile, HPLC-grade formic acid, and water needed for this experiment. Glenmark in Mumbai provided the APIs for the standards for Aceclofenac and Misoprostol.

2.4.2 Instrumentation:

For this work, we employed a Waters Alliance liquid chromatography (model e- 2695) that was monitored by the empower 2.0 data management system and a light diode array detector (model 2998).

2.4.3 Preparation of standard solution

Use 50 mg Aceclofenac and 50 mg Misoprostol as working standards, and put them in a 100 ml flask before diluting them with diluents to the desired concentration. Dilute the prepared solution to a final volume of 50 ml by adding diluents to a further 5 ml.

Preparation of sample solution

Assemble a flask with 100 ml of diluents and 69.5 mg of Aceclofenac and Misoprostol sample in it, then sonicate to dissolve it. A further 5 ml of the sample solution is diluted to 50 ml by adding diluents to the sample solution.

2.5 Method Development Analytical method development:

Accuracy for Aceclofenac and Misoprostol analysis has been established using HPLC in the proposed research.

2.5.1 Method development parameters:

Selection of following parameters in method development is very important.

- Mode of chromatography
- Wavelength
- Column
- Mobile phase composition
- Solvent delivery system
- Flow rate
- Injection volume

2.5.1.1 Selection of mode of chromatography:

Selected mode of chromatography	:	Reversed phase chromatography Basis of
selection	:	polarity of the molecule
Reason for selection elutes	:	as Aceclofenac and Misoprostol is
polar molecule it faster along with mobile phase		

2.5.1.2 Detector wavelength selection:

The choice of a detector wavelength is critical to completing the analytical procedure. PDA detector and wavelength are used to determine precise wavelength of the standard API, which is manufactured and injected into the chromatographic system using PDA detector and wavelength.

Selected wave length: 227 nm

Basis for selection: Maximum absorbance of analytes and impurities

Reason for selection: Aceclofenac and Misoprostol having maximum absorbance 227 nm.

2.5.1.3 Selection of column:

Column selected: Inertsil ODS (150x4.6mm, 3.5 µ)

Basis for selection: Based on the polarity, and chemical differences among analytics

Reason for selection:

Excellent physiochemical surface characteristics and compatibility with a wide variety of organic solvents are only some of the advantages of these materials.

2.5.1.4 Selection of the mobile phase composition and of the buffer:

Peak symmetries and separation are heavily influenced by the buffer and its intensity. There are a number of factors that must be taken into consideration when selecting the proper buffer strength for a chromatographic injection load.

Mobile phase preparation:

Solution A: Acetonitrile

Solution B: 0.1percent formic acid

2.5.1.5 Selection of the rate of flow:

Even in reverse phase separation for the resolution of tiny molecules, flow rate is a crucial element. However, in large-scale inverted phase chromatography, adding the sample solution at a high flow rate has a significant impact on the analytical results. The dynamic binding capacity of a sample might vary depending on the flow rate employed for sample loading. When scaling up the purification process, the dynamic binding capacity must be estimated to determine the optimal flow rate for loading. In this system, the flow rate is set to 1 ml/min and is dependent on factors such as flow factor, retention duration, column composition, separation impurity, and peak symmetrical symmetry.

2.5.1.6 Selection of injection volume:

For API estimate, a volume of 10 to 20 µL is often advised. Extraction proved problematic, thus the test concentration may be reduced and the injection volume increased to 50 µL in the end. As long as the specified column volume isn't overflowing, it's all ok. 10 µL of Aceclofenac and Misoprostol are injected in this approach.

2.6 RESULTS AND DISCUSSION

2.6.1 Trials in optimization of chromatographic condition: Trial-1 [Fig 2.03]

Buffer	:	Ammonium acetate pH-4 adjusted with OPA Mobile
phase	:	Acetonitrile and Buffer (80:20)
Column	:	Agilent eclipse XDB (250x 4.6mm, 5µ)

Rate of flow : 1ml/min Volume of injection : 10µl Period of run : 15 min

Wavelength : 200-400 nm Observation : Only one peak observed

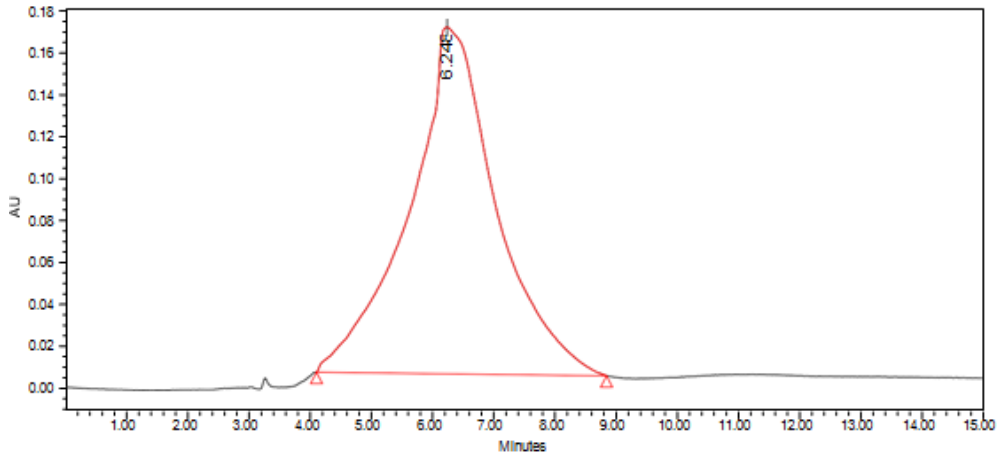

Figure 2.03: chromatogram of trial-1

Trial-2 [Fig 2.04]

Buffer : Ammonium acetate pH-4 adjusted with OPA Mobile phase : Acetonitrile and Buffer (70:30)

Column : Agilent eclipse XDB (250x 4.6mm, 5µ)

Rate of flow : 1ml/min Volume of injection : 10µl Period of run : 9 min

Wavelength : 227 nm

Observation : Merged peak is obtained

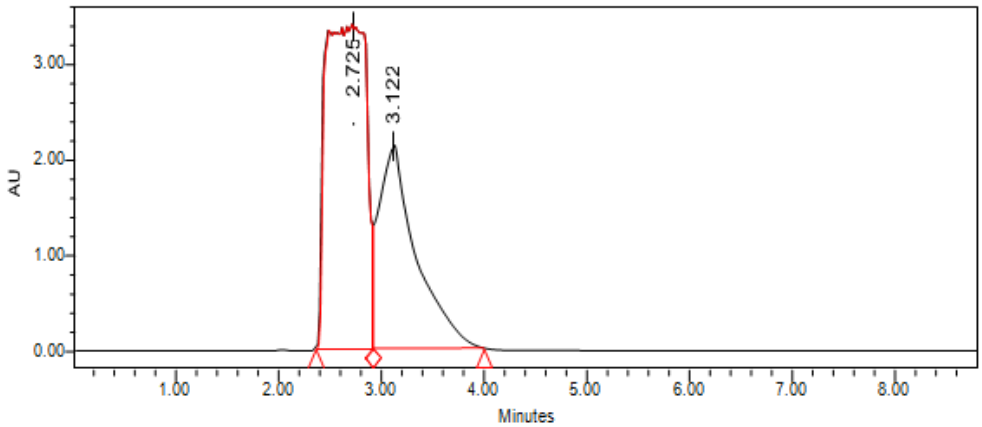

Figure 2.04: Chromatogram of trial-2

Trial-3 [Fig 2.05]

Buffer : Ammonium acetate pH-4 adjusted with OPA

Mobile phase : Acetonitrile and Buffer (60:40)

Column	:	Agilent eclipse XDB (250x 4.6mm, 5µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	15min
Wavelength	:	227 nm
Observation	:	Base line is not sufficient

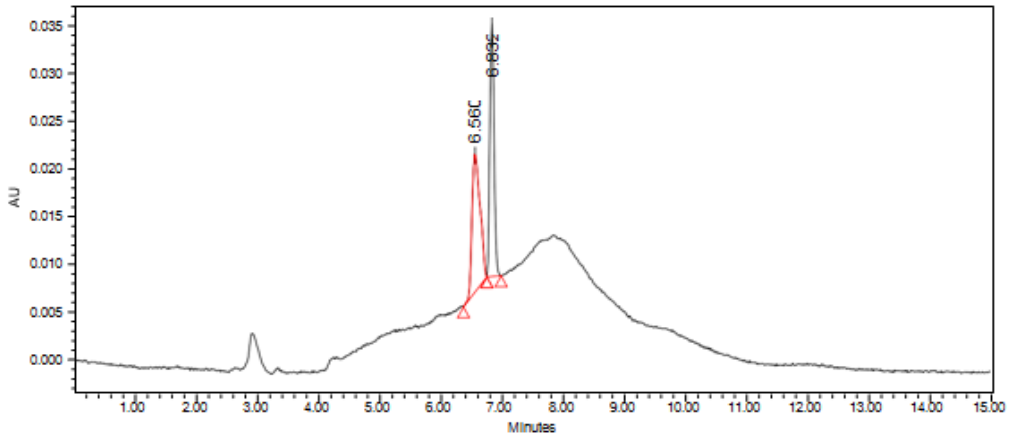

Figure 2.05: Chromatogram of trial-3

Trial-4 [Fig 2.06]

Mobile phase	:	Acetonitrile and 0.1% OPA (35:65)
Column	:	Agilent eclipse XDB (250x 4.6mm, 5µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	7min
Wavelength	:	227 nm
Observation	:	Broad peaks are obtained

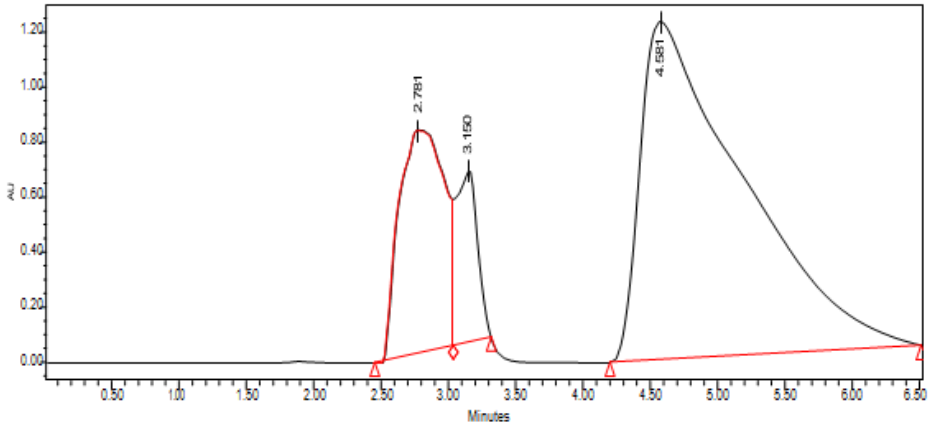

Figure 2.06: Chromatogram of trial-4

Trial-5 [Fig 2.07]

Mobile phase	:	Acetonitrile: 0.1% OPA (70:30)
Column	:	Inertsil ODS (150x4.6mm, 3.5 µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	7min
Wavelength	:	227 nm
Observation	:	Resolution is not within the limit

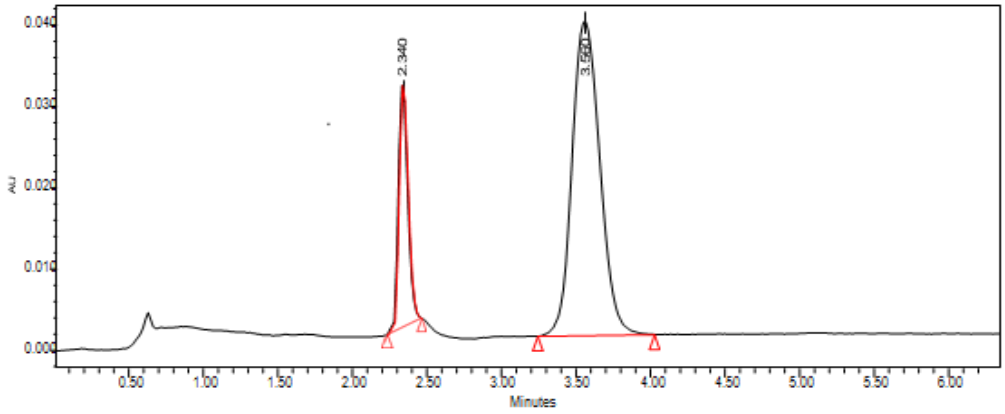

Figure 2.07: Chromatogram of trial-5

Trial-6 [Fig 2.08]

Mobile phase	:	Acetonitrile: 0.1% OPA (50:50)
Column	:	Inertsil ODS (150x4.6mm, 3.5 µ)
Rate of flow	:	1 ml/min
Volume of injection	:	10µl
Period of run	:	10 min
Wavelength	:	227 nm
Observation	:	This method is suitable for validation

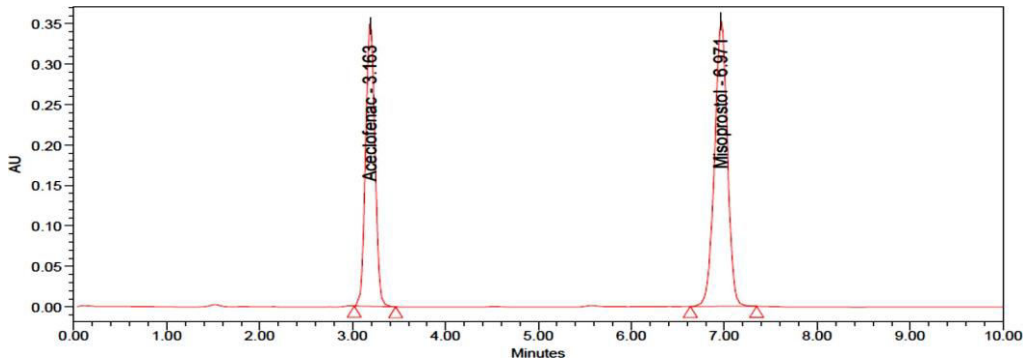

Figure 2.08: Chromatogram of trial-6

The chromatographic results of all trials were represented in the following **table 2.01.**

Trial No.	RT	Area	% Area	USP Resolution	USP Tailing	USP Plate count
1	6.248	16375076	100.00		1.06	91
2	2.725	684523	72.64		2.54	682
	3.122	274563	24.37	4.28	3.42	1785
3	6.560	131075	50.57		1.19	8734
	6.832	128116	49.43	1.29	1.15	44525
4	2.781	257415	18.74		1.08	1826
	3.150	481136	75.26	4.15	1.02	5263
	4.581	321523	23.27	5.22	3.55	1863
5	2.340	130185	20.67		1.23	6318
	3.560	499668	79.33	5.37	1.13	1806
6	3.163	3365478	1.15		0.96	3654
	6.971	3469581	1.08	5.27	1.14	8547

Table 2.01: Chromatographic results of all trials

2.6.2 Optimized method [Table 2.02, Fig 2.09]

S.NO	Parameter	Chromatographic condition
1	Mobile phase	Acetonitrile: 0.1% OPA (50:50)
2	Column	Inertsil ODS (150x4.6mm, 3.5 µ)
3	Rate of flow	1ml/min
4	Column temperature	Ambient temperature
5	Sample temperature	Ambient temperature
6	Wavelength	227 nm
7	Volume of injection	10µl
8	Period of run	10 min
9	Retention time	Aceclofenac Retention time-3.163 Misoprostol retention time-6.971

Table 2.02: Optimized method chromatographic conditions

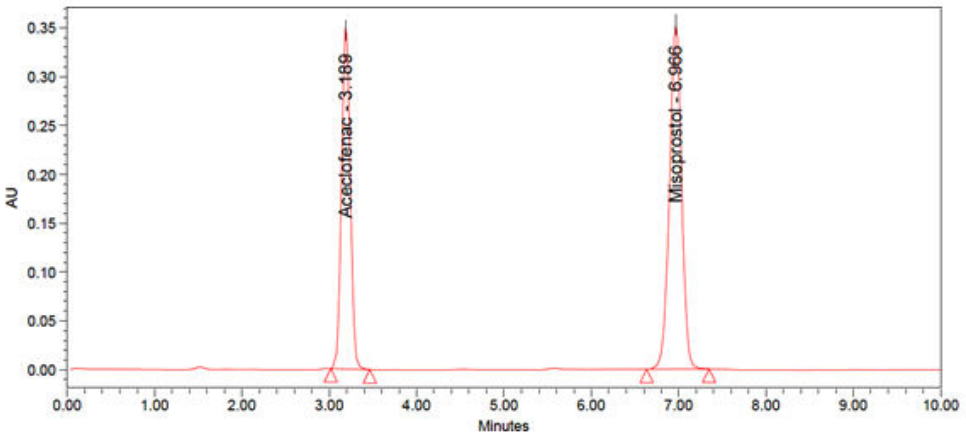

Figure 2.09: Chromatogram of standard

2.7 Validation of method

It has been shown to be accurate, specific and precise in compliance with the ICH Q2 (R1) requirements for device appropriateness, as well as robustness, LOD, LOQ and stability.

2.7.1 Specificity [Fig 2.10]

Specificity is the ability to test the analyte in the presence of other components, such as contaminants or excitements, that may be presumed to be present in the sample solution and the norm solution, without any interference. A blank sample was used as a control and spiked with Aceclofenac and Misoprostol.

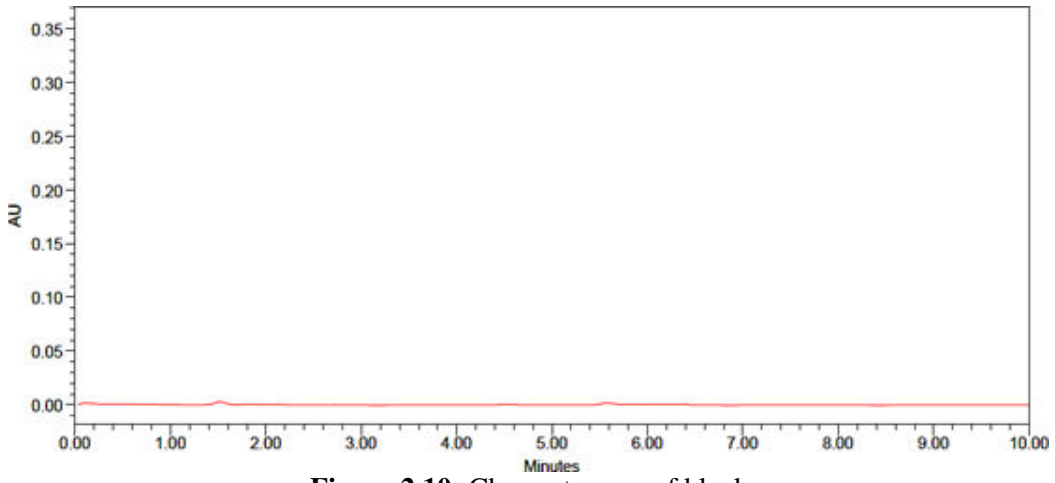

Figure 2.10: Chromatogram of blank

2.7.2 Linearity:

As a consequence of its capacity to deliver findings within a predetermined context, the empirical technique is linear. It was determined that the peak area correlated with the concentration of analytes in the sample: six standard solutions were used. The peak area was displayed on the calibration curve and the regression equations were derived using the normal solution concentration as a reference point. The least squares technique of least squares was used to calculate the slope, intercept, and correlation coefficient.

Linearity stock solution preparation:

Use 50 mg Aceclofenac and 50 mg Misoprostol as working standards, and put them in a 100 ml flask before diluting them with diluents to the desired concentration.

10 percent solution preparation:

Dilution of the stock solution in a new 50 ml volumetric flask was carried out using the diluents stated above.

25 percent solution preparation:

One of the stock solutions was diluted in a 50 ml volumetric flask with diluents to the mark in another 50 ml volumetric flask.

50 percent solution preparation:

2.5 ml of the aforesaid stock solution was diluted with the diluents up to the mark in a separate 50 ml volumetric flask.

100 percent solution preparation:

Stock solution was diluted in a 50-ml volumetric flask with the diluents up to the mark in a separate 50-ml volumetric flask.

125 percent solution preparation:

6.25 ml of the above-mentioned stock solution was diluted with the diluents to the appropriate concentration in a separate 50 ml volumetric flask.

150 percent solution preparation:
7.5ml of the above-mentioned stock solution was diluted with the diluents to the appropriate concentration in another 50 ml volumetric flask.

Procedure:
Using a chromatographic technique, measure the peak area for each degree. Plotting the peak area against the concentration (on the X-axis) and then calculating the correlation coefficient is what this method entails. Results of linearity is in **table 2.03** and calibration plots were in **fig 2.11, 2.12**.

Range:
To put it another way: The range of analytic approaches encompasses the gap between the top and lower levels of analysis.

Acceptance criteria:
The correlation coefficient should not be less than 0.999

Analyte	Range of Linearity	calibration curve equation	Correlation coefficient
Aceclofenac	5-75 µg/ml	Y=68390.79x+14397.06	0.99998
Misoprostol	5-75 µg/ml	Y=70351.52x+4707.45	0.9999

Table 2.03: Results of linearity

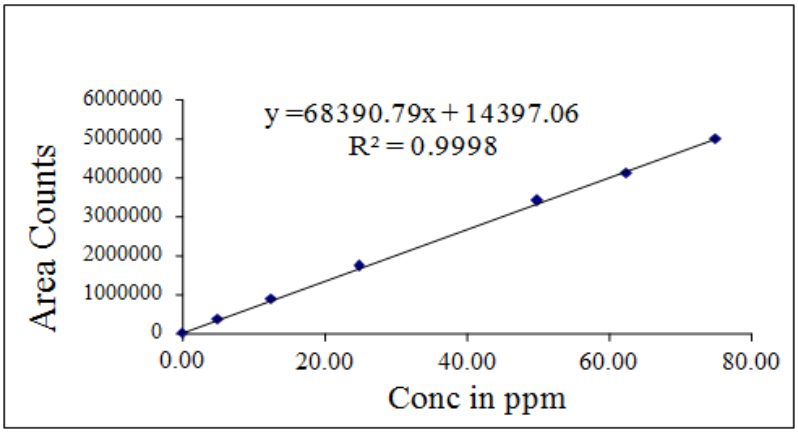

Figure 2.11: Calibration plot of Aceclofenac

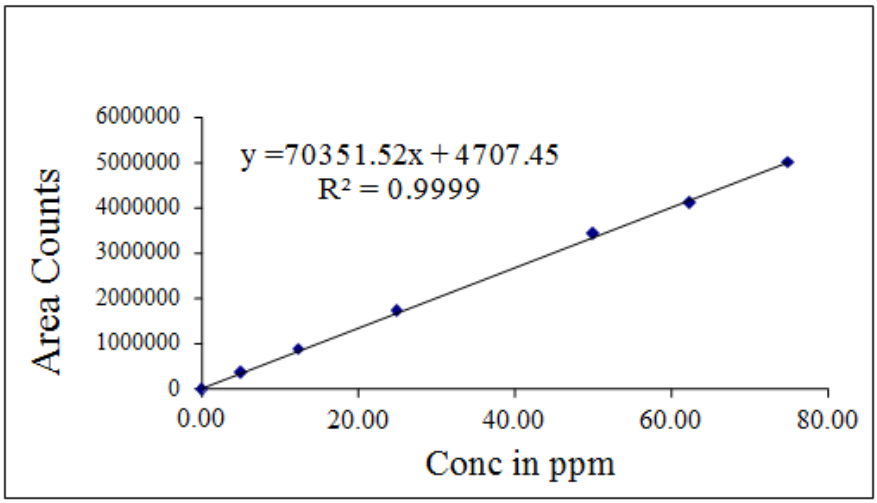

Figure 2.12: Calibration plot of Misoprostol

2.7.3 ACCURACY:

50 percent solution preparation (with respect to the concentration of the target assay)
Recovery trials at three different concentration levels determined the accuracy (50 percent , 100 percent and 150 percent). APIs were produced at concentrations of 25, 50, and 75 g/ml. The assay was carried out in accordance with the test procedure, in which the test solution was injected into three preparations at each spike level. Aceclofenac and Misoprostol had percentage recovery values of 99.5-100.6 and 99.4- 100.4, respectively. Accuracy results were in **table 2.04.**

Acceptance criteria:
The rate of recovery for each stage should be between 98-102 percent

% of target concentration	Aceclofenac		Misoprostol	
	% Recovery	% RSD	% Recovery	% RSD
50	99.6	1.19	99.4	0.39
100	100.6	0.64	100.2	0.47
150	99.5	0.35	100.4	0.58

Table 2.04: Accuracy results of Aceclofenac and Misoprostol

2.7.4 Precision:
In an analytical procedure, the rate at which repeated homogenous samplings provide similar results is a measure of accuracy. Six injections of Aceclofenac (50ppm) and Misoprostol (50ppm) were spiked to ensure the correctness of the injection method. **Table 2.05** gives results of system precision and the **figures** from **2.13-2.18** shows system precision chromatograms.

S.NO	Standard results	
	Aceclofenac	Misoprostol
1	3352468	3496582
2	3324175	3478512
3	3302659	3465001
4	3354628	3485796
5	3346291	3477519
6	3344570	3425631
Mean	3337465	3471507
Std dev	20179.790	24759.163
% RSD	0.60	0.71

Table 2.05: Results of system precision

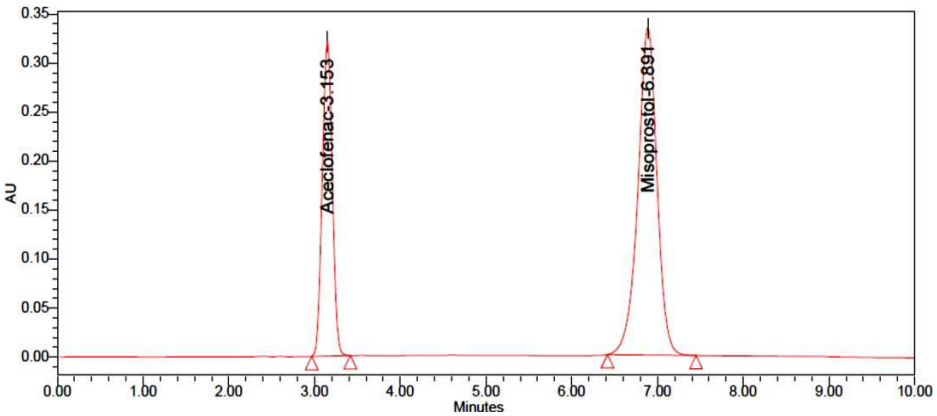

Figure 2.13: Chromatogram of system precision-1

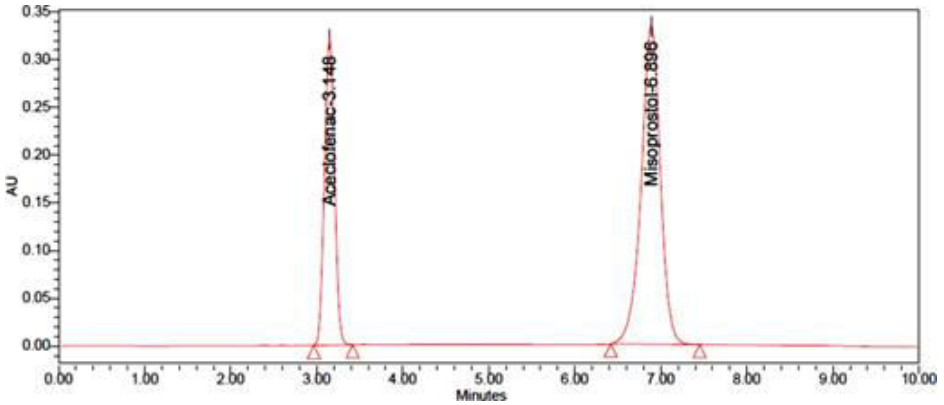

Figure 2.14: Chromatogram of system precision-2

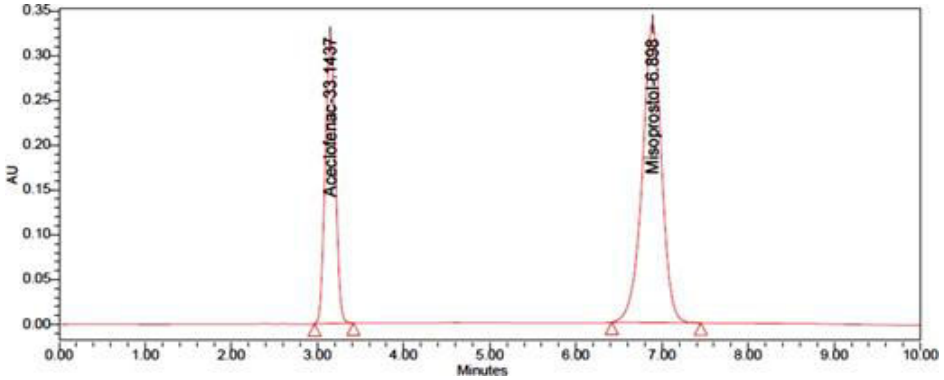

Figure 2.15: Chromatogram of system precision-3

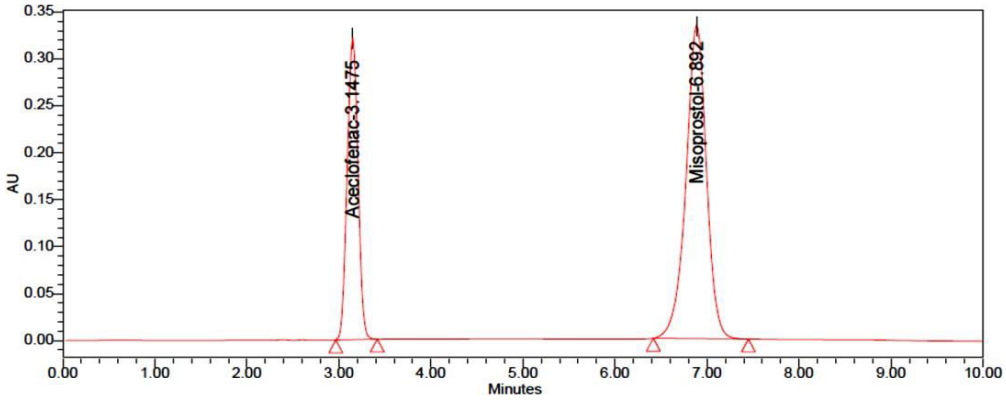

Figure 2.16: Chromatogram of system precision-4

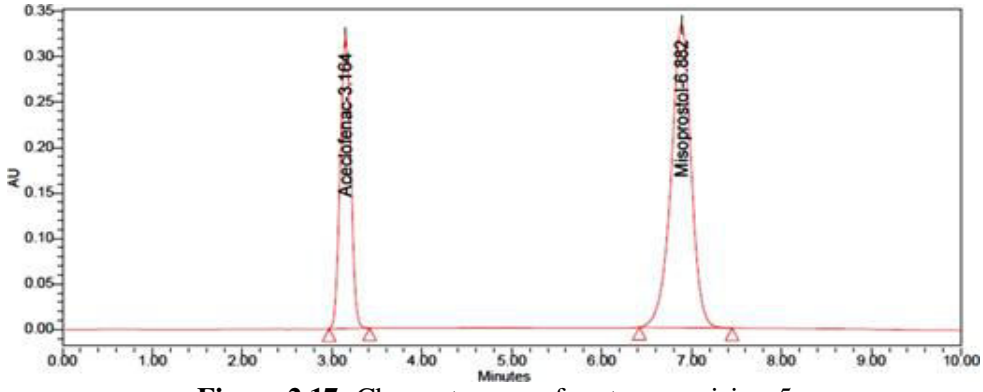

Figure 2.17: Chromatogram of system precision-5

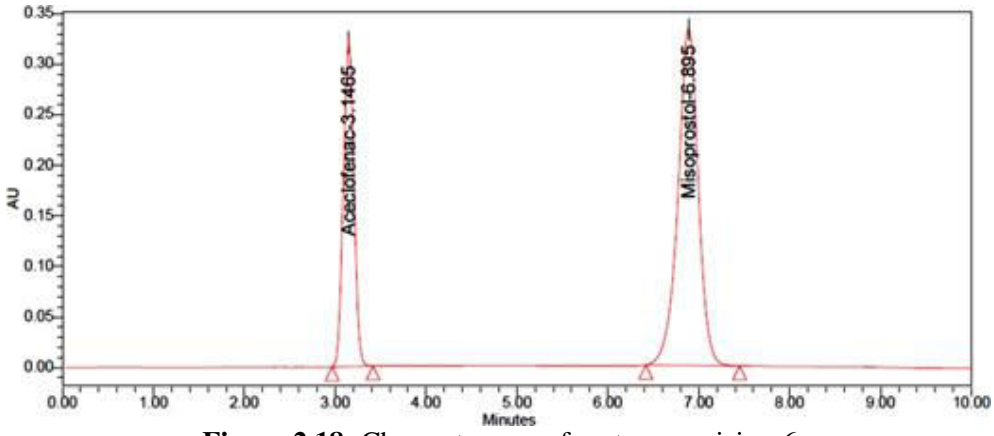

Figure 2.18: Chromatogram of system precision-6

Method precision [Table 2.06 and Fig 2.19]

S.NO	Aceclofenac area	Misoprostol area
1	3365104	3485796
2	3385676	3475162
3	3395864	3455216
4	3374856	3485126
5	3320156	3421365
6	3395694	3425163
Mean	3372892	3457971
Std dev	28483.927	29089.893
% RSD	0.84	0.84

Table 2.06: Results of method precision

Acceptance criteria: The RSD percent for the area six standard injection results should be more than 2 %.

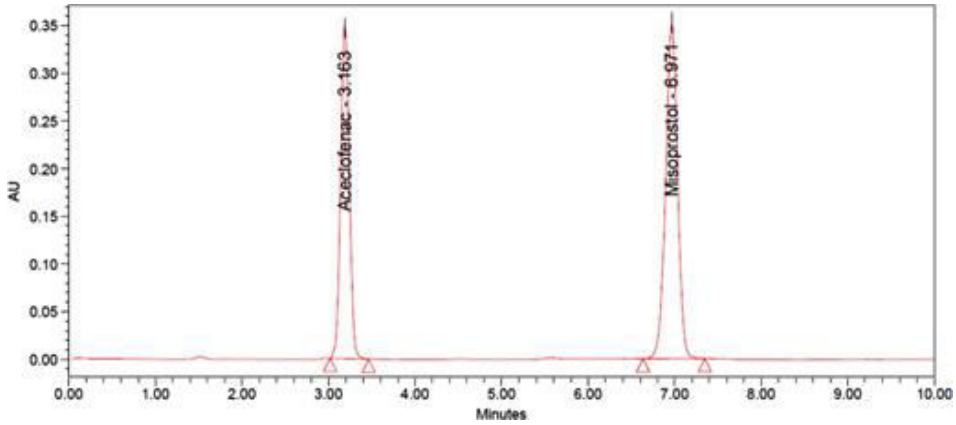

Figure 2.19: Chromatogram of method precision

Acceptance criteria: The RSD percentage for the six normal injection results should not be more than 2%.

2.7.5 Limit of detection (LOD) and limit of quantification (LOQ):

Using quality formulae, the limit of detection and quantification (LOQ) may be calculated as the concentration below which an analyte can be reliably identified. Aceclofenac and Misoprostol had LOD values of 0.06 g/ml and 0.06 g/ml, respectively, and s/n values of 5, 5. As for

Aceclofenac and Misoprostol, the LOQ was 0.208 g/mL for both drugs, and the s/n was 26 for both.

2.7.6 Robustness:

The tactic's robustness was shown to attract RSD at a rate of just 2%. The optimum technique parameters, such as flow (0.2 ml/min) and organic content in mobile phase (10 percent), were wiped away by little adjustments. **Table 2.07** gives results of robustness and the **figures** from **2.20-2.23** shows robustness chromatograms.

Table 2.07: Robustness study of Aceclofenac and Misoprostol

Drug name	**Flow Plus (1.2 ml/min)**	**Flow Minus (0.8 ml/min)**	**Org Plus (55:45)**	**Org Minus (45:55)**
	% RSD			
Aceclofenac	0.35	0.19	0.69	0.54
Misoprostol	0.27	0.62	0.42	1.36

Table 2.07: Robustness study of Aceclofenac and Misoprostol

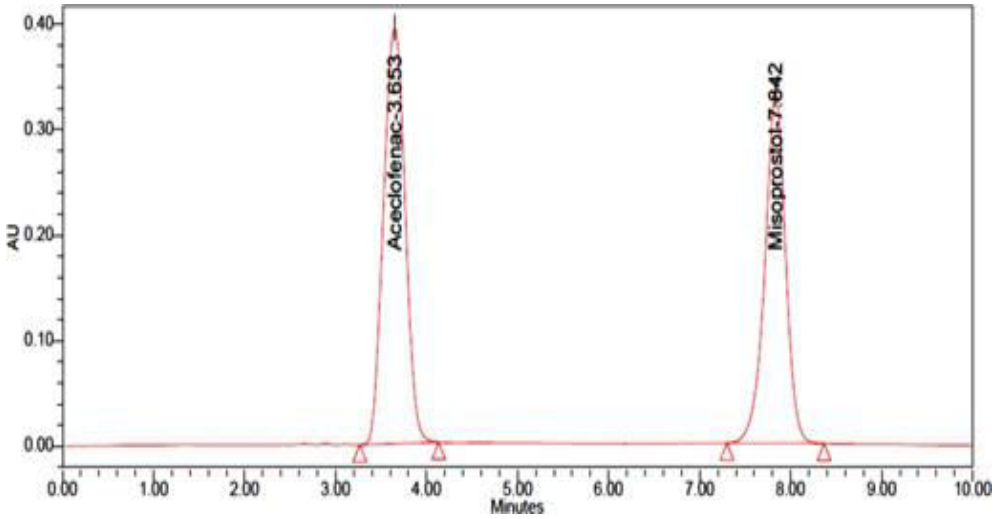

Figure 2.20: Less flow rate chromatogram (0.8ml/min)

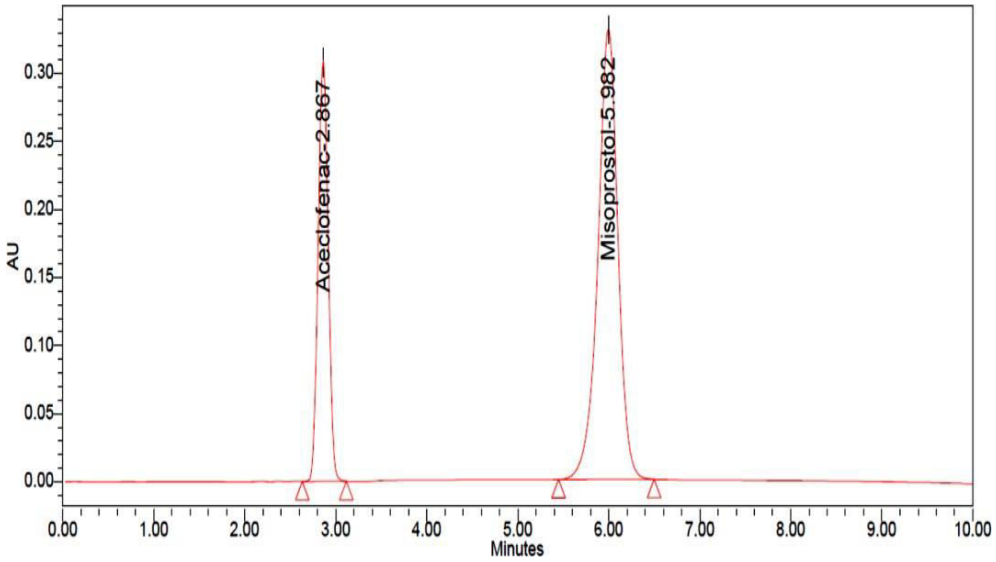

Figure 2.21: More flow rate chromatogram (1.2ml/min)

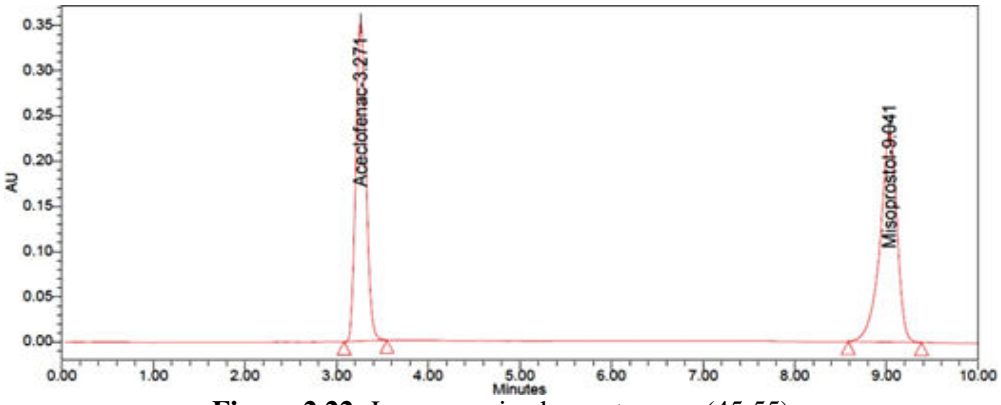

Figure 2.22: Less organic chromatogram (45:55)

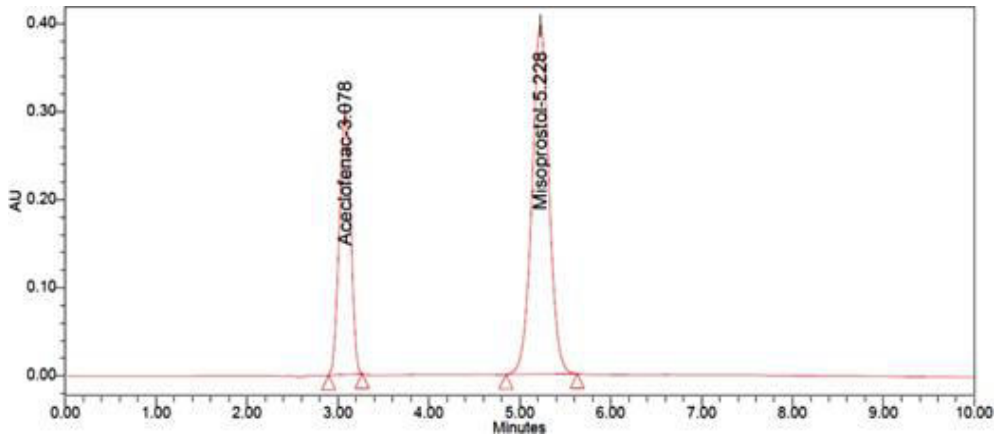

Figure 2.23: More organic chromatogram (55:45)

2.7.7 Forced degradation studies:

Studying 1 N concentrations of forced degradation settings that included acidic and basic peroxides as well as hydrolysis and reduction, as well as thermal stress was done.

Stock solution preparation:

After correctly weighing and transferring the 50 mg of Aceclofenac and the 50 mg of Misoprostol to the 100 ml volumetric flask, it is necessary to add the 70 ml of diluents and sonicate for 30 minutes to completely dissolve the diluents.

Acid degradation:

For 15 minutes, add 1 ml of 1N Hcl to 1 ml of the sample stock solution in a volumetric flask with a capacity of 10 ml. Add 1ml of 1N NaOH after 15 minutes and dilute to the desired strength.

Alkali degradation:

For 15 minutes, 1 ml of sample stock solution is transferred to a 10 ml volumetric flask and 1 millilitre of 1N NaOH is added. After 15 minutes, add 1 ml of 1N Hcl and diluents to get the solution to the desired volume.

Peroxide degradation:

Transferring 1ml of the sample stock solution to a 10ml volumetric flask, a 30 percent hydrogen peroxide solution was added, and the volume was brought up to capacity using various diluents.

Reduction degradation:

It was transferred to a 10-ml volumetric flask, where it was diluted with 30 percent sodium bi sulphate solution and brought up to the diluents mark.

Thermal degradation:
In the oven for six hours, the sample solution was kept at 105°C. HPLC was used to purify the final product.

Hydrolysis degradation:
Sample stock was transferred to a volumetric flask of 10ml and diluted with 1ml of water to get the desired amount of water dilution.

Table 2.08 gives results of forced degradation and the **figures** from **2.24-2.30** shows forced degradation chromatograms.

Degradation	Aceclofenac (% Degradation)	Misoprostol (% Degradation)
Control	0.1	0.2
Acid deg	17.1	16.1
Alkali deg	16.4	15.9
Peroxide deg	14.6	13.5
Reduction deg	12.7	12.7
Thermal deg	13.2	10.8
Hydrolysis deg	11.8	11.9

Table 2.08: Forced degradation data of Aceclofenac and Misoprostol

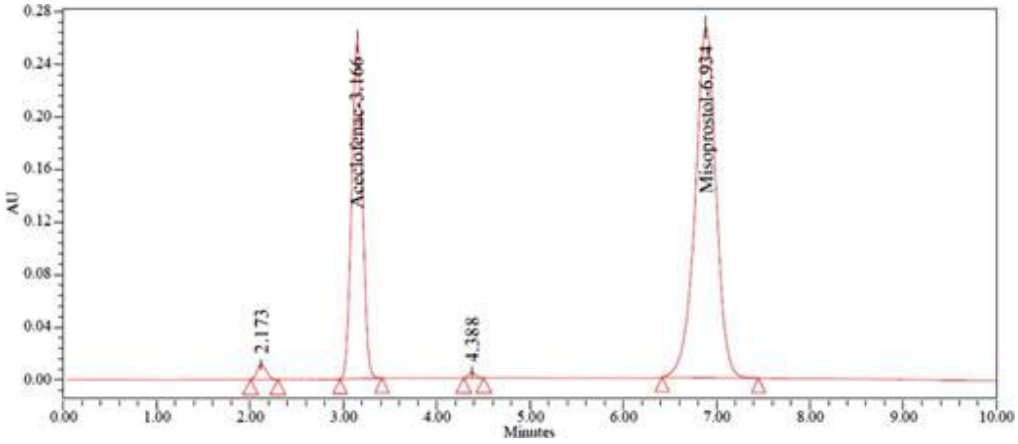

Figure 2.24: Chromatogram of acid degradation

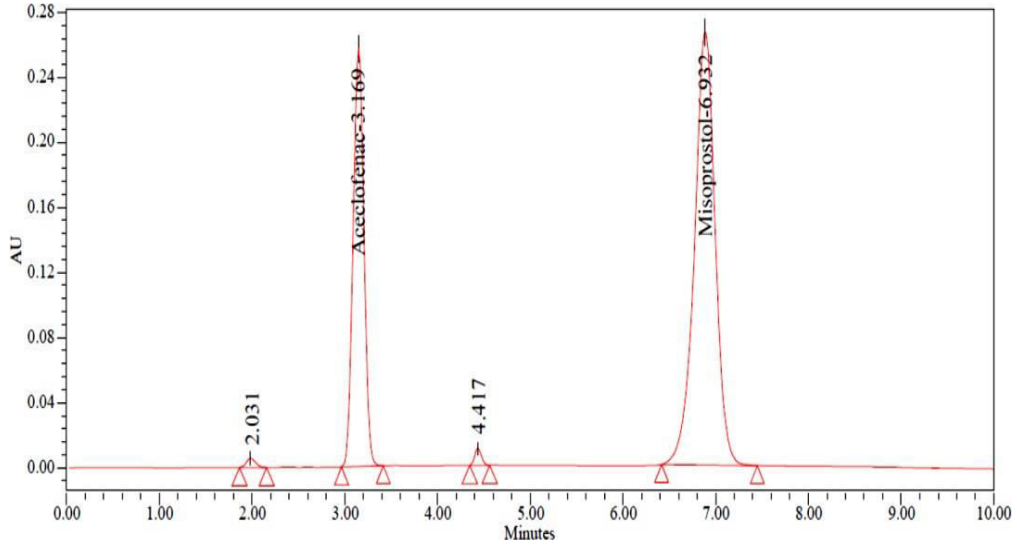

Figure 2.25: Chromatogram of alkali degradation

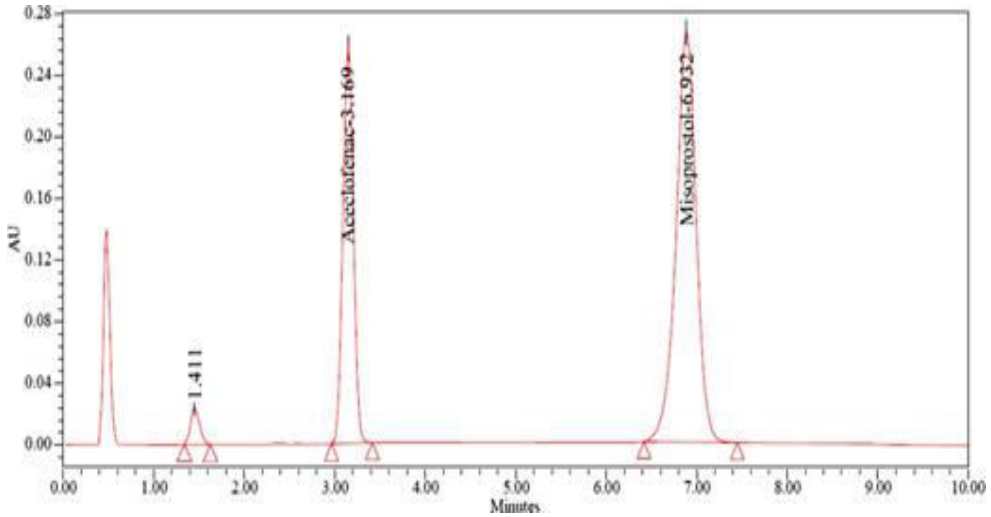

Figure 2.26: Chromatogram of peroxide degradation

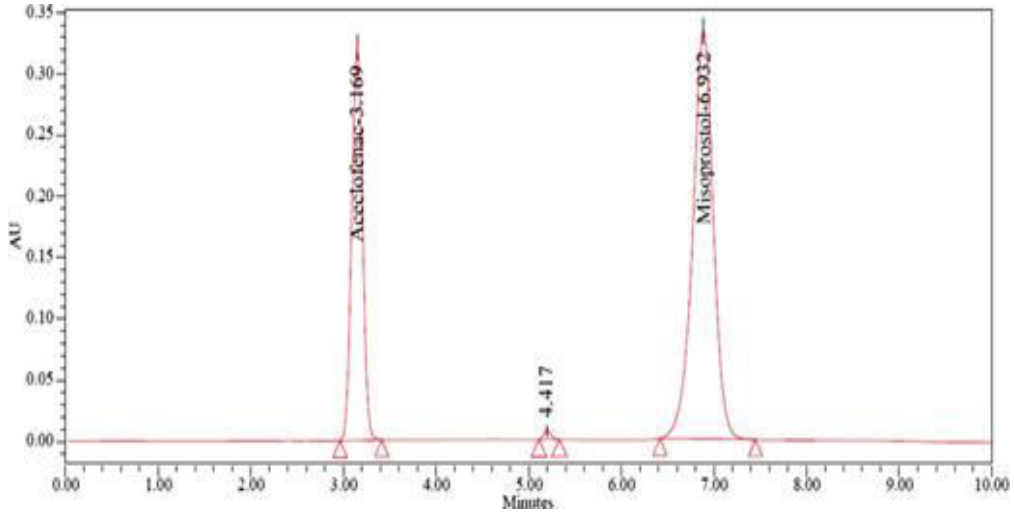

Figure 2.27: Chromatogram of reduction degradation

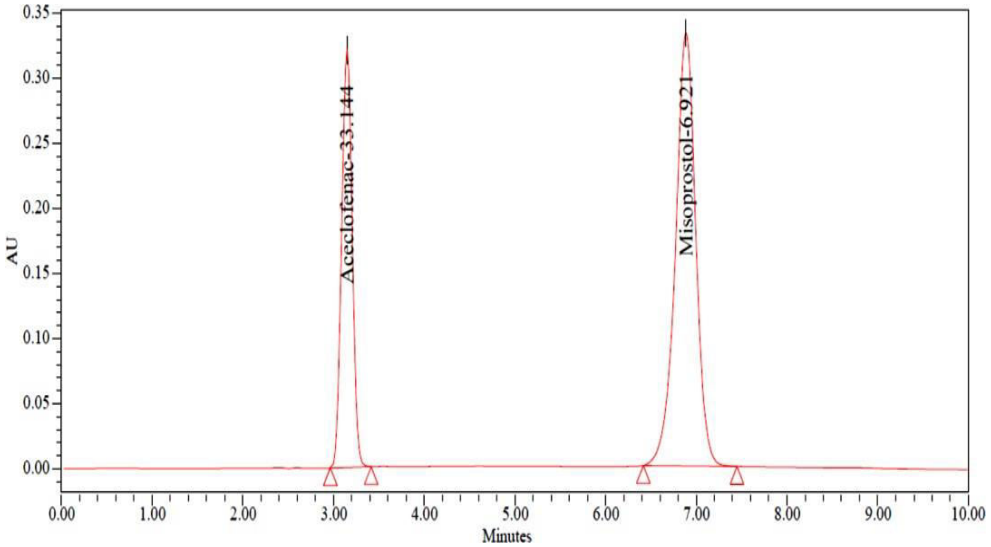

Figure 2.28: Chromatogram of Hydrolysis degradation

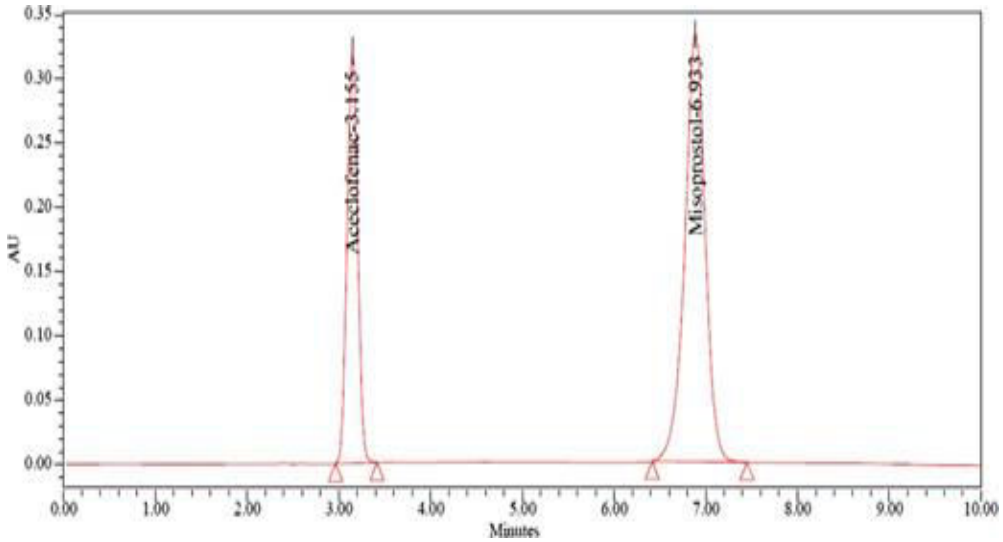

Figure 2.29: Chromatogram of Thermal degradation

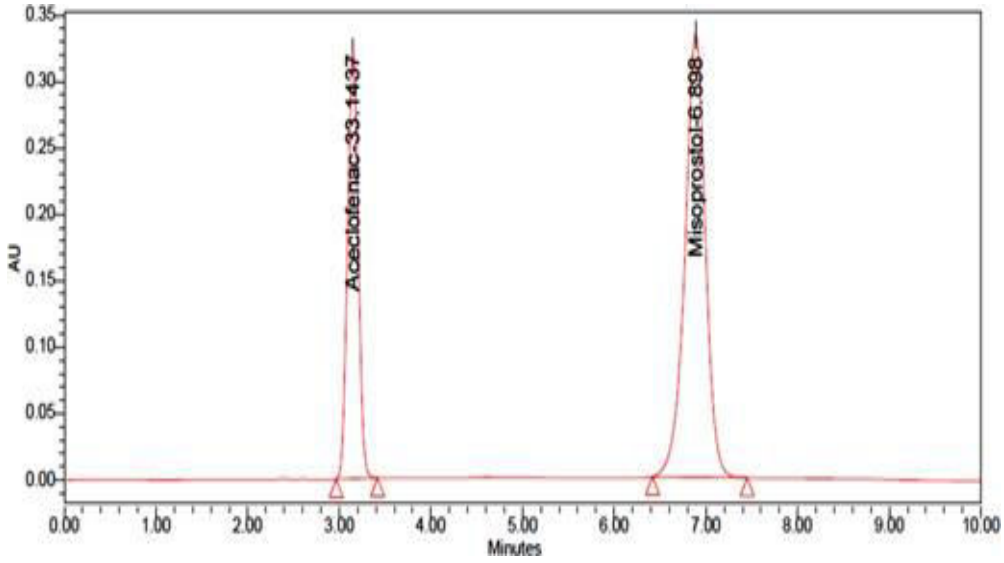

Figure 2.30: Chromatogram of control degradation

2.7.8 Stability:

The stability of ordinary and sample solutions was examined from the beginning to the end of a 24-hour storage period at room temperature by monitoring the stability methodologies. They were given at various intervals, and the assay percentage variation between the original and 24 hour injections was just around 2%. Aceclofenac and Misoprostol are not affected by storage conditions. **Table 2.09** gives results of stability and the **figures** from **2.31-2.35** shows stability chromatograms.

Time intervals	Aceclofenac (% Difference)	Misoprostol % Difference
Initial	0.00	0.00
6 Hrs	-0.9	-0.7
12 Hrs	-1.1	-0.9
18 Hrs	-1.7	-1.5
24 Hrs	-2.8	-2.2

Table 2.09: Stability data of Aceclofenac and Misoprostol

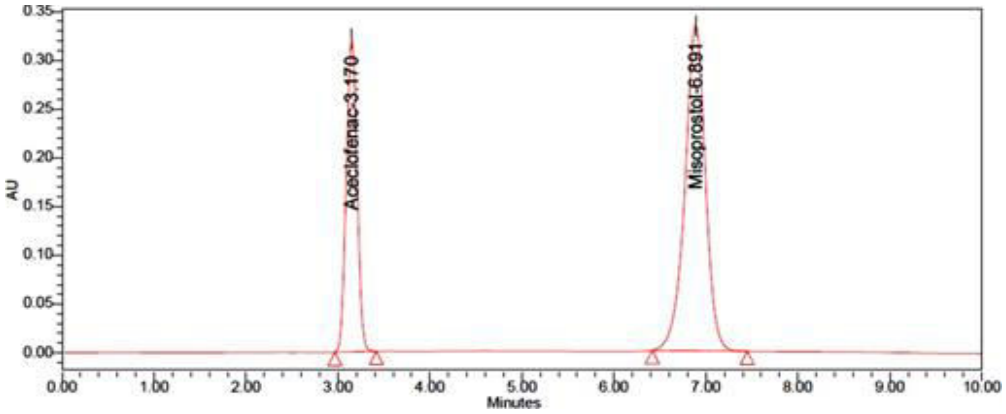

Figure 2.31: Chromatogram of stability initial RT

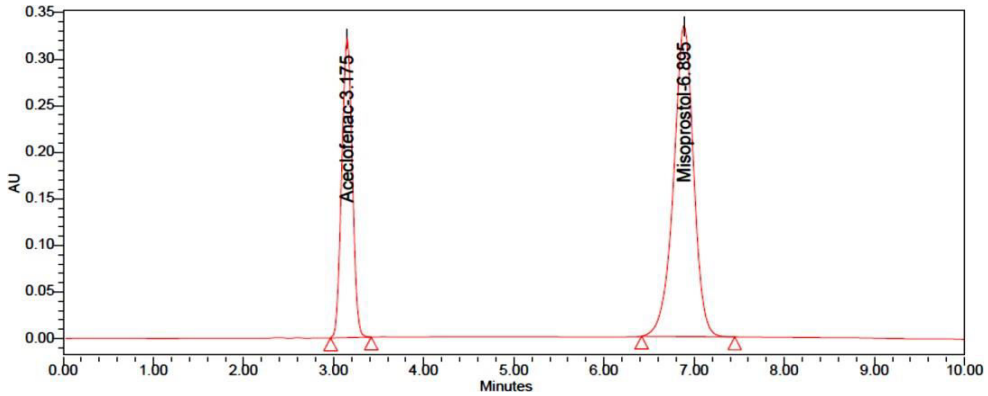

Figure 2.32: Chromatogram of stability 6 Hrs RT

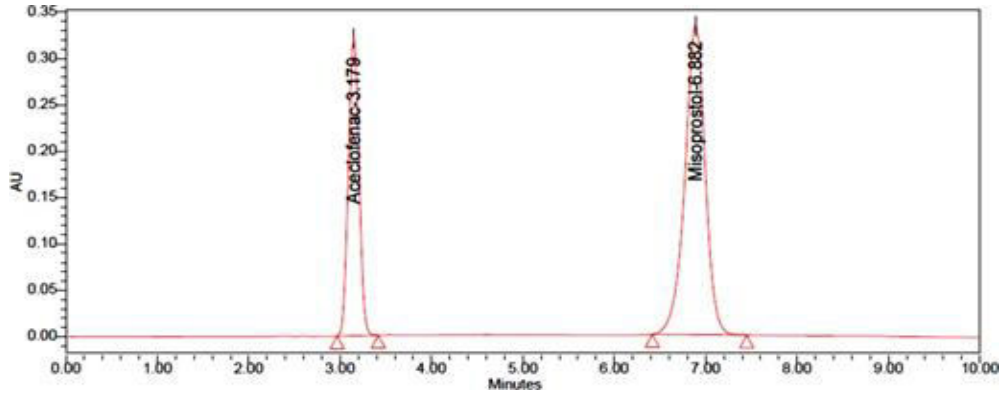

Figure 2.33: Chromatogram of stability 12 Hrs RT

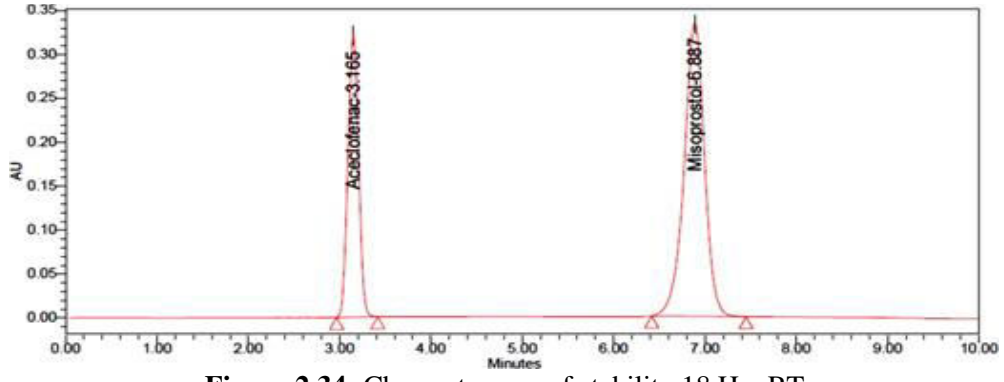

Figure 2.34: Chromatogram of stability 18 Hrs RT

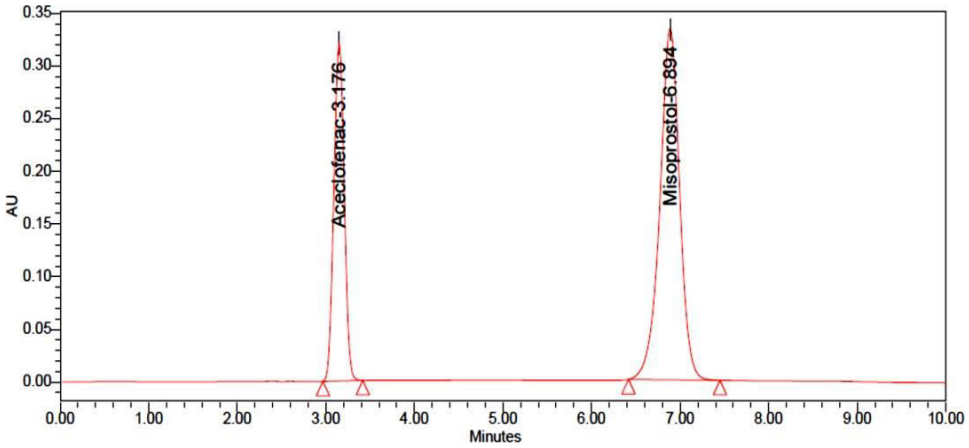

Figure 2.35: Chromatogram of stability 24 Hrs RT

2.8 CONCLUSION:

In this procedure, Aceclofenac and Misoprostol are quantified according to ICH criteria in bulk and pharmaceutical formulations. It was discovered that the newly developed method was exact, accurate, linear, and dependable. The convenience and lower cost of the chemicals used make this method more cost effective. To achieve reliable chemical quantification, the recommended HPLC settings provide appropriate resolution. According to the findings of the tests, the precision and repeatability data are good. The chromatographic technology that was developed was extensively utilised in regular drug testing.

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3.

BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF AVELUMAB, AXITINIB AND ITS APPLICATION TO PHARMACOKINETIC STUDIES IN RABBIT PLASMA BY USING LCMS/MS

3.1 AVELUMAB

3.1.1 Drug Profile of Avelumab

Avelumab is a fully human monoclonal antibody medication for the treatment of merkel cell carcinoma, urothelial carcinoma, and renal cell carcinoma (**1**). In adults and children at least 12 years of age, treatment of a particular form of skin cancer, metastatic merkel cell carcinoma (MCC) (**2**). Up to 12% of patients with MCC have incorrectly prognosed distant metastatic disease (mMcc). And progression to mMcc is fruequentin upto 21 percent in patients with local or regional disease (**3**). Although no prospective clinical chemotherapy (**4**) have been performed and no regime has been officially approved for mMCC treatment, combinations of platinum/ etoposide have been commonly used and reasonably high objective response rates (ORRs) have been achieved, response time, however, is limited and no siginificant survival benefit has been reported. Highlighting the need for alternative treatments. Recently, clinical trials with immune checkpoint inhibitors targeting the programmed death ligand 1(PD-L1)/programmed death 1(PD-1) interaction have shown clinical activity and durable responses in patients with advanced MCC (**5**). Avelumab is given by an infusion into vein through a special filter over 60 minutes every two weeks. Avelumab gives side effects to a few patients after discontinuation, such as immune-related side effects and other common side effects, such as feeling tired, muscle pain, muscles, joints, tendons, ligaments, nerves, and increased liver enzymes (**6-10**).

Structure of Avelumab [Fig 3.01]

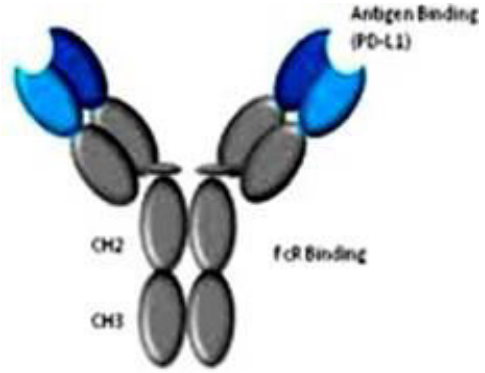


Figure 3.01: Structure of Avelumab

3.1.2 Total mass of the Drug: 143831.79 $g \cdot mol^{-1}$

3.1.3 Empirical Formula: $C_{6374}H_{9898}N_{1694}O_{2010}S_{44}$

3.1.4 Category: Avelumab injection is a monoclonal antibody, which is a kind of drug.

3.1.5 Mechanism of Action of Avelumab

Anti-tumor immune response may be inhibited in the tumour microenvironment if PD-L1 is expressed on tumour cells and tumor-infiltrating immune cells. T-cell cytotoxicity, T-cell proliferation, and cytokine production are all inhibited when PD-L1 binds to the PD-1 and B7.1 receptors on T cells and antigen-presenting cells. There are two ways to prevent the binding of Avelumab: by binding to PD-L1 via loops 7 and by blocking the interaction between the receptors of the protein (PD-1 and B7.1) and its substrate (PD-L1). Immunological responses,

particularly anti-tumor immune responses, are restored as a consequence of the relaxation of inhibitory effects of PD-L1 via this interaction. Additionally, Avelumab has been shown to cause antibody-dependent cell-mediated cytotoxicity (ADCC). Blocking the activity of PD-L1 in syngeneic mice tumour models resulted in reduced tumour development.

3.2 AXITINIB

3.2.1 Drug profile of Axitinib

Axitinib is a small molecule Tyrosine kinase inhibitor (**11**) developed by Pfizer under trade name Inlyta which take orally. It has been shown significantly inhibit growth of breast cancer in animal models (**12**). And has shown partial response in clinical trails with Renal cell carcinoma (RCC) and several other tumour types (**13**). It was approved for RCC by the U.S. Food and drug Administration after showing a modest increase in Progression free survival. There have been reports of fatal adverse effects. Most common effects are Diarrhea, High blood pressure (**14**), Fatigue, Loss of appetite, Anemia (**15**).

Structure of Axitinib [Fig 3.02]

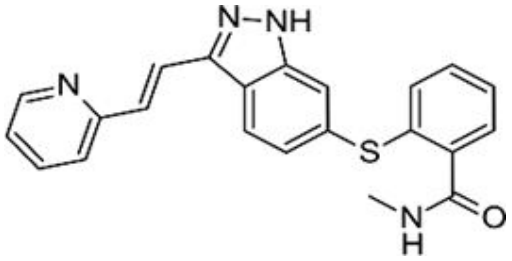


Figure 3.02: Structure of Axitinib

3.2.2 IUPACName:N-methyl-2-[[3-[(E)-2-pyridin-2-ylethenyl]-1H-indazol-6-yl]sulfanyl]benzamide

3.2.3 Total mass of the Drug: 386.5

3.2.4 Empirical Formula: $C_{22}H_{18}N_4OS$

3.2.5 Solubility

Axitinib is more than 0.2 g/mL soluble in aqueous environments with a pH ranging from 1.1 to 7.8.

3.2.6 Category

Kinase inhibitors, such as Axitinib, are a kind of drug. It does its job by inhibiting the activity of a mutant protein that instructs cancer cells to proliferate.

3.2.7 Mechanism of Action of Axitinib

Angiogenesis inhibition is the principal mechanism of action, which is hypothesised to be achieved via inhibiting vascular endothelial growth factor receptor 1-3, c-KIT and PDGFR (the formation of new blood vessels by tumours). Other tyrosine-kinase inhibitors like as sorafenib have been shown to induce autophagy. Additionally, it has been shown to inhibit the drug-resistant T315I mutant version of the BCR-ABL fusion protein by attaching to it in a different conformation than the VEGF binding.

3.2.8 Introduction

Avoiding co-administration with strong CYP3A4/CYP3A5 inhibitors may lower plasma clearance of axitinib, hence it should be avoided whenever feasible.

3.2.9 Side Effects:

- Diarrhoea.

- High blood pressure (hypertension)
- Fatigue.
- Loss of appetite.
- Nausea.
- Dysphonia (hoarse or soft voice)
- Decrease in kidney function(increase in creatinine blood test)
- Anemia (decrease in red blood cells)

3.3 CYTARABINE

3.3.1 Drug profile of Cytarabine

Cytarabine is a chemotherapy medication used to treat acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelogenous leukemia (CML), and non-Hodgkin's lymphoma. It is given by injection into a vein, under the skin, or into the cerebrospinal fluid. There is a liposomal formulation for which there is tentative evidence of better outcomes in lymphoma involving the meninges.

Structure of Cytarabine [Fig 3.03]

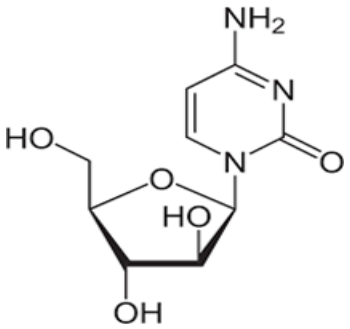


Figure 3.03: Structure of Cytarabine's

3.3.2 IUPAC Name: 4-amino-1-[(2R,3S,4S,5R)-3,4-dihydroxy-5 (hydroxymethyl)oxolan-2-yl]pyrimidin-2-one

3.3.3 Total mass of the Drug: 243.22

3.3.4 Empirical Formula: $C_9H_{13}N_3O_5$

3.3.5 Solubility

The organic solvents DMSO and DMF should be flushed with an inert gas in order to remove the cyclabine. Cytarabine's solubility in DMSO and DMF is about

0.2 mg/ml and 0.1 mg/ml, respectively.

3.3.6 Category

Drug type: Antiineoplastic or cytotoxic chemotherapy medication cytarabine. Antimetabolite is a term used to describe this drug. Cytarabine is a drug that has been shown to be effective in the treatment of cancer.

3.3.7 Mechanism of Action of Cytarabine

Cytarabine works by causing damage to DNA and then incorporating itself into the strand. All growing mammary cells in vitro may be cytotoxic to cytarabine. In particular, it targets cells in the S-phase, when DNA synthesis is taking place, and kills those cells preferentially. It may also prevent cells from progressing from the

G1 phase into the S-phase under certain circumstances. Cytarabine seems to inhibit DNA polymerase, despite the fact that its method of action is still a mystery. Cytarabine has also been shown to be incorporated into DNA and RNA in small but substantial amounts.

3.4 Literature Resurvey

Deiyao Lu, Jiahao Li, Weiting Liang, Yanming Hong, Chen, Tao Liu, Zhuojia ei Dong, Tiantian Zhang, Hongbing Huang, and Jie Jiang, A Cost-Effectiveness Analysis: First-Line Avelumab Plus Axitinib Versus Sunitinib for Advanced Renal-Cell Carcinoma: Progression-free survival (PFS) can be improved with avelumab and axitinib in the first-line therapy of advanced renal cell carcinoma (RCC), however the medication's cost impact is uncertain. avelumab with axitinib Vs sunitinib as first-line therapy for advanced RCC was evaluated from a US payer viewpoint for its cost-effectiveness

Robert J. Motzer, Paul B. Robbins, Laurence Albiges, Thomas Powles, John B. Haanen, Xinmeng Jasmine Mu, James Larkin, Keith A. Ching, Motohide Uemura, Sumanta K. Pal, Gwenaelle Gravis, Boris Alekseev, Matthew T.Campbell, KonstantinPenkov, JaeLyun Lee, Xiao Wang, Weidong Zhang, Jing Wang, Aleksander Chudnovsky, Subramanian Hariharan, Alessandra di Pietro, Amber C. Donahue & Toni K. Choueiri, Avelumab plus axitinib versus sunitinib in advanced renal cell carcinoma: biomarker analysis of the phase 3 JAVELIN Renal 101 trial: The JAVELIN Renal 101 study (n = 886; NCT02684006) indicated a substantially longer progression-free survival (PFS) with first-line avelumab + axitinib vs sunitinib in advanced renal cell carcinoma. We report on genetic analysis of baseline tumour samples (aRCC). We showed that neither PD-L1 expression nor tumour mutational load differentiated PFS in either trial arm. A single nucleotide variation in the FcR gene had no effect on the study results. We discovered novel immunomodulatory and angiogenesis gene expression signatures (GESs), previously unknown mutational profiles and their GESs, and many HLA types that were related with differences in progression-free survival (PFS) across treatment groups. As a result of these results, new techniques for improving patient care in renal cell carcinoma (RCC) may be developed based on this information.

Rebecca Shay Andrew Nicklawsky Dexiang Gao Elaine T.Lam, **A Cost- Effectiveness Analysis of Nivolumab Plus Ipilimumab Versus Pembrolizumab Plus Axitinib and Versus Avelumab Plus Axitinib in First-Line Treatment of Advanced Renal Cell Carcinoma:** For metastatic renal cell carcinoma, there are currently a number of feasible first-line therapeutic alternatives, making the decision of early therapy more challenging. Choosing the best treatment may need consideration of measures other than patient characteristics. Three combination treatments presently licenced for use in treatment-nave advanced or metastatic renal cellcarcinoma were evaluated for their cost-effectiveness in this study A US payer viewpoint on manivolumab + ipilimumab, pembrolizumab + axitinib (PA), and the combination of avelumab + axitinib (AA).

T.K.ChoueiriJ.LarkinS.PalR.J.MotzerB.I.RinlB.VenugopalB.AlekseevH. MiyakeG.GravisM.A.BilenS.HariharanA.ChudnovskyK.A.ChingX.J.MuM.Mar ianiP.B.RobbinsB.HuangA.di PietroL.AlbigesEfficacy and correlative analyses of avelumab plus axitinib versus sunitinib in sarcomatoid renal cell carcinoma: post hoc analysis of a randomized clinical trialPatients with sarcomatoid histology (sRCC) in their advanced renal cell carcinoma (RCC) have the worst prognosis. Patients with advanced sRCC were compared to sunitinib in this study, which evaluated the effectiveness of the combination of avelumab and axitinib. Patients with treatment-naive advanced RCC were recruited in the randomised, open- label, multicenter phase III JAVELIN Renal 101 Study (NCT02684006). Avelumab with axitinib or sunitinib were administered to patients in a 1:1 randomization.

Efficacy (including progression-free survival) and biomarker analyses were also included in this post hoc study of patients with RCC.

3.5 Experimental

3.5.1 Chemical and reagents:

There were no issues with the quality of the acetonitrile or Tri fluoro acetic acid, water (HPLC grade). Spectrum pharma research solutions pvt ltd, Hyderabad, provided all APIs for Avelumab and Axitinib used as reference standards.

3.5.2 Instrumentation:

Waters alliance e2695 type HPLC system linked to QTRAP 5500 triple quadrupole instrument (sciex) mass spectrometer was used. [16-18] the Empower

2.0 Programme was used to carry out the surgery.

3.5.3 Preparation of standard and internal control samples Preparation of standard stock solution

After dissolving the contents of a 100ml volumetric flask and 70ml of diluent in sonication for 10 minutes, the volumetric flask is filled to the mark with 70ml of diluent. Add 0.1 ml to a 100 ml volumetric flask for further dilution. 4ml of the aforesaid solution is put into a 10ml volumetric flask and diluent is added to bring the volume to the mark.

Preparation of internal standard

Dissolve 50mg of Cytarabine internal standard fully in 100ml of diluent in a volumetric flask by ultrasonically dissolving the solution for ten minutes. You'll need 50ml of volumetric flask for this solution. Diluent is added to the 10ml volumetric flask to bring the solution to a final volume of 10ml.

Preparation of standard solution

200 l of plasma and 300 l of ACN were put to a 2ml centrifuge tube, and 500 l of standard stock solutions, 500 l of IS and 500 l of diluents were added and vortexed for 10 minutes. Afterward, the centrifuge tube was centrifuged for 10 minutes. A centrifuge at 5000 rpm for 30 minutes was then applied to these samples. The clear solution was put into a vial and injected into a system after being filtered with a 0.45 nylon syringe filter.

3.6 METHOD DEVELOPMENT

3.6.1 Biological Source:

Specimens were calibrated and controlled using a buffered blank plasma obtained from a rabbit. Vivo Bio Tech Ltd., Hyderabad, provided plasma samples for the specificity check experiment.

3.6.2 Method Optimization:

At the LQC level in rabbit plasma, Axitinib and Avelumab were to be tested for accuracy, precision, and specificity. The optimization experiment for the following variables.

3.6.3 Protein Precipitation:

Using a micro pipette, transfer 200 l of plasma sample (system suitability, zero standards, calibration standard, quality control samples and validation samples) into an Eppendrof tube. Axitinib and Avelumab standard stock and IS stock are now added, as well as 800 l of organic solvent vortexing for 10 minutes. Centrifuge at 5000 RPM for 20 minutes more, then use the resultant solution for testing.

3.6.4. Optimization Process

Explain a functional approach for enhancing analytical efficiency that organises the selection, execution, and optimization of the processes involved. Methods for characterising tests for the

medication components Avelumab and Axitinib need to be developed that are both simple to use and highly efficient.

3.6.4.1 Selection of Mobile phase

The chemical characteristics of axitinib and avelumab were supported by the mobile procedure. The large ratio of organic modifiers and buffers utilised might affect peak elution. Solubility in organic processes will also play an important function in the analytical system in this situation.

3.6.4.2 Selection of Column

An iterative process of trial and error was used once again once the right mobile phase had been selected. LCMS bioanalytical analysis differs from traditional HPLC drug analysis. LCMS analysts have a hurdle when working with bioanalytical matrices since most of the time the matrix components co-elute with the analyte. Analyte and interfering peak were separated by using stationary stages such as cyano, amino, and nitrile. All other chromatographic conditions, save for the column, remained constant throughout the operation.

3.6.4.3 Optimization of the final mobile phase

Final adjustments were made to the mobile phase composition and buffer concentration following the determination of the internal standard for Axitinib and Avelumab's retention time. So that Axitinib and Avelumab could be eluted with the optimum peak separation was selected for the final mobile stage.

3.6.4.5 Optimization of flow rate

To guarantee optimum RT, peak asymmetry, and resolution, several flow rates are examined once the mobile phase composition has been optimised. It is possible to calculate the ultimate flow rate using just the RT, correct peak asymmetry, and enough resolution.

3.7 RESULTS AND DISCUSSION

3.7.1 Trials in optimisation of chromatographic conditions

There have been a number of studies to try to differentiate these medications, ranging from Tril-1 to Trial-10, and

Trial-1 [Fig 3.04]

Chromatographic conditions

Column	:	Waters symmetry C18 150 x4.6 mm, 3.5µ
Mobile Phase	:	0.1% OPA+ ACN (40+60)
Volume of injection	:	10 µl
Rate of flow	:	1ml / min
Observation	:	Only two peaks are observed.

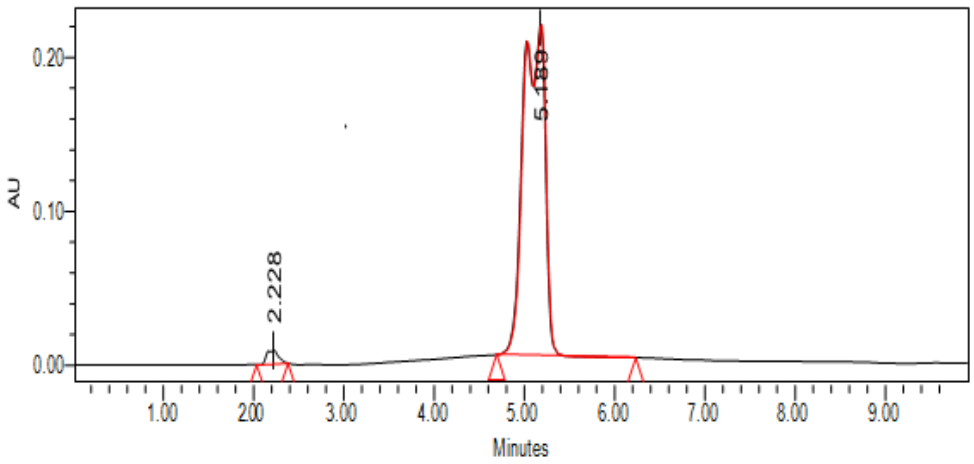


Figure 3.04: Chromatogram of trial-1

TriaL-2 [Fig 3.05]

Chromatographic conditions

Column	:	Waters symmetry C18 150 x4.6 mm, 3.5µ
Mobile Phase	:	0.1% OPA+ ACN (35+65)
Volume of injection	:	10 µl
Rate of flow	:	1ml / min
Observation	:	Only three peaks are observed.

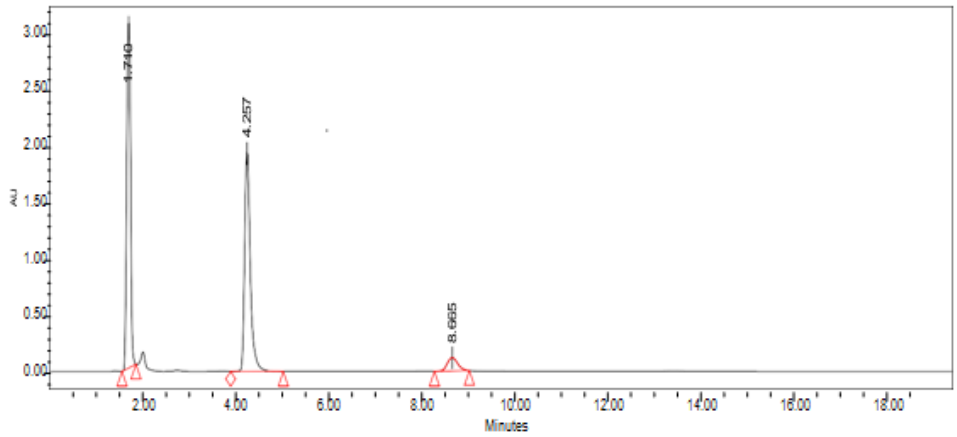


Figure 3.05: Chromatogram of trial-2

Trial-3 [Fig 3.06]

Chromatographic conditions

Column	:	Waters symmetry C18 150 x4.6 mm, 3.5µ
Buffer	:	2.5ml Hexane Sulphonic Acid in 1lt water and adjust p^H-5 with OPA

Mobile Phase	:	Buffer + ACN (60+40)
Volume of injection:		10µl
Rate of flow	:	1ml / min
Observation	:	Extra peak is observed.

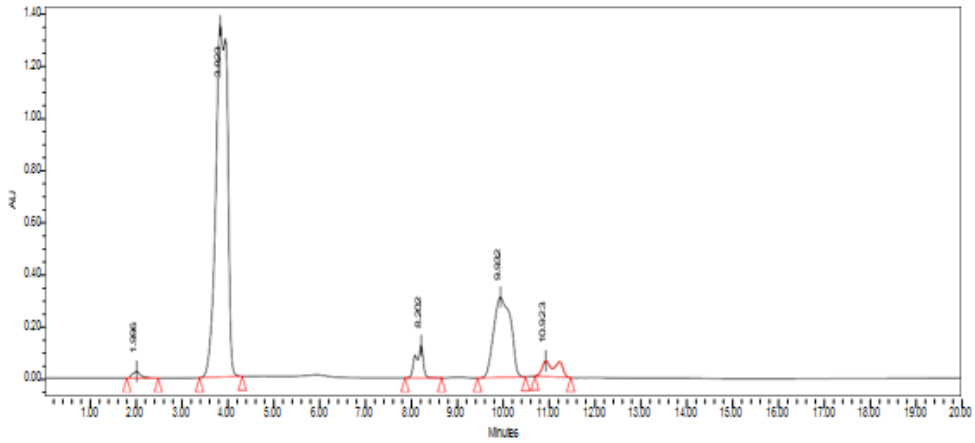


Figure 3.06: Chromatogram of trial-3

Trial-4 [Fig 3.07]

Chromatographic conditions

Column	:	Waters symmetry C18 150 x4.6 mm, 3.5µ
Buffer	:	2.5ml Hexane Sulphonic Acid in 1lt water and adjust p^H-5 with OPA
Mobile Phase	:	Buffer + ACN (70+30)
Volume of injection	:	10µl
Rate of flow	:	1ml / min
Observation	:	Resolution is not within the limit.

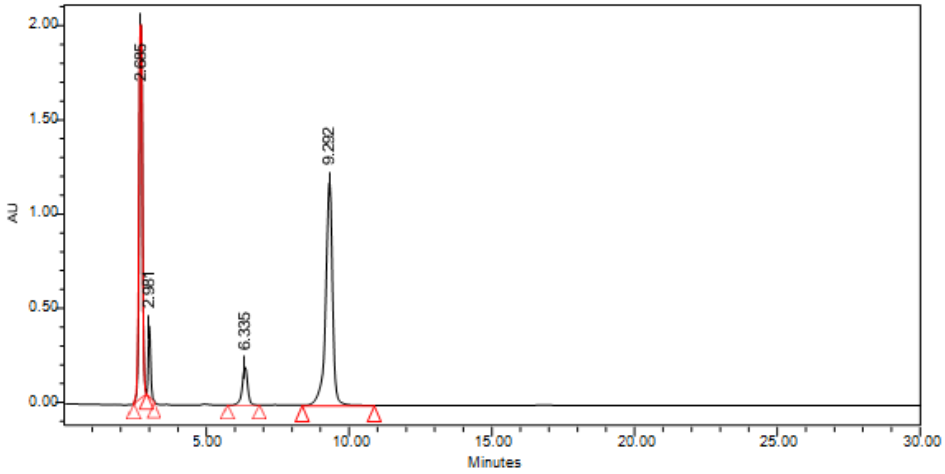


Figure 3.07: Chromatogram of trial-4

Trial-5 [Fig 3.08]

Chromatographic conditions

Column	:	X- bridge phenyl (150 x 4.6 mm, 3.5 micron)
Buffer	:	2.5ml Hexane Sulphonic Acid in 1lt water and adjust p^H-5 with OPA
Mobile Phase	:	Buffer + ACN (80+20)
Volume of injection	:	10 µl
Rate of flow	:	1ml / min
Observation	:	Base line is not sufficient

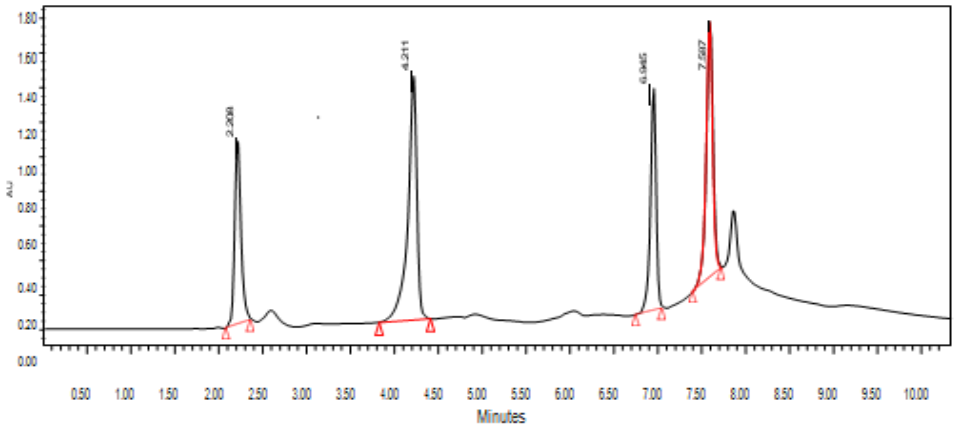


Figure 3.08: Chromatogram of trial-5

Trial-6 [Fig 3.09]

Chromatographic conditions

Column	:	X- bridge phenyl (150 x 4.6 mm, 3.5 micron)
Mobile Phase	:	0.1%TFA+ ACN (70+30)
Volume of injection	:	10 µl
Rate of flow	:	1ml / min
Observation	:	Peak shape is not good.

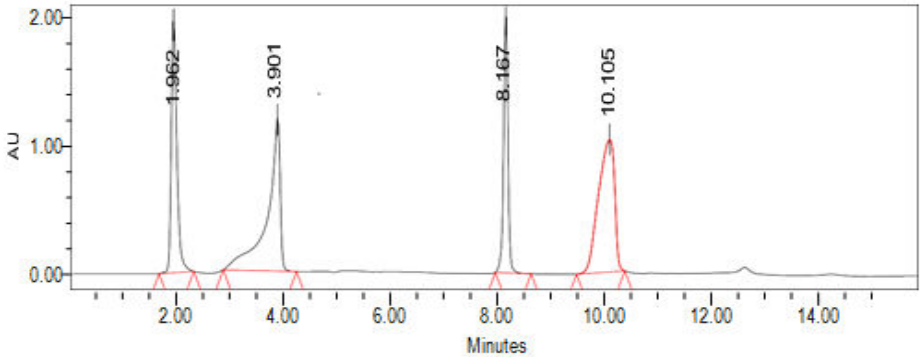


Figure 3.09: Chromatogram of trial-6

Trial 7 [Fig 3.10]

Chromatographic conditions

Column	:	X- bridge phenyl (150 x 4.6 mm, 3.5 micron)
Mobile Phase	:	0.1% TFA+ ACN (60+40)
Volume of injection	:	10 µl
Rate of flow	:	1ml / min
Observation	:	Response of the peak is very low.

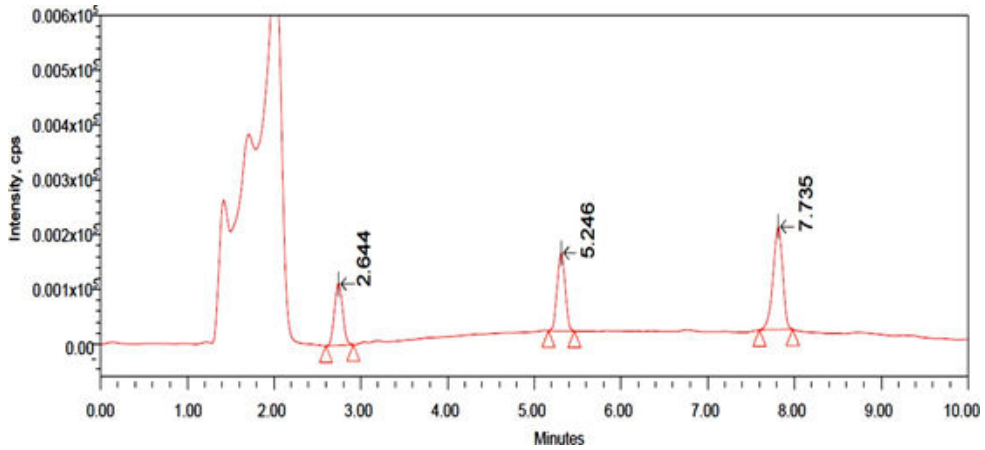


Figure 3.10: Chromatogram of trial-7

Trial 8 [Fig 3.11]

Chromatographic conditions

Column	:	X- bridge phenyl (150 x 4.6 mm, 3.5 micron)
Mobile Phase	:	0.1% TFA+ ACN (55+45)
Volume of injection	:	10 µl
Rate of flow	:	1ml / min
Observation	:	More response of the peak is observed.

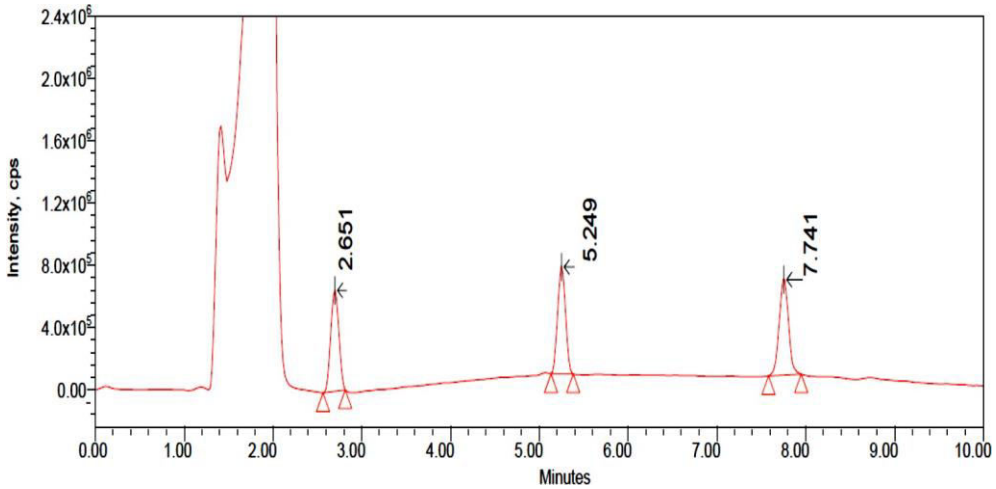


Figure 3.11: Chromatogram of trial-8

Trial 9 (Optimised chromatography) [Fig 3.12] Chromatographic conditions

Column	:	X- bridge phenyl (150 x 4.6 mm, 3.5 micron)
Mobile Phase	:	0.1% TFA+ ACN (50+50)
Volume of injection	:	10 µl
Rate of flow	:	1ml / min
Observation	:	This method is suitable.

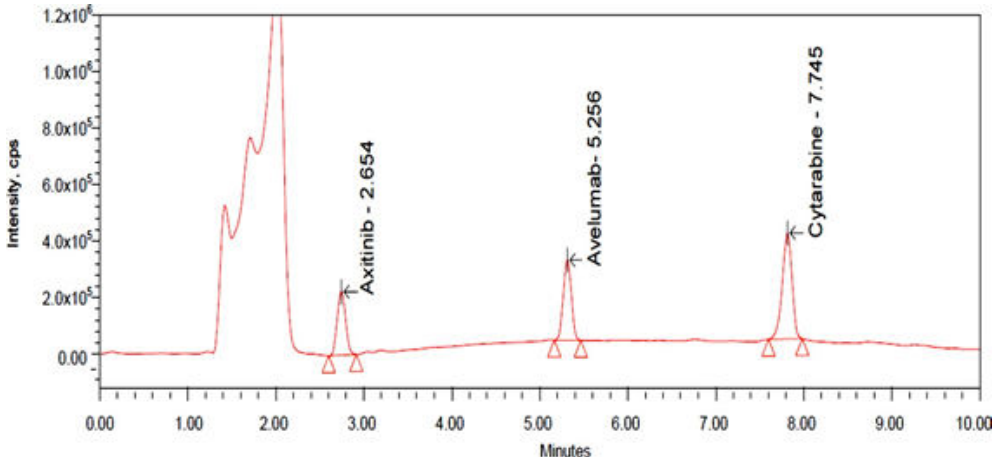


Figure 3.12: Chromatogram of trial-9

Chromatographic results of all trials were shown in **table 3.01.**

Trial No.	RT	Area	% Area	USP Resolution	USP Tailing	USP Plate count
1	2.228	76420	2.05		1.68	3485
	5.189	3655890	97.95	15.22	2.44	4866
2	1.710	17612916	48.90		1.18	5820
	4.257	16562688	45.99	18.55	2.12	8751
	8.665	1839800	5.11	16.77	2.14	4852
3	1.996	312625	0.89		1.02	8425
	3.823	24200371	68.61	14.26	1.06	8769
	8.202	1387900	3.93	15.28	1.18	10282
	9.932	7910531	22.43	16.44	1.26	12246
	10.923	1459211	4.14	18.22	1.54	7863
4	2.685	16423846	39.82		0.96	3321
	2.981	2161545	5.24	1.742	1.30	6023
	6.335	2684506	6.51	13.837	0.88	6080
	9.292	19979349	48.44	7.883	0.82	8145
5	2.208	5366838	18.33		2.14	8469
	4.211	9992659	34.13	5.85	2.28	9856
	6.945	5865898	20.03	5.55	1.44	15058
	7.587	8057025	27.51	6.44	1.56	8671
6	1.962	14112544	21.23		1.12	5428
	3.901	19657580	29.57	4.54	3.14	8460
	8.167	11168949	16.80	5.22	1.18	7880
	10.105	21534024	32.40	4.68	2.16	8555

7	2.644	$0.0010x10^5$	-	-	-	-
	5.246	$0.0019x10^5$	-	-	-	-
	7.735	$0.0023x10^5$	-	-	-	-
8	2.651	$6.228x10^5$	-	-	-	-
	5.249	$8.116x10^5$	-	-	-	-
	7.741	$8.092x10^5$	-	-	-	-
9	2.654	$1.912x10^5$	-	-	-	-
	5.256	$3.224x10^5$	-	-	-	-
	7.745	$3.751x10^5$	-	-	-	-

Table 3.01: Chromatographic data of all trials

3.7.2 Optimised process:

In this trial the peaks have good resolution and clear separation was achieved in Trial-9. Optimized method gives **table 3.02** and **fig 3.13** gives optimized chromatogram.

S.NO	Parameter	Chromatographic condition
1	Movable phase	Acetonitrile: 0.1% TFA (50:50)
2	Column	X-Bridge phenyl 150mm x 4.6 mm, 3.5µm
3	Rate of flow	1 ml / min
4	Temperature of the column	Room temperature
5	Temperature of the sample	Room temperature
6	Volume of injection	10 µl
7	Period of run	10min

Table 3.02: Optimised method chromatographic conditions

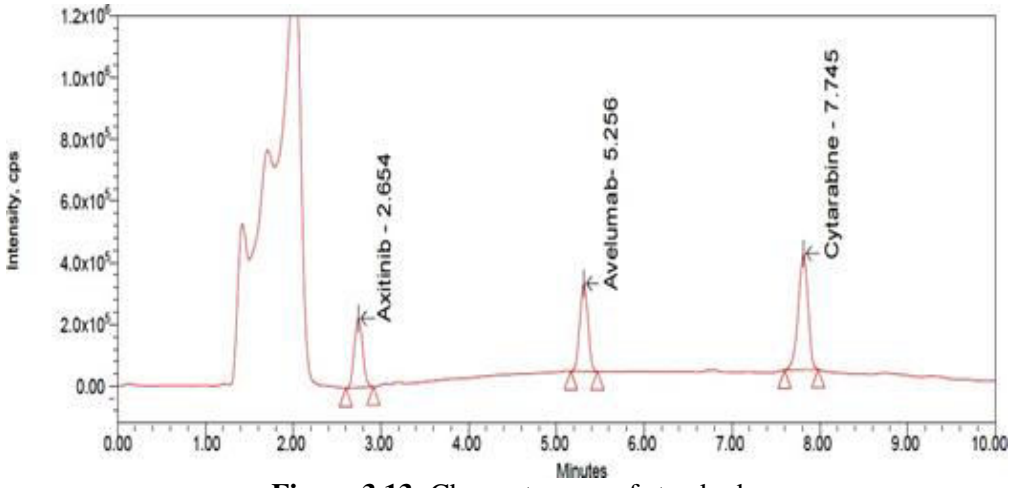


Figure 3.13: Chromatogram of standard

3.7.3 Validation of Method

The bio analytical process was verified in accordance with ICH guidelines using the suggested method.

3.7.3.1 Specificity and Selectivity

Retention times of Axitinib and Avelumab, both at their ISTD, did not provide any contradictory signals in six different randomly blank rabbit plasma specimens. Their chromatogram's lower limit of quantification should be known. **Fig**

3.14 gives blank chromatogram, and **Fig 3.15** gives blank with internal standard and **Fig 3.16** gives LLOQ chromatogram.

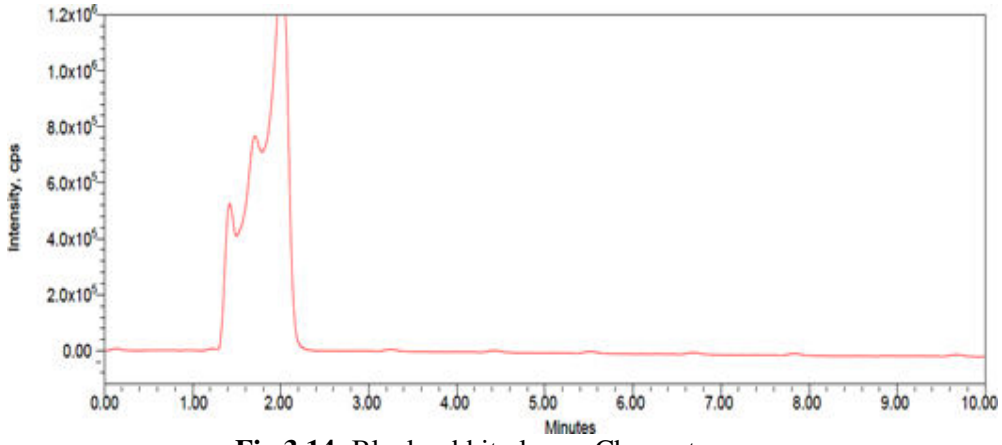


Fig.3.14: Blank rabbit plasma Chromatogram

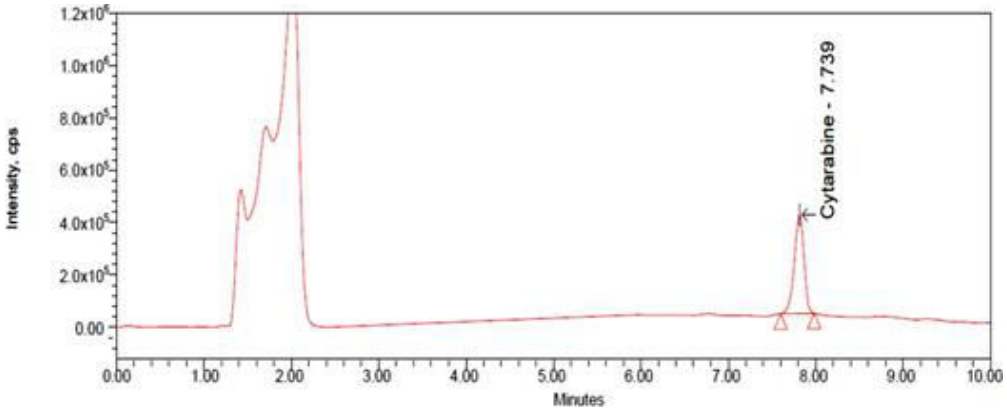


Fig. 3.15: Blank-IS Chromatogram

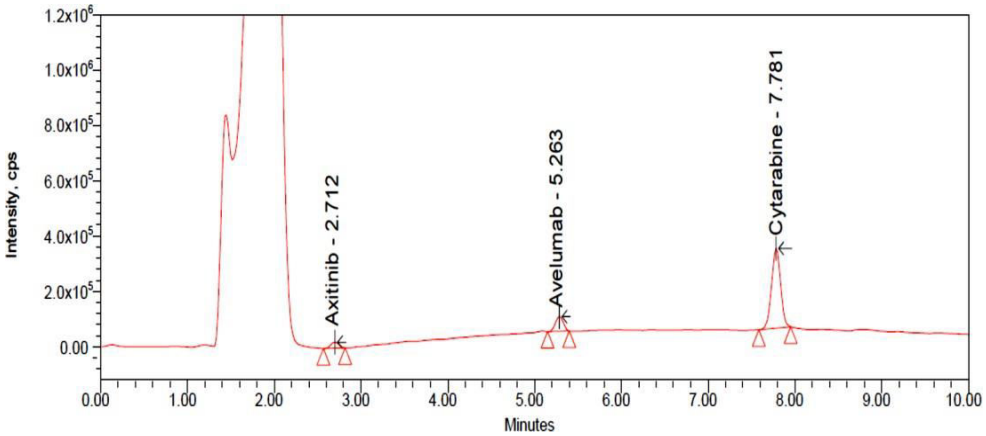


Fig. 3.16: LLOQ Chromatogram

3.7.3.2 System suitability

System appropriateness is a measure that predicts the chromatographic state under diverse analytical settings with a high degree of accuracy and precision. The chromatogram may be analysed by doing six replications of the MQC-1 through MQC-6 chromatogram. It passed the appropriateness test. **Table 3.03**, **3.04** gives suitability results and **figures** from **3.17-3.22** gives suitability chromatograms.

Sample Name MQC (5ng/ml)	Analyte Region(cps)	Analyte RT(min)	ISTD Region (200ng/ml)	ISTD RT (min)	Region Ratio
MQC-1	$1.945x10^5$	2.654	$3.754x10^5$	7.745	0.5181
MQC-2	$1.957x10^5$	2.658	$3.762x10^5$	7.758	0.5202
MQC-3	$1.938x10^5$	2.646	$3.747x10^5$	7.768	0.5172
MQC-4	$1.941x10^5$	2.649	$3.755x10^5$	7.757	0.5169
MQC-5	$1.959x10^5$	2.621	$3.762x10^5$	7.745	0.5207
MQC-6	$1.947x10^5$	2.648	$3.771x10^5$	7.765	0.5163
Mean	$1.948x10^5$	2.646	$3.759x10^5$	7.756	0.5182
SD	0.008	0.013	0.008	0.010	0.002
%CV	0.44	0.49	0.22	0.13	0.35

Table 3.03: System suitability Results of Axitinib

Sample Name (20 ng/ml)	Analyte Region(cps)	Analyte RT(min)	ISTD Region (200ng/ml)	ISTD RT (min)	Region Ratio
MQC	$3.219x10^5$	5.256	$3.754x10^5$	7.745	0.8575
MQC	$3.246x10^5$	5.249	$3.762x10^5$	7.758	0.8628
MQC	$3.235x10^5$	5.265	$3.747x10^5$	7.768	0.8634
MQC	$3.224x10^5$	5.269	$3.755x10^5$	7.757	0.8586
MQC	$3.240x10^5$	5.225	$3.762x10^5$	7.745	0.8612
MQC	$3.228x10^5$	5.221	$3.771x10^5$	7.765	0.8560
Mean	$3.232x10^5$	5.248	$3.759x10^5$	7.756	0.8599
SD	0.010	0.020	0.008	0.010	0.003
%CV	0.31	0.39	0.22	0.13	0.35

Table 3.04: System suitability Results of Avelumab

Benchmark for approval

In the proposed approach the CV % of the RT should be less than 2.00 percent. The percent CV of the response ratio should be ≤ 5.00 percent

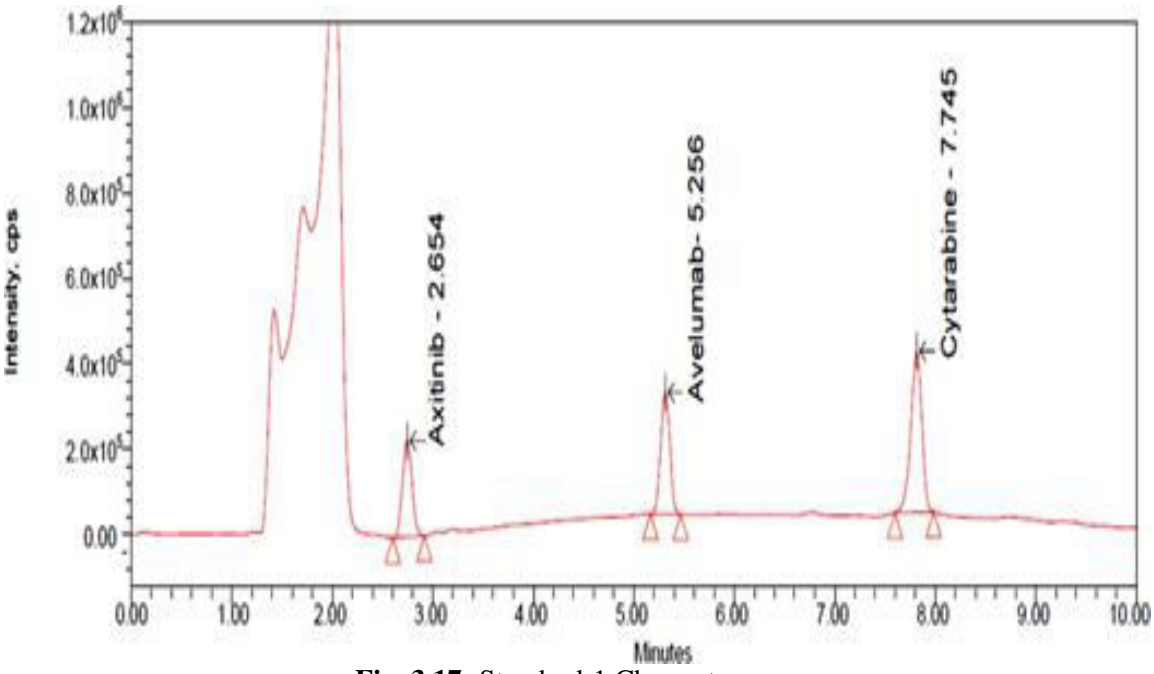


Fig. 3.17: Standard-1 Chromatogram

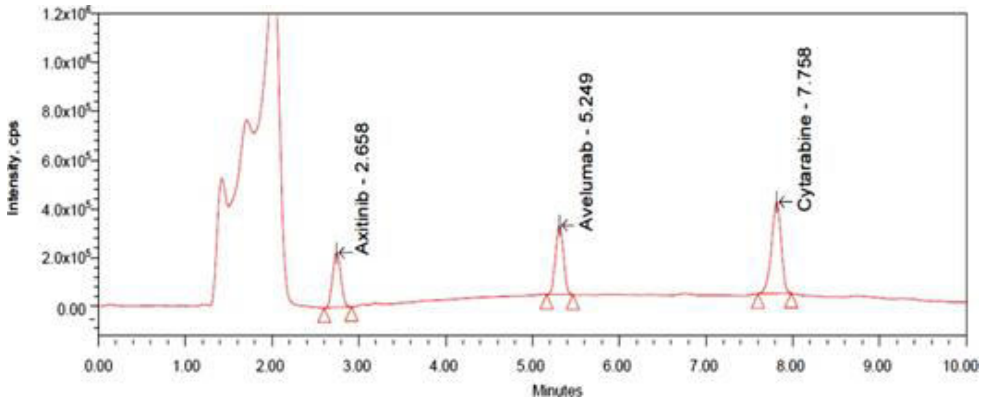


Fig. 3.18: Standard-2 Chromatogram

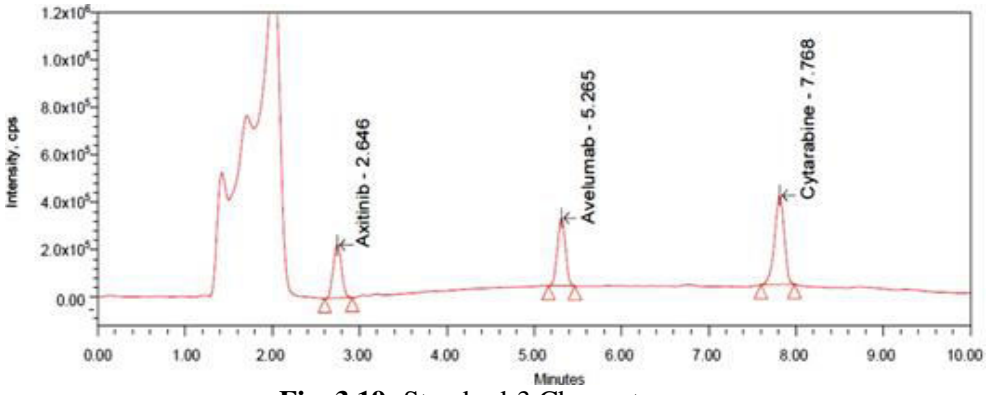


Fig. 3.19: Standard-3 Chromatogram

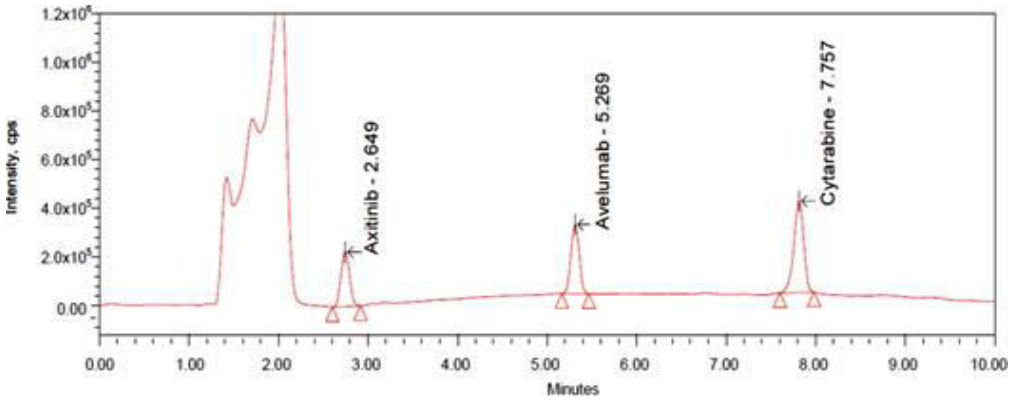


Fig.3.20: Standard-4 Chromatogram

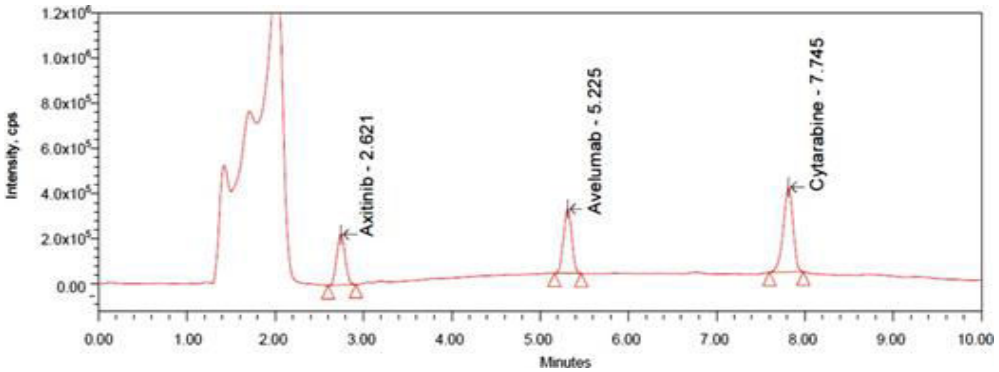


Fig.3.21: Standard-5 Chromatogram

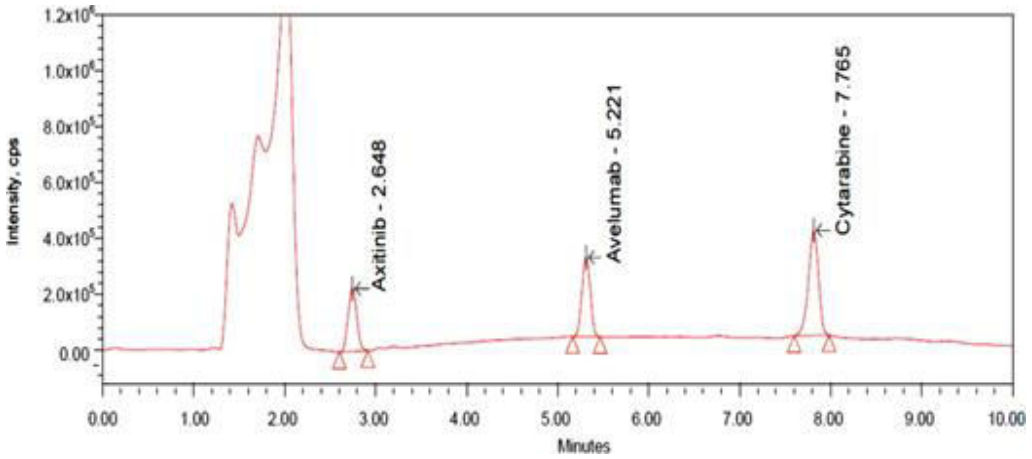


Fig.3.22: Standard-6 Chromatogram

3.7.3.3 Linearity [Fig 3.23-3.32]

The graphical depiction indicates a linear relationship between the concentrations of 2-40 ng/ml and 0.5-10 ng/ml of various medications. It had an average coefficient of 0.999. The analyte peak to IS peak ratio was used to determine the samples' concentrations. About eight times, the test was run through its paces.

Acceptance criteria

The linearity regression coefficient should be 0.999. It was accepted.

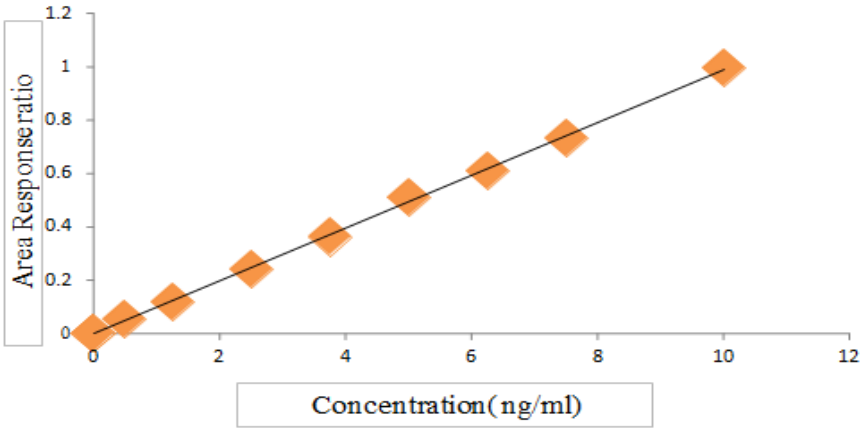


Figure 3.23: Calibration plot for concentration Vs area ratio of Axitinib

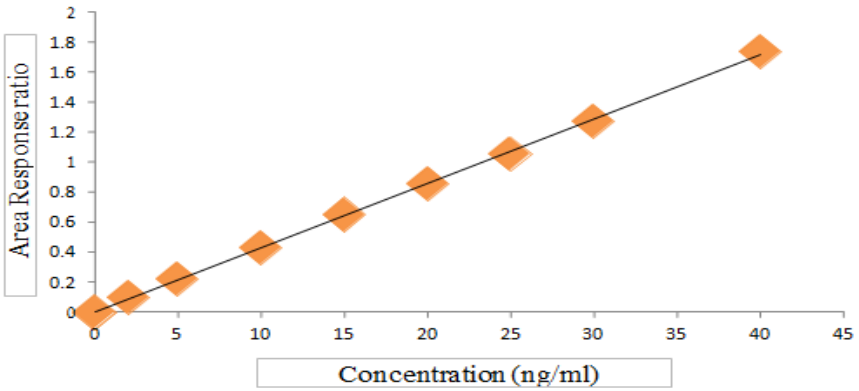


Figure 3.24: Calibration plot for concentration Vs area ratio of Avelumab

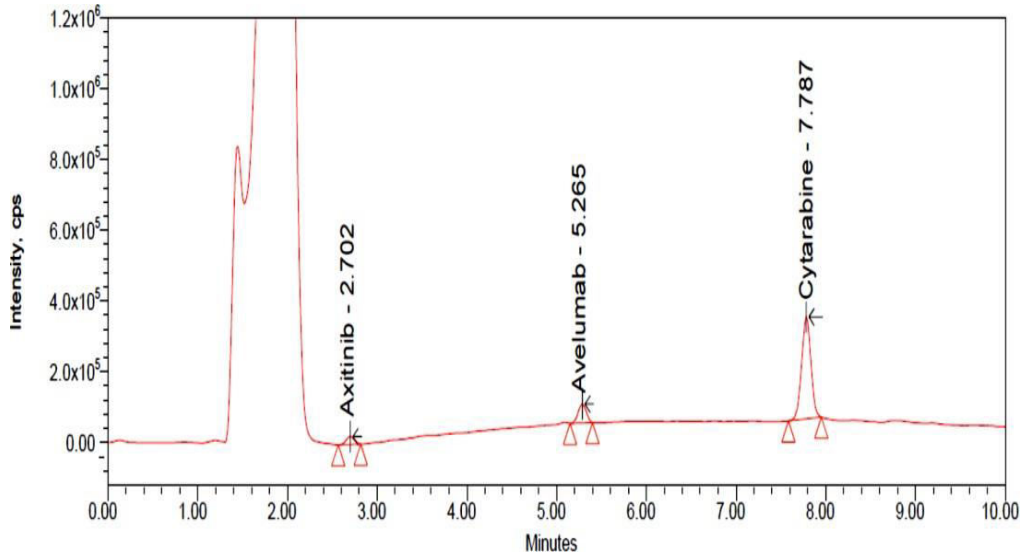


Figure 3.25: Chromatogram of linearity-1

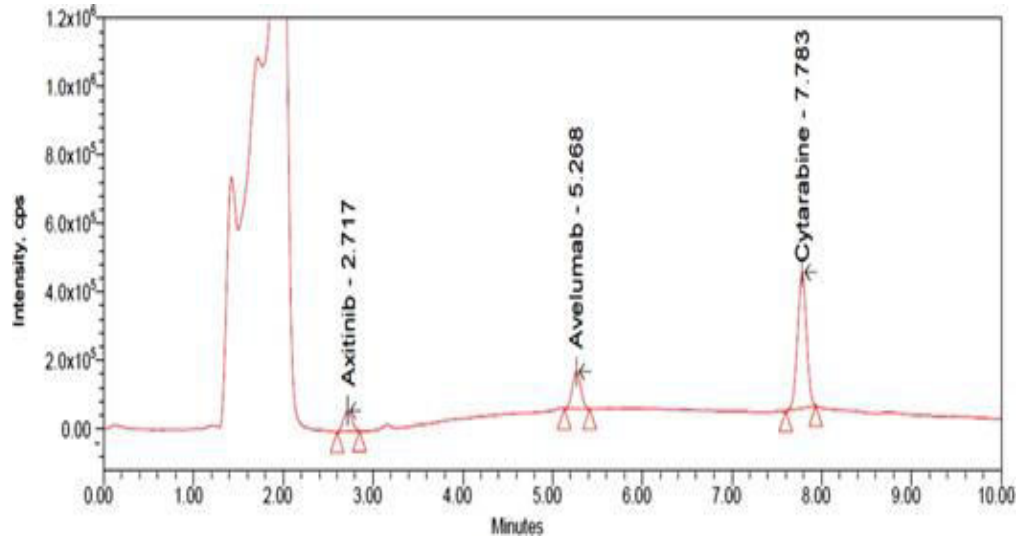


Figure 3.26: Chromatogram of linearity-2

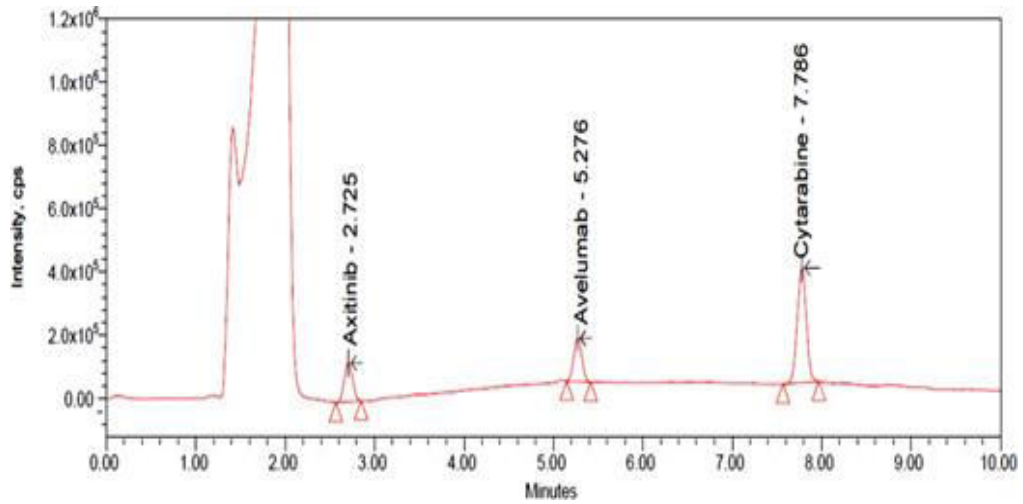


Figure 3.27: Chromatogram of linearity-3

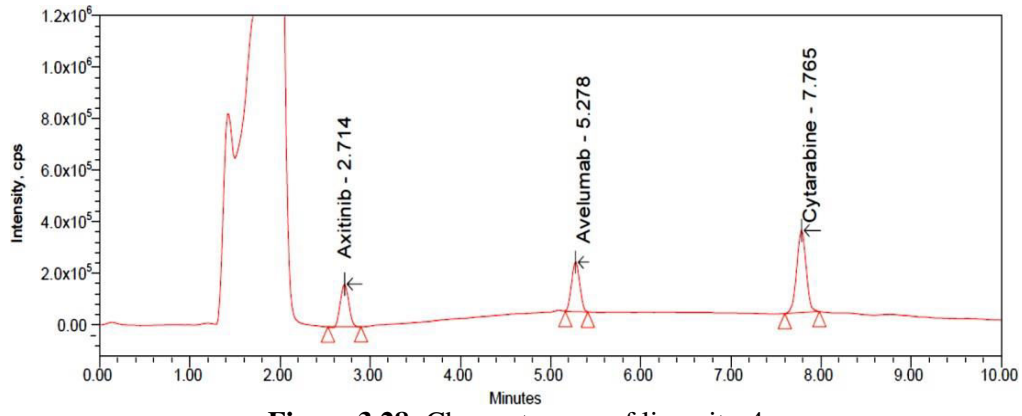


Figure 3.28: Chromatogram of linearity-4

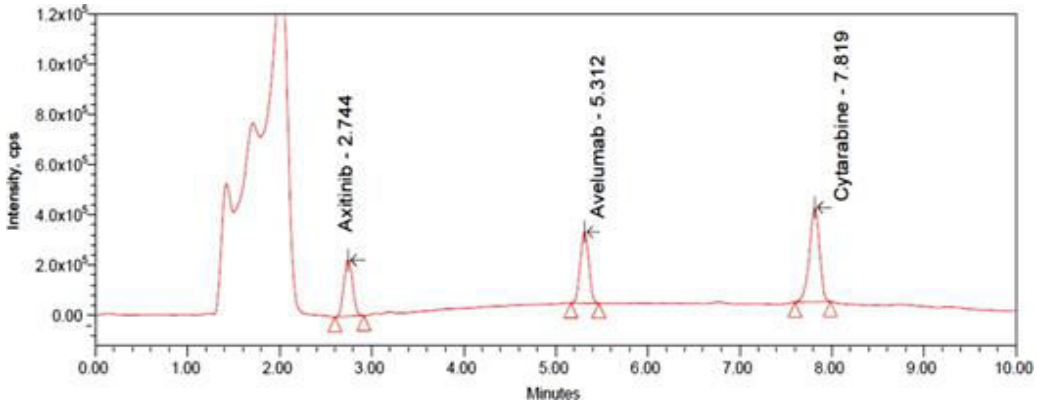


Figure 3.29: Chromatogram of linearity-5

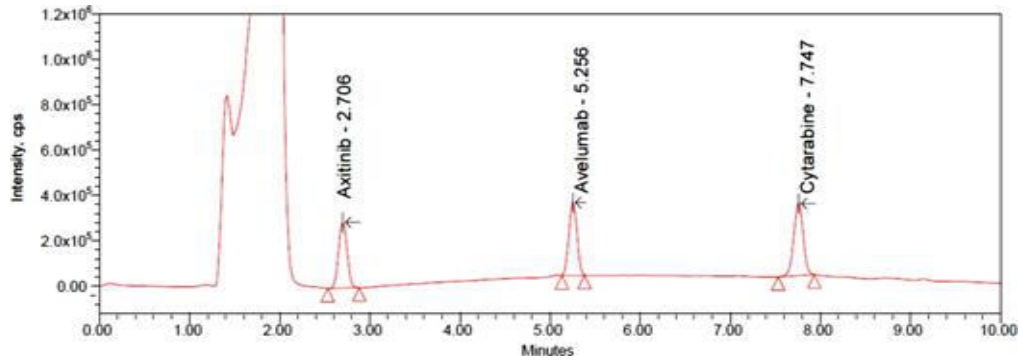


Figure 3.30: Chromatogram of linearity-6

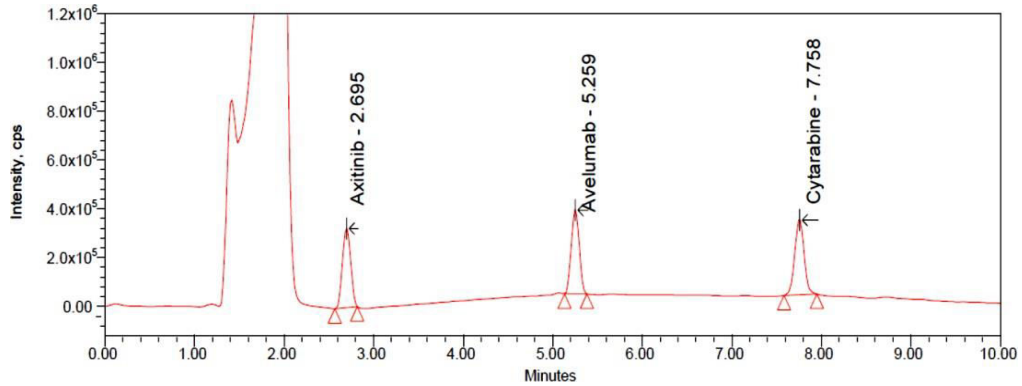


Figure 3.31: Chromatogram of linearity-7

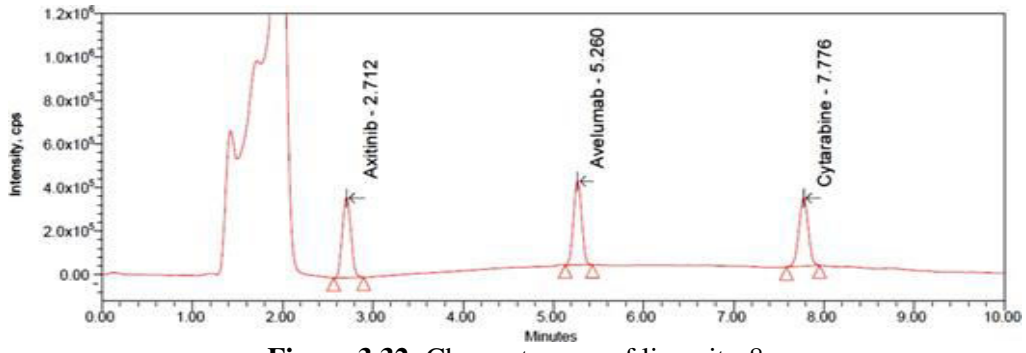


Figure 3.32: Chromatogram of linearity-8

3.7.3.4 Sensitivity

Identifying the chromatographic sensitivities is an important step in achieving firmness, authenticity and reducing system uncertainty by doing six replications with varied chromatographic strengths results in the table below. **Table 3.05, 3.06** gives sensitivity results and **Fig 3.33** gives LLOQ chromatogram.

Number of repetitions	**LLOQ**
	Ostensible strength in ng/ml
	0.5
	Analyte peak area
1	$0.216x10^5$
2	$0.213x10^5$
3	$0.202x10^5$
4	$0.209x10^5$
5	$0.211x10^5$
6	$0.205x10^5$
n	6
Average	$0.209x10^5$
SD	0.005
% CV	2.47
% Average Accuracy	107.29%

Table no 3.05: Axitinib Sensitivity Results

Number of repetitions	**LLOQ**
	Ostensible strength in ng/ml
	2.0
	Analyte peak area
1	$0.364x10^5$
2	$0.352x10^5$
3	$0.386x10^5$
4	$0.377x10^5$
5	$0.365x10^5$
6	$0.343x10^5$
n	6
Average	$0.365x10^5$
Standard deviation	0.016
% CV	4.32
% Average Accuracy	112.93%

Table no 3.06: AvelumabSensitivity Results

The benchmark for approval

For four of the six samples, the average percent exactness is between 80 and 120. CV's accuracy percentage might be as low as 20, 00 percent.

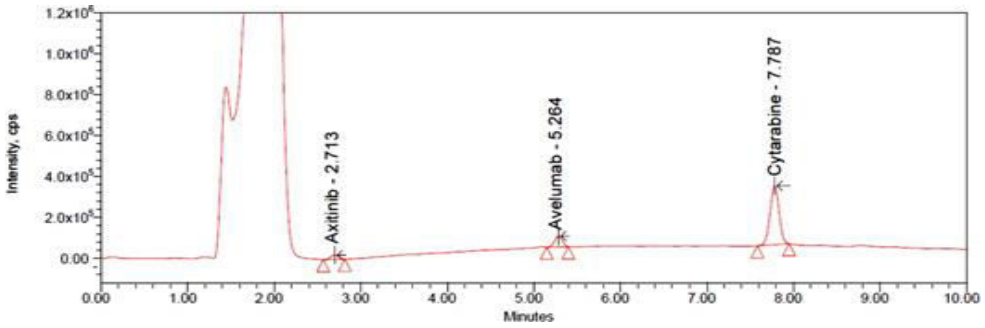


Fig 3.33: LLOQ Sensitivity chromatogram

3.7.3.5 Matrix effect

Sample response after plasma extraction and analyte compatibility at identical intensities are examined in this research. A chromatographic technique is used to detect the plasma and ionised ions in the extracted plasma. **Table 3.07, 3.08** gives matrix effect results. **Fig 3.34, 3.35** gives matrix effect chromatograms.

Repetition count	**Plasma-portion**	**HQC**	**LQC**
		Ostensible strength in ng/ml	
		7.50	2.50
		Analyte peak area	
1.	Lot 1	$2.757x10^5$	$0.859x10^5$
		$2.732x10^5$	$0.862x10^5$
		$2.744x10^5$	$0.846x10^5$
2.	Lot 2	$2.763x10^5$	$0.871x10^5$
		$2.747x10^5$	$0.868x10^5$
		$2.738x10^5$	$0.855x10^5$
3.	Lot 3	$2.747x10^5$	$0.847x10^5$
		$2.751x10^5$	$0.856x10^5$
		$2.722x10^5$	$0.865x10^5$
4.	Lot 4	$2.741x10^5$	$0.879x10^5$
		$2.748x10^5$	$0.885x10^5$
		$2.735x10^5$	$0.888x10^5$
5.	Lot 5	$2.721x10^5$	$0.844x10^5$
		$2.725x10^5$	$0.851x10^5$
		$2.739x10^5$	$0.837x10^5$
6.	Lot 6	$2.707x10^5$	$0.854x10^5$
		$2.713x10^5$	$0.836x10^5$
		$2.722x10^5$	$0.847x10^5$
n		18	18
Mean		$2.736x10^5$	$0.858x10^5$
SD		0.015	0.015
%CV		0.56	1.78
Percent Mean Accuracy		93.63%	88.09%
No. of QC Failed		0	0

Table No 3.07: Matrix effect Results of Axitinib (HQC-7.50ng/ml, LQC- 2.50ng/ml)

Repetition count	**Plasma- portion**	**HQC**	**LQC**
		Ostensible strength in ng/ml	
		30.0	10.0
		Analyte peak range	
1.	Lot 1	$4.849x10^5$	$1.617x10^5$
		$4.826x10^5$	$1.632x10^5$
		$4.857x10^5$	$1.608x10^5$
2.	Lot 2	$4.840x10^5$	$1.631x10^5$
		$4.841x10^5$	$1.625x10^5$
		$4.833x10^5$	$1.633x10^5$
3.	Lot 3	$4.822x10^5$	$1.622x10^5$
		$4.828x10^5$	$1.605x10^5$
		$4.831x10^5$	$1.611x10^5$
4.	Lot 4	$4.858x10^5$	$1.632x10^5$
		$4.833x10^5$	$1.624x10^5$
		$4.841x10^5$	$1.627x10^5$
5.	Lot 5	$4.835x10^5$	$1.649x10^5$
		$4.827x10^5$	$1.626x10^5$
		$4.822x10^5$	$1.614x10^5$
6.	Lot 6	$4.821x10^5$	$1.624x10^5$
		$4.810x10^5$	$1.618x10^5$
		$4.829x10^5$	$1.626x10^5$
n		18	18
Mean		$4.834x10^5$	$1.624x10^5$
SD		0.013	0.010
%CV		0.26	0.65
% Mean Accuracy		99.71%	100.50%
QC failures		0	0

Table No 3.08: Matrix effect Results of Avelumab (HQC-30ng/ml, LQC-10ng/ml)

The benchmark for approval

Two out of three samples had a percent average exactness of 85-115. 80% of the matrices must fulfil the standards for approval. LQC is 85% and HQC is 115 percent for numerous biologic-matrix back-measured concentration accuracy percentages.

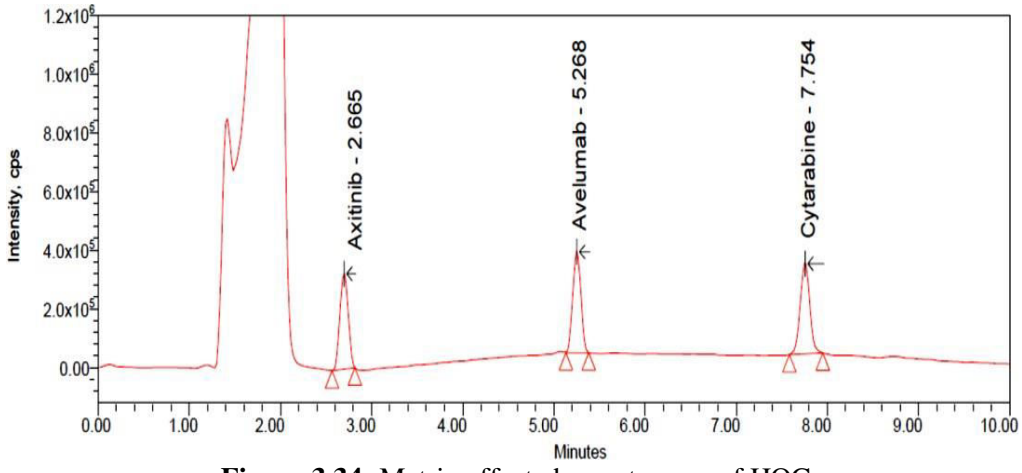


Figure 3.34: Matrix effect chromatogram of HQC

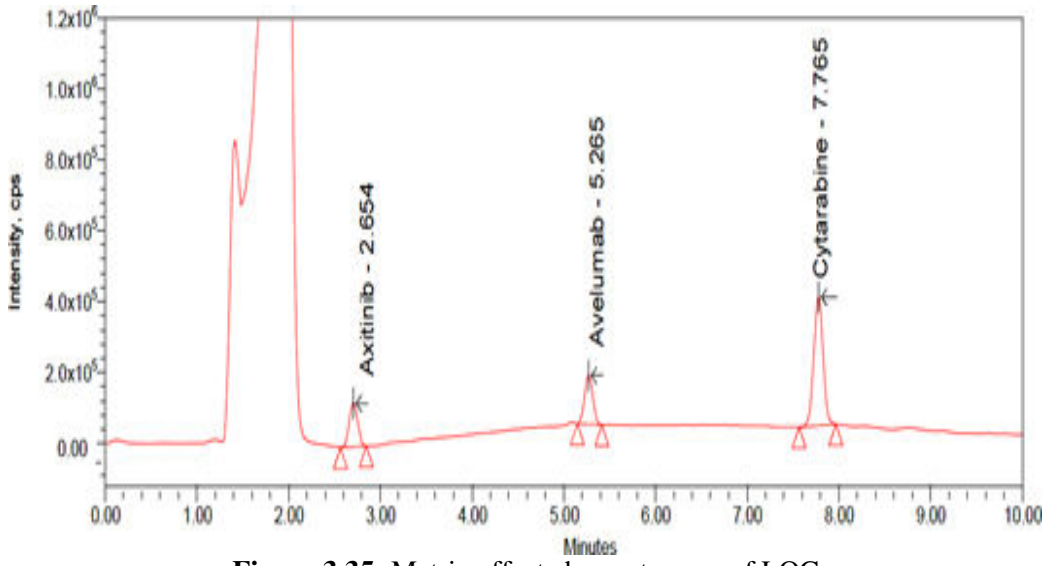


Figure 3.35: Matrix effect chromatogram of LQC

3.7.3.6 LOD and LOQ

In order to anticipate the bounds of both availability and quantity, an LCMS method was developed that involves injecting progressively decreasing concentrations of standard solutions into a calibration curve approach. Axitinib and Avelumab had LOD values of 0.15 ng/ml and 0.6 ng/ml, respectively, with s/n of 3 and 9 respectively. Axitinib and Avelumab are anticipated to have LOQ values of 0.5 ng/ml and 2 ng/ml, respectively, with s/n of 22 and 28. **Table 3.09** gives detection and quantification limit. **Fig 3.36 and 3.37** gives LOD and LOQ chromatograms.

Specimen name	LOD		LOQ	
	Strength in ng/ml	s/b factor	Strength in ng/ml	s/b factor
Axitinib	0.15	3	0.5	22
Avelumab	0.6	9	2	28

Table 3.09: Detection & quantification limits

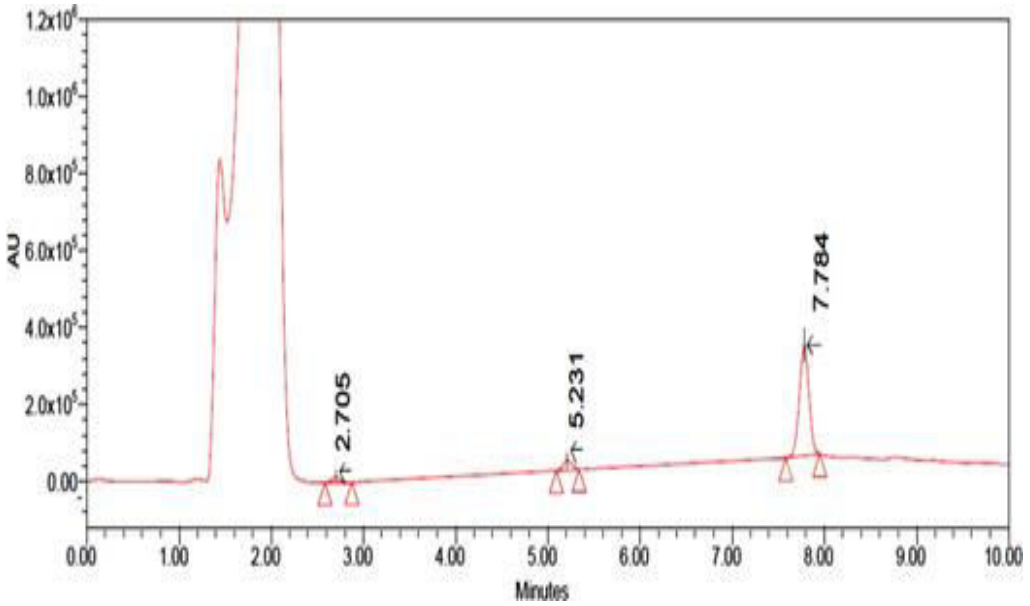


Figure 3.36: Chromatogram of LOD

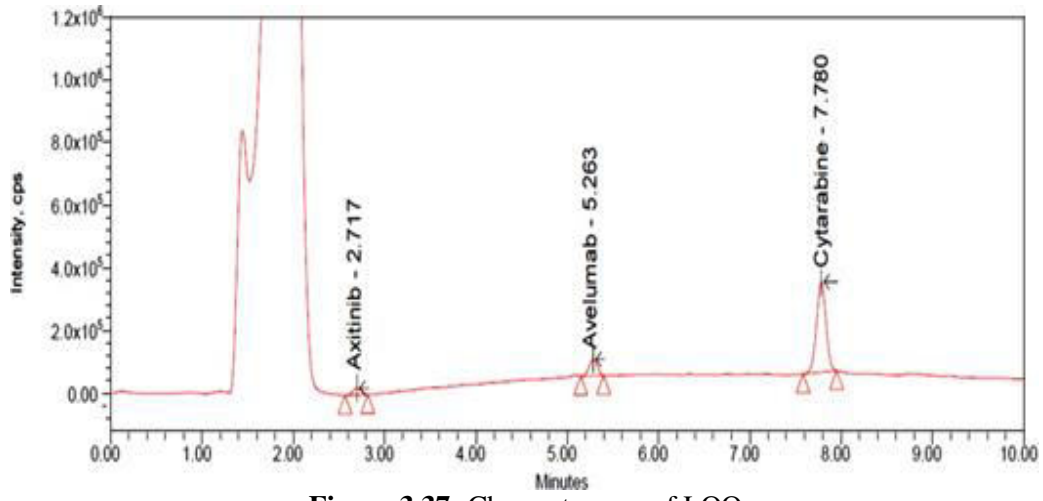


Figure 3.37: Chromatogram of LOQ

3.7.3.7 Precision and Accuracy

The accuracy and precision of internal control samples were determined by combining the findings of all individual assays. Based on the information presented, it was clear that the plan was both accurate and successful. Table 3.10 and 3.11 demonstrate the precision findings of avelumab and axitinib. Quality control samples for avelumab and axitinib had accuracy values of 98.8-99.9 and 99.4-99.8, respectively. Of the total samples tested, less than 5% come from Avelumab and Axitinib CV. Precision and accuracy results were given in **table 3.10 and 3.11**. **Fig 3.38-3.41** gives the precision and accuracy chromatograms.

Table No 3.10: Precision and accuracy Results of Axitinib

Qc Name	LLQC	LQC	MQC	HQC
Conc.(ng/ml)	0.5	2.5	5	7.5
QC sample -1	0.512	2.528	5.1	7.526
QC sample -2	0.534	2.678	5.248	7.589
QC sample -3	0.538	2.798	5.384	7.682
QC sample -4	0.548	2.854	5.468	7.763
QC sample -5	0.553	2.93	5.528	7.542
QC sample -6	0.567	2.961	5.687	7.524
Mean	0.542	2.791	5.402	7.604
Stddev	0.0171	0.149	0.190	0.089
%CV	1.895	0.985	0.885	0.985
Accuracy %	99.145	98.354	99.568	100.128

Table No 3.11: Precision and accuracy Results of Avelumab

QC Name	LLQC	LQC	MQC	HQC
Conc.(ng/ml)	2 ng/ml	10 ng/ml	20 ng/ml	30 ng/ml
QC sample -1	2.182	10.194	20.165	30.138
QC sample -2	2.314	10.652	20.125	30.125
QC sample -3	2.568	10.353	20.145	30.242
QC sample -4	2.478	10.485	20.256	30.356
QC sample -5	2.121	10.865	20.569	30.458
QC sample -6	2.957	10.586	20.685	30.567
QC Name	**LLQC**	**LQC**	**MQC**	**HQC**
Mean	2.4133	10.506	20.313	30.308
SD	0.302	0.262	0.251	0.185

%CV	1.124	0.965	0.975	0.856
Accuracy	99	99.624	99.182	100

The benchmark for approval

A accuracy of 15% for LQC, MQC, and HQC samples is acceptable, whereas LLQC samples should have a precision of 20% is acceptable under the present technique.

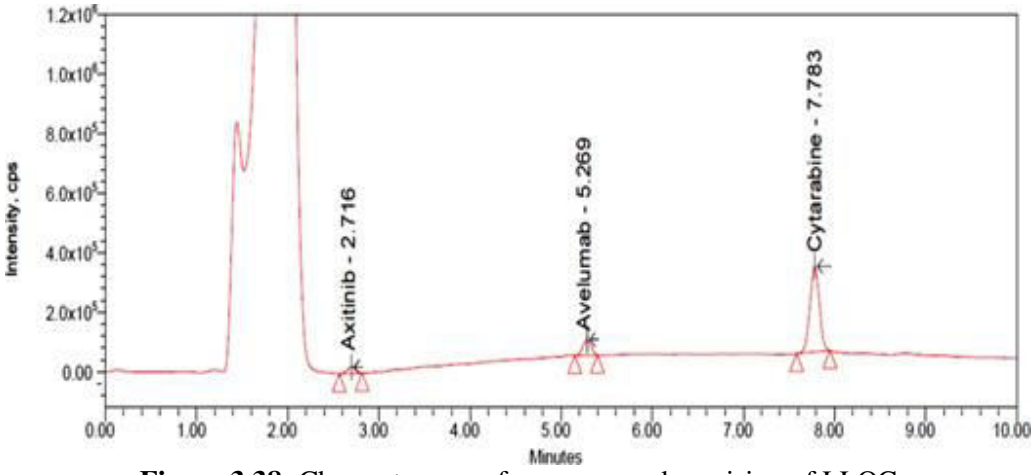


Figure 3.38: Chromatogram of accuracy and precision of LLQC

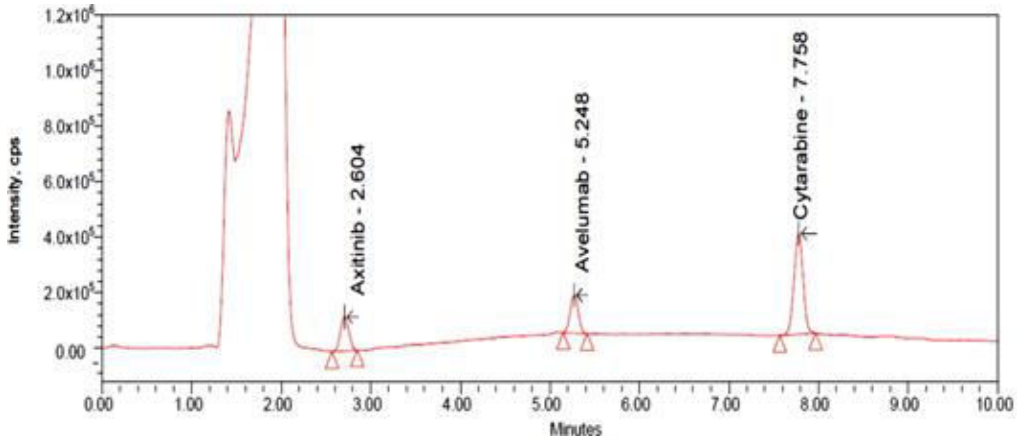


Figure 3.39: Chromatogram of accuracy and precision of LQC

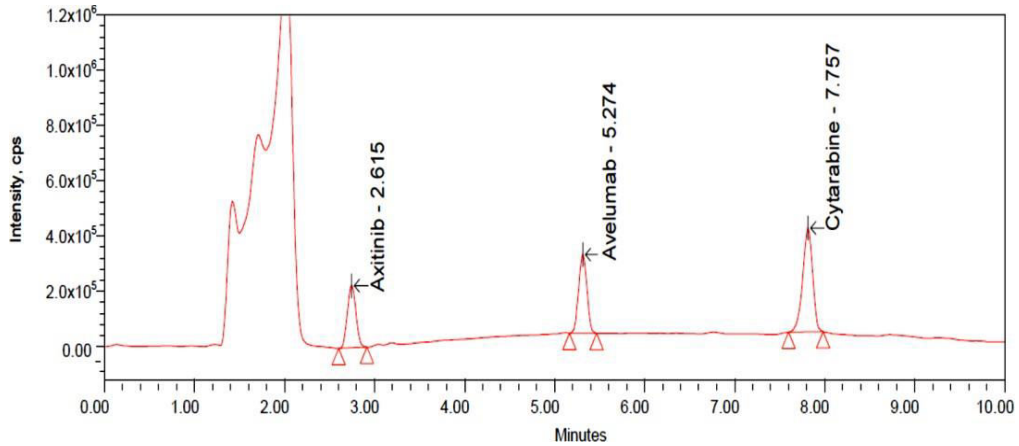


Figure 3.40: Chromatogram of accuracy and precision of MQC

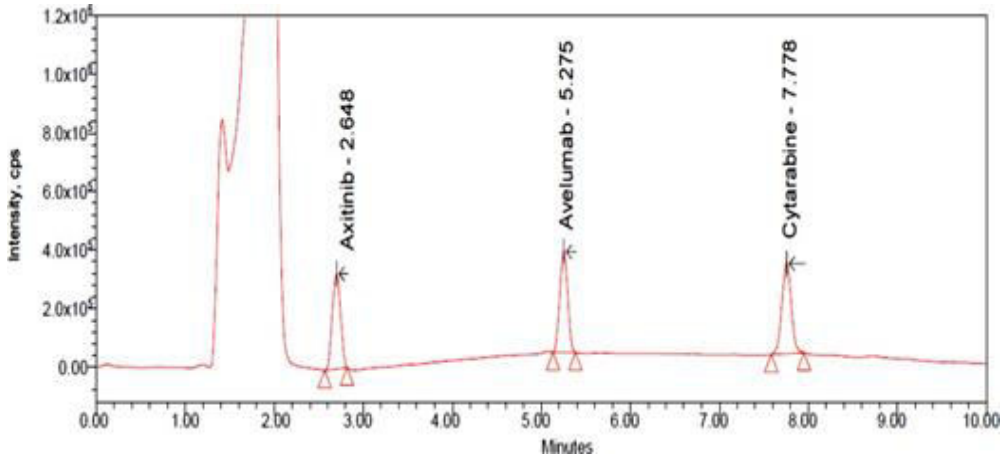


Figure 3.41: Chromatogram of accuracy and precision of HQC

3.7.3.8 Recovery of analyte

Qualitative and quantitative assessments of medication and IS recovery were performed at three distinct concentrations (low, medium, and high). By comparing the sample's reaction to a normal solution, recovery is determined. The amount of analyte utilised affects the extraction's effectiveness, and the findings are equivalent to those obtained by analytical methods. Following are the extracted and un- extracted chromatograms for the chromatograms of LQC, MQC, and HQC. **Table**

3.12 and 3.13 gives recovery results. **Fig 3.42-3.47** gives recovery chromatograms.

Table No 3.12: Recovery of analyte of Axitinib

Replicate trial	HQC (7.5 ng/ml)		
	Results for extraction part	Results for un- extracted part	Matrix Factor
1.	$2.775x10^5$	$2.847x10^5$	0.9747
2.	$2.767x10^5$	$2.858x10^5$	0.9682
3.	$2.746x10^5$	$2.863x10^5$	0.9591
4.	$2.759x10^5$	$2.841x10^5$	0.9711
5.	$2.764x10^5$	$2.850x10^5$	0.9698
6.	$2.750x10^5$	$2.868x10^5$	0.9589
n	6	6	6
Mean	$2.760x10^5$	$2.855x10^5$	0.9670
SD	0.011	0.010	0.007
%CV	0.39	0.36	0.68
%Mean Recovery	94.46%	97.71%	-

Replicate trial	MQC(5.0 ng/ml)		
	Extracted Response	Un Extracted Response	Matrix Factor
1.	$1.924x10^5$	$2.054x10^5$	0.9367
2.	$1.945x10^5$	$2.063x10^5$	0.9428
3.	$1.931x10^5$	$2.047x10^5$	0.9433
4.	$1.944x10^5$	$2.069x10^5$	0.9396
5.	$1.925x10^5$	$2.052x10^5$	0.9381
6.	$1.919x10^5$	$2.068x10^5$	0.9279
n	6	6	6

Mean	$1.931x10^5$	$2.059x10^5$	0.9381
SD	0.011	0.009	0.006
%CV	0.56	0.44	0.60
%Mean Recovery	99.13%	105.70%	-

LQC (2.5ng/ml)		
Extracted Response	**Un Extracted Response**	**Matrix Factor**
$0.898x10^5$	$1.014x10^5$	0.8856
$0.883x10^5$	$1.008x10^5$	0.8760
$0.889x10^5$	$1.024x10^5$	0.8682
$0.872x10^5$	$1.031x10^5$	0.8458
$0.897x10^5$	$1.013x10^5$	0.8855
$0.866x10^5$	$1.029x10^5$	0.8416
6	6	6
$0.884x10^5$	$1.020x10^5$	0.8671
0.013	0.009	0.019
1.48	0.93	2.23
90.76%	104.72%	-

Table No 3.13: Recovery of analyte of Avelumab

Replicate trial	**HQC (30ng/ml)**		
	Results for extraction part	**Results for un-extracted part**	**Matrix Factor**
1.	$4.819x10^5$	$4.967x10^5$	0.9702
2.	$4.806x10^5$	$4.982x10^5$	0.9647
3.	$4.828x10^5$	$4.978x10^5$	0.9699
4.	$4.800x10^5$	$4.974x10^5$	0.9650
5.	$4.831x10^5$	$4.965x10^5$	0.9730
6.	$4.826x10^5$	$4.991x10^5$	0.9669
n	6	6	6
Mean	$4.818x10^5$	$4.976x10^5$	0.9683
SD	0.013	0.010	0.003
%CV	0.26	0.20	0.34
%Mean Recovery	99.38%	102.64%	-

Replicate trial	**MQC (20ng/ml)**		
	Results for extraction part	**Results for un-extracted part**	**Matrix Factor**
1.	$3.214x10^5$	$3.424x10^5$	0.9387
2.	$3.237x10^5$	$3.412x10^5$	0.9487
3.	$3.219x10^5$	$3.437x10^5$	0.9366
4.	$3.228x10^5$	$3.416x10^5$	0.9450
5.	$3.207x10^5$	$3.404x10^5$	0.9421
6.	$3.240x10^5$	$3.429x10^5$	0.9449
n	6	6	6

Mean	$3.224x10^5$	$3.420x10^5$	0.9427
SD	0.013	0.012	0.004
%CV	0.41	0.35	0.47
%Mean Recovery	99.75%	105.82%	-

LQC (10ng/ml)		
Results for extraction part	**Results for un- extracted part**	**Matrix Factor**
$1.621x10^5$	$1.795x10^5$	0.9031
$1.627x10^5$	$1.774x10^5$	0.9171
$1.635x10^5$	$1.781x10^5$	0.9180
$1.642x10^5$	$1.758x10^5$	0.9340
$1.639x10^5$	$1.763x10^5$	0.9297
$1.612x10^5$	$1.786x10^5$	0.9026
6	6	6
$1.629x10^5$	$1.776x10^5$	0.9174
0.012	0.014	0.013
0.71	0.79	1.42
100.80%	109.90%	-

The benchmark for approval

Specific stages such as QC and ISTD must have a recovered percentage (percent RSD) of less than or equal to 15%. Replicas of QC should provide net average recovery findings of less than or equal to 20%.

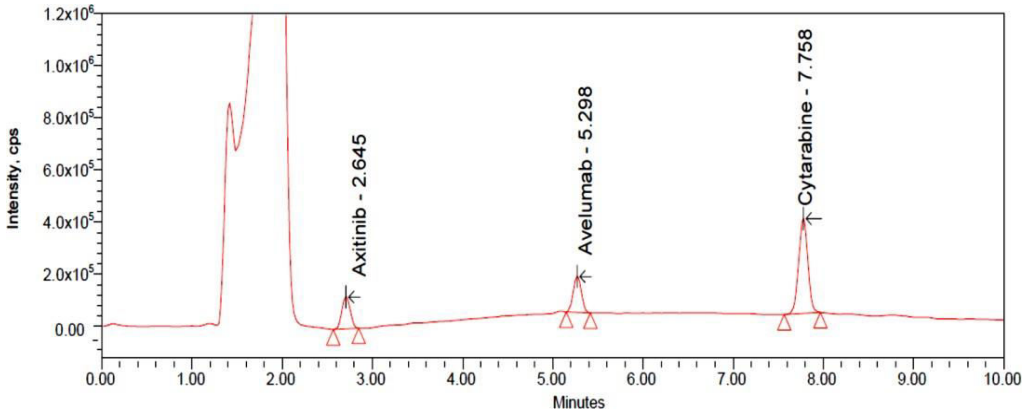


Figure 3.42: Chromatogram of recovery extracted LQC

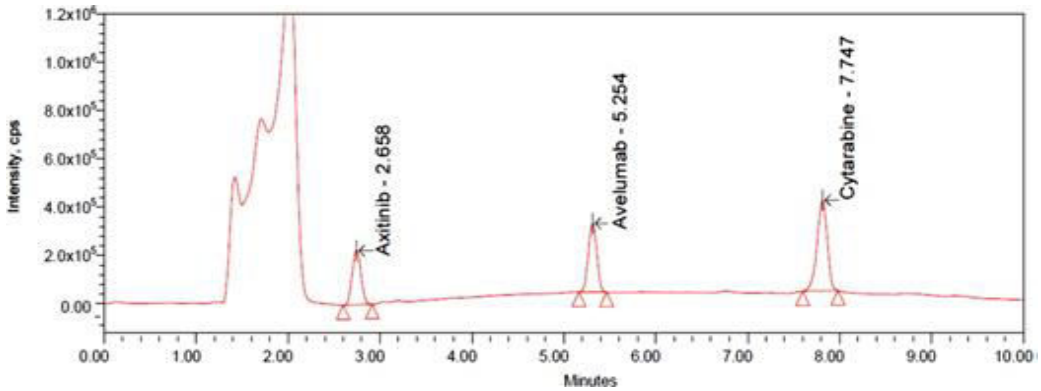


Figure 3.43: Chromatogram of recovery extracted MQC

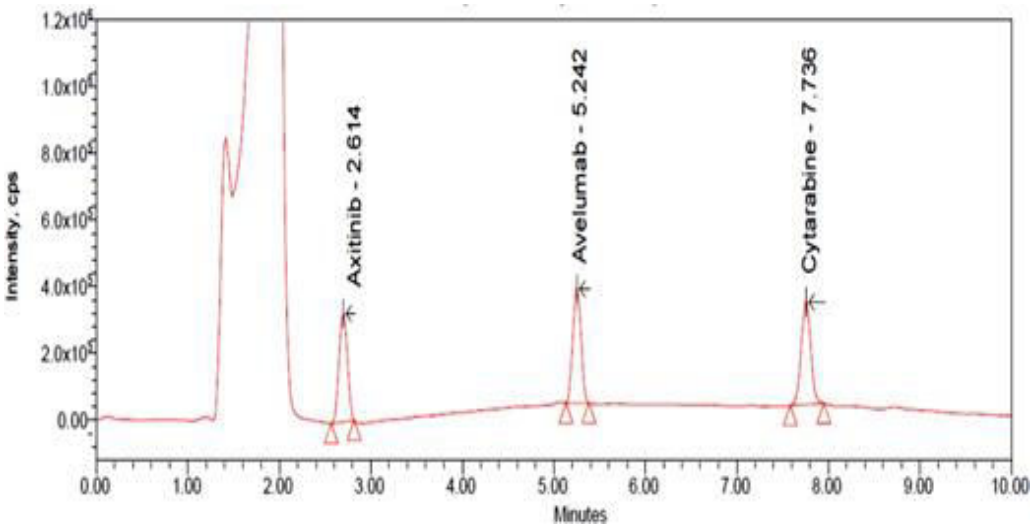


Figure 3.44: Chromatogram of recovery extracted HQC

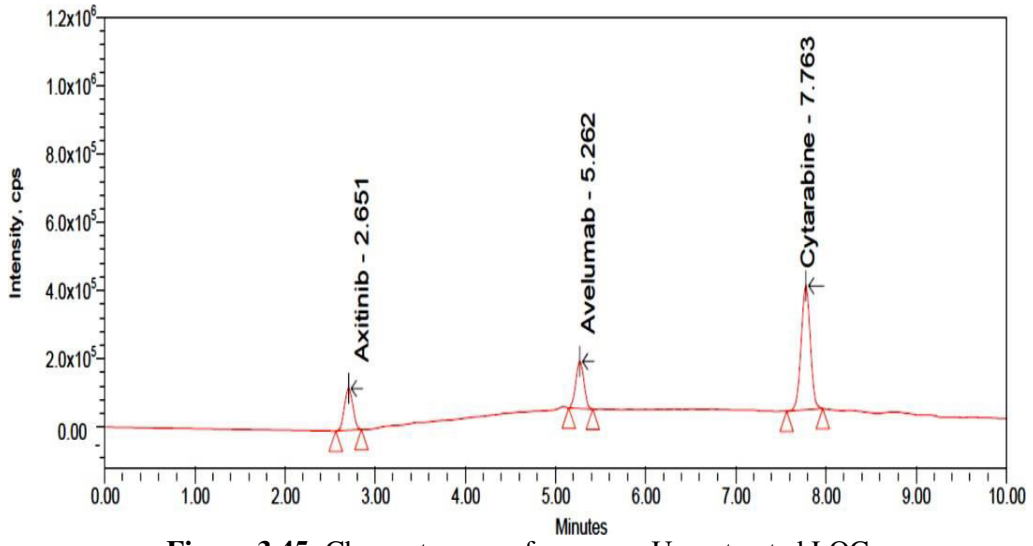


Figure 3.45: Chromatogram of recovery Un-extracted LQC

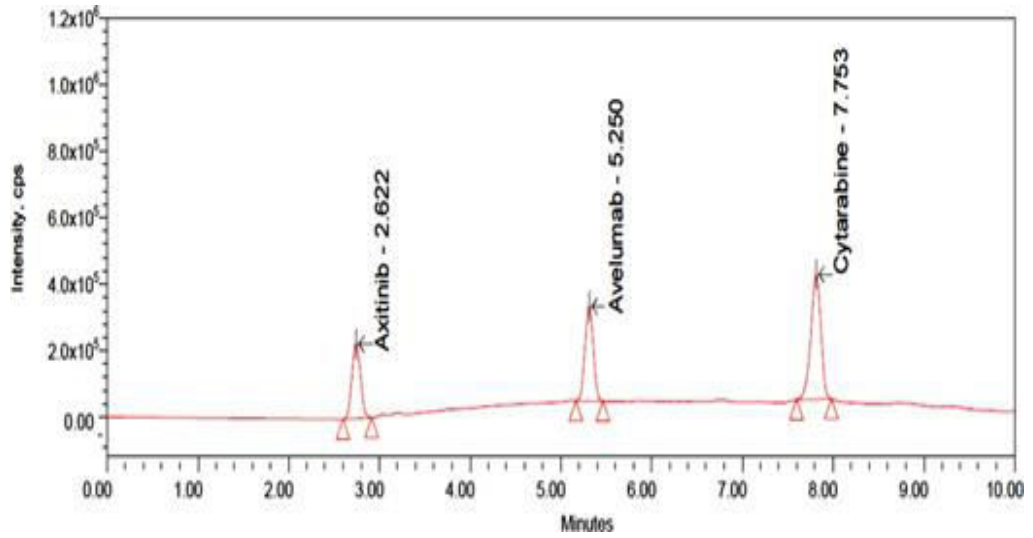


Figure 3.46: Chromatogram of recovery Un-extracted MQC

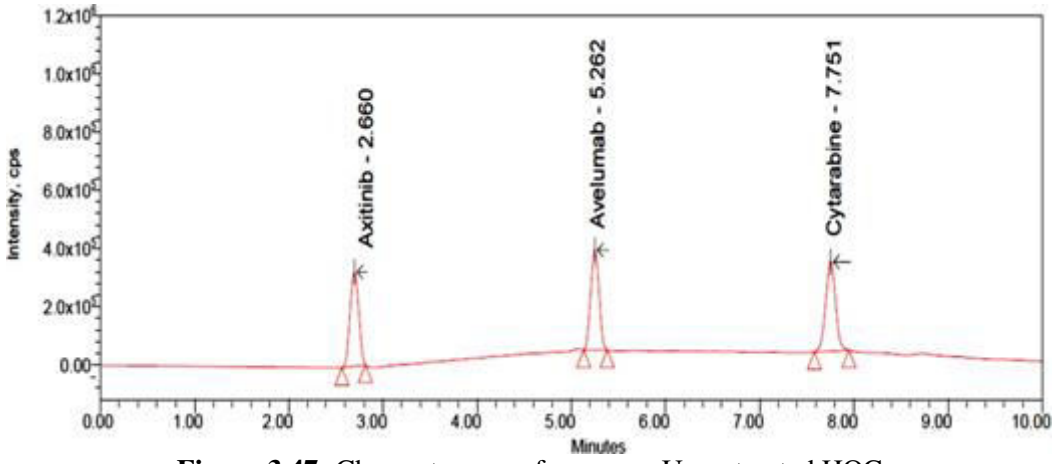


Figure 3.47: Chromatogram of recovery Un-extracted HQC

3.7.3.9 Ruggedness on precision accuracy

For six replications of varied strengths, precision and accuracy metrics are used to evaluate the proposed approach's correctness and effectiveness. Based on the percentage of SD, CV, and accuracy findings presented in the table below, Axitinib and Avelumab passed the Ruggedness on precision accuracy. **Table 3.14 and 3.15** gives ruggedness data.

P& A ID	Acquisition Batch ID	HQC (7.5 ng/ml)	MQC (5 ng/ml)	LQC (2.5ng/ml)	LLQC (0.5ng/ml)
		Ostensible strength in ng/ml			
		7.5	5.0	2.5	0.5
		Analyte peak region			
Different Column		$2.770x10^5$	$1.945x10^5$	$0.897x10^5$	$0.204x10^5$
		$2.749x10^5$	$1.922x10^5$	$0.864x10^5$	$0.217x10^5$
		$2.760x10^5$	$1.938x10^5$	$0.871x10^5$	$0.232x10^5$
		$2.756x10^5$	$1.911x10^5$	$0.879x10^5$	$0.224x10^5$
		$2.745x10^5$	$1.929x10^5$	$0.892x10^5$	$0.212x10^5$
		$2.768x10^5$	$1.917x10^5$	$0.881x10^5$	$0.220x10^5$
n		6	6	6	6
Average		$2.758x10^5$	$1.927x10^5$	$0.881x10^5$	$0.218x10^5$
SD		0.010	0.013	0.012	0.010
% CV		0.36	0.67	1.41	4.44
% Average Accuracy		94.39%	98.92%	90.45%	111.91%

Table No 3.14: Ruggedness on precision accuracy of Results of Axitinib

P& A ID	Acquisition Batch ID	HQC	MQC	LQC	LLQC
		Ostensible strength in ng/ml			
		30.0	20.0	10.0	2.0
		Analyte peak region			
Different Column		$4.811x10^5$	$3.214x10^5$	$1.612x10^5$	$0.389x10^5$
		$4.836x10^5$	$3.246x10^5$	$1.634x10^5$	$0.364x10^5$
		$4.824x10^5$	$3.232x10^5$	$1.600x10^5$	$0.391x10^5$
		$4.809x10^5$	$3.223x10^5$	$1.619x10^5$	$0.378x10^5$
		$4.813x10^5$	$3.218x10^5$	$1.627x10^5$	$0.383x10^5$
		$4.835x10^5$	$3.209x10^5$	$1.638x10^5$	$0.376x10^5$

n	6	6	6	6
Average	$4.821x10^5$	$3.224x10^5$	$1.622x10^5$	$0.380x10^5$
SD	0.012	0.013	0.014	0.010
% CV	0.25	0.42	0.88	2.60
% Average Accuracy	99.44%	99.75%	100.37%	117.57%

Table No 3.15: Ruggedness on precision accuracy of Results of Avelumab

3.7.3.10Stability

Diluents were added to Avelumab and Axitinib solutions and stored in a refrigerator at 2-8°C for solution stability analysis. 24-hour stock solutions were related with stock solutions that had been created the previous day. The bench top and auto sampler plasmas were stable for 24 hours, and 24 hours at 20°C in the auto sampler. Future stability indicated that Avelumab and Axitinib may be stored at - 30°C for up to 24 hours without deterioration. **Table 3.16 and 3.17** gives stability results.

Table no 3.16: Stability results of Avelumab

Stability experiment spiked plasma		**Spiked plasma conc.(n=6,ng/ml)**	**Conc.measured (n=6,ng/ml)**	**%CV**
Bench top stability	LQC	10	10.135	1.246
	MQC	20	20.257	0.858
	HQC	30	30.458	0.968
Auto sampler stability	LQC	10	10.897	0.952
	MQC	20	20.589	0.856
	HQC	30	30.124	0.977
Long term(Day28) stability	LQC	10	10.368	0.985
	MQC	20	20.354	0.856
	HQC	30	30.126	0.746
Wet extract stability	LQC	10	10.328	0.789
	MQC	20	20.856	0.852
	HQC	30	30.175	0.845
Dry extract stability	LQC	10	10.689	0.963
	MQC	20	20.657	0.784
	HQC	30	30.821	0.894
Freeze thaw stability	LQC	10	10.628	0.854
	MQC	20	20.145	0.874
	HQC	30	30.286	0.745
Short term stability	LQC	10	10.369	0.841
	MQC	20	20.486	1.456
	HQC	30	30.289	1.025

Table No 3.17: Stability results of Axitinib

Stability experiment spiked plasma		**Spiked plasma conc.(n=6,ng/ml)**	**Conc.measured (n=6,ng/ml)**	**%CV**
Bench top stability	LQC	2.5	2.534	1.042
	MQC	5	5.12	0.986
	HQC	7.5	7.548	0.974
Auto sampler stability	LQC	2.5	2.525	0.981
	MQC	5	5.321	0.874
	HQC	7.5	7.584	0.954
Long term (Day	LQC	2.5	2.587	0.845
	MQC	5	5.874	0.768

28)stability	HQC	7.5	7.582	0.734
Wet extract stability	LQC	2.5	2.574	0.861
	MQC	5	5.369	0.827
	HQC	7.5	7.514	0.965
Dry extract stability	LQC	2.5	2.542	1.142
	MQC	5	5.841	1.254
	HQC	7.5	7.586	0.964
Freeze thaw stability	LQC	2.5	2.564	0.985
	MQC	5	5.684	1.246
	HQC	7.5	7.521	1.103
Short term stability	LQC	2.5	2.574	0.824
	MQC	5	5.231	0.987
	HQC	7.5	7.541	1.485

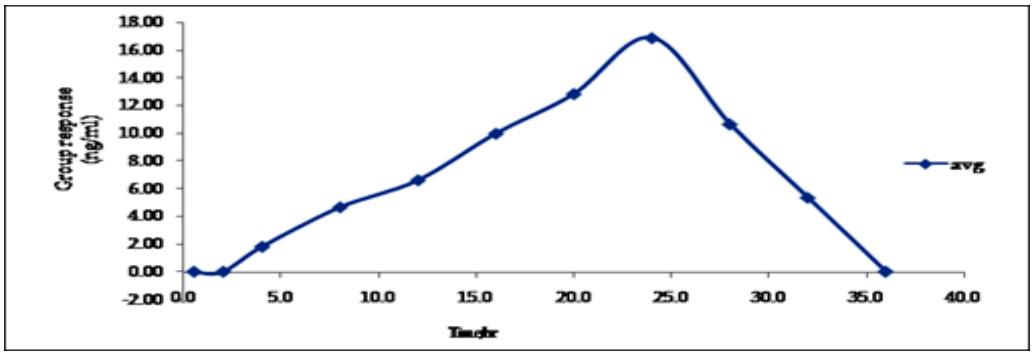
3.7.3.11Pharmacokinetic Studies

Cmax, Tmax, T1/2, Kel, Ka, AUC0-t, AUC0-, AUMC 0-24, AUMCt-,

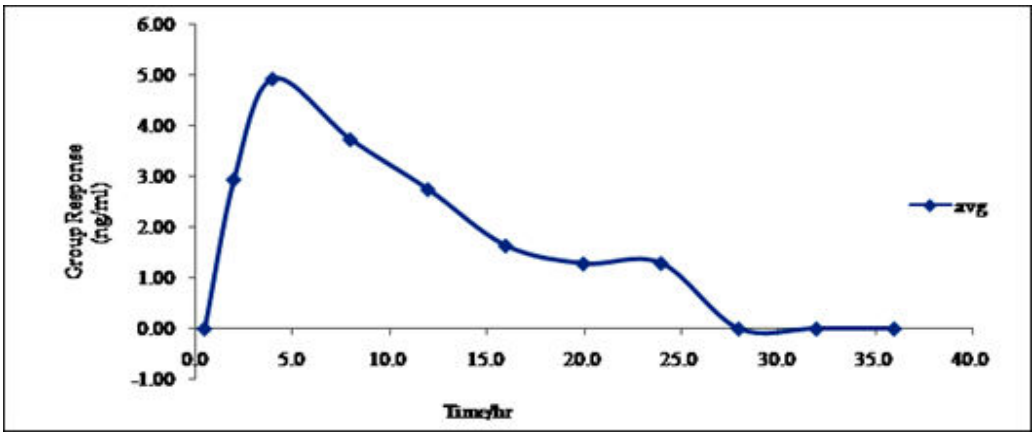
MRT0-24, MRT0- were computed and the data is displayed in Table 6. They observed that the Cmax of the two drugs was 16.9 ng/ml for avelumab whereas the Cmax of the other drug was 4.9 ng/ml, respectively. Avelumab and axitiniib had Tmaxes of 24 and 4 hours, respectively. The t12 values for avelumab and axitiniib were 32 and 24 hours, respectively. It is 0.41 for avelumab and 0.005 for axitiniib. Each of the two drugs studied had an estimated half-life of 0.13 hours and 1.41 hours, respectively, according to the results of this study. These two drugs had AUC0-t values that were 273 and 63 ng-hr/ml, respectively, at the time of the study. It was determined that AUMC0-t and AUMC0-t for ng-hr/ml were 352.18, 141.11 and 273.06, 63.Cmax, Tmax, T1/2, Kel, Ka, AUC0-t, AUC0-, AUMC 0-24, AUMCt-, MRT0-24, MRT0- Cmax, Tmax, T1/2, Kel, Ka, AUC0-t, AUC0-, AUMC

0-24, AUMCt-, MRT0-24, MRT0-were computed and the data is displayed in Table

6. They observed that the Cmax of the two drugs was 16.9 ng/ml for avelumab whereas the Cmax of the other drug was 4.9 ng/ml, respectively. Avelumab and axitiniib had Tmaxes of 24 and 4 hours, respectively. The t12 values for avelumab and axitiniib were 32 and 24 hours, respectively. It is 0.41 for avelumab and 0.005 for axitiniib. Each of the two drugs studied had an estimated half-life of 0.13 hours and 1.41 hours, respectively, according to the results of this study. These two drugs had AUC0-t values that were 273 and 63 ng-hr/ml, respectively, at the time of the study. It was determined that AUMC0-t and AUMC0-t for ng-hr/ml were 352.18, 141.11 and 273.06, 63.18 ug/h ml-1, respectively. Table 6 shows that the MRT0-24 and MRT0- for ng-hr/ml were 24.17, 6.31 and 36.0, 36.0 respectively. **Table 3.16** gives pharmacokinetic parameter results and **fig 3.48** gives recovery plots.



A



B

Figure 3.48: Recovery plot of (A) Axitinib and (B) Avelumab

Table No 3.18: Pharmacokinetic parameters of Axitinib and Avelumab

Pharmacokinetic parameters	**Avelumab**	**Axitinib**
AUC_{0-t}	273 ng-hr/ml	63 ng-hr/ml
C_{max}	16.9 ng/ml	4.9 ng/ml
$AUC_{0-\infty}$	352 ng-hr/ml	141 ng-hr/ml
t_{max}	24 hr	4hr
$T_{1/2}$	32 hr	24hr
k_{el}	0.41 h-1	0.005 h-1
Ka	0.13 h-1	1.41 h-1
MRT0-24	24.17 ng-hr/ml	6.31 ng-hr/ml
MRT0-□	36.0 ng-hr/ml	36.0 ng-hr/ml

3.8 CONCLUSIONS

For the first time, a more sensitive HPLC-ESI-LCMS/MS technique for the detection of Avelumab and Axitinib in rabbit plasma was developed and validated. The bio analytical approach presented here is robust, quick, and repeatable. The FDA has deemed this approach to be safe and effective. Pharmacokinetic and analytic research may benefit from the simple and efficient technique that has been devised.

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4.

NEW VALIDATED REVERSE PHASE ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY METHOD

4.1DROSPIRENONE

4.1.1Drug Profile of Drospirenone

Drospirenone is a progestin (**1**) medication which is used in birth control pills to prevent pregnancy and in menopausal hormone therapy (**2, 3**), among other uses

(**4**). It is available both alone under the brand name **Slynd** and in combination with an estrogen under the brand name **Yasmin** among others. The medication is taken by mouth. Common side effects include acne (**5**), headache, breast tenderness (**6**), weight increase, and menstrual changes (**7, 8**). Rare side effects may include high potassium levels and blood clots, among others (**9**). Drospirenone is a progestin (**10**), or a synthetic progestogen, and hence is an agonist of the progesterone receptor, the biological target of progestogens like progesterone. It has additional antimineralocorticoid **(11, 12**) and antiandrogenic activity (**13**) and no other important hormonal activity. Because of its antimineralocorticoid activity and lack of undesirable off-target activity, drospirenone is said to more closely resemble bioidentical progesterone than other progestins (**14, 15**). Drospirenone (DRSP) is used by itself as a progestogen-only birth control pill, in combination with the estrogens ethinylestradiol (EE) or estetrol (E4), with or without supplemental folic acid (vitamin B_9), as a combined birth control pill, and in combination with the estrogen estradiol (E2) for use in menopausal hormone therapy. A birth control pill with low-dose ethinylestradiol is also indicated for the treatment of moderate acne, premenstrual syndrome (PMS) (**16**), premenstrual dysphoric disorder (PMDD) (**17, 18**), and dysmenorrhea (**19**) (painful menstruation). For use in menopausal hormone therapy, E2/DRSP is specifically approved to treat moderate to severe vasomotor symptoms (**20**) (hot flashes), vaginal atrophy, and postmenopausal osteoporosis (**21, 22**). The drospirenone component in this formulation is included specifically to prevent estrogen-induced endometrial hyperplasia (**23**). Drospirenone has also been used in combination with an estrogen as a component of hormone therapy for transgender women (**24, 25**).

Structure of Drospirenone [Fig 4.01]

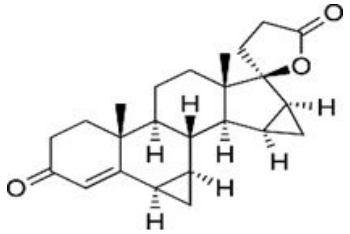

Figure 4.01: Drospirenone's chemical structure

4.1.2Name of the IUPAC:

(1R,2R,4R,10R,11S,14S,15S,16S,18S,19S)-10,14-

dimethylspiro[hexacyclo[9.8.0.0^{2,4}.0^{5,10}.0^{14,19}.0^{16,18}]nonadec-5-ene-15,5'-oxolane]-2',7- dione

4.1.3Molecularformula: $C_{24}H_{30}O_3$

4.1.4Molecular weight: 366.501 g·mol^{-1}

4.1.5Category: A progestin drug, drospirenone is prescribed to women.

4.1.6Mechanism of action:

Fetal stimulating hormone and luteinizing hormone (LH) production are prevented when Drospirenone and Ethinyl Estradiol are taken together. In addition to altering the nature of cervical mucus and impeding sperm motility, this medication may also help prevent pregnancy by reducing the likelihood of embryo implantation. An derivative of the diuretic spironolactone, Drospirenone has anti-mineralocorticoid action, inhibiting aldosterone receptors, which increases sodium and water excretion. Drospirenone treatment has been shown to have antiandrogenic effects in animal studies. Inhibiting the binding of dihydrotestosterone (DHT) to its receptor and preventing the generation of androgens in the ovaries, this action helps cure acne and hirsutism. At this point in the menstrual cycle, drospirenone may also help to lessen the swelling in the sebaceous follicles.

- Headache, mood changes, feeling tired or irritable;
- Weight gain.

4.1.8 Contraindications:

Progestogen-sensitive malignancies such as cervical cancer and other progestogen sensitive malignancy, benign or malignant liver tumours, and undetected irregular uterine haemorrhage are all contraindications to drospirenone use (high potassium levels).

4.1.9 Absorption:

Drospirenone's first-pass actions result in a bioavailability of around 76%. 13,19 Drospirenone's plasma concentration reaches its peak between 60 to 87 ng/mL within 1 to 2 hours after oral dosing. 1 In a European prescribing monograph for a combination product of estradiol and drospirenone, drospirenone is absorbed entirely and fast. A Cmax of 21.9 ng/ml was reached roughly one hour after injection, according to this study. Bioavailability is claimed to be between 76% and 84%.'

4.1.10 Uses:

Ethinyl estradiol and progestin are the two hormones in this medicine (drospirenone). To avoid becoming pregnant, women take this medication. Ovulation (the release of an egg) is inhibited throughout the menstrual cycle, which is how it works. To prevent sperm from reaching an egg (fertilisation), it thickens the vaginal fluid and alters the lining of the uterus (womb). A fertilised egg will be expelled from the body if it does not connect to the uterus. Your periods will be more regular, you will have less blood loss and discomfort, and your risk of ovarian cysts will be reduced by using birth control. It does not protect you or your partner against sexually transmitted illnesses if you use this medicine (such as HIV, gonorrhea, chlamydia).

4.1.11 Adult dose:

- 3mg

4.2ESTETROL

4.2.1Drug Profile of Estetrol:

Estetrol (**E4**), or **oestetrol**, is a weak estrogen steroid hormone, which is found in detectable levels only during pregnancy in humans (**26**). It is produced exclusively by the fetal liver (**27**). Estetrol is closely related to estriol (E3), which is also a weak estrogen that is found in high quantities only during pregnancy (**28**). Along with estradiol (E2), estrone (E1), and E3, estetrol (E4) is a major estrogen in the body,

although only during pregnancy. Estetrol is an estrogen and has estrogenic effects in various tissues. Estetrol interacts with nuclear Estrogen Receptor (ERα) (**29**) in a manner identical to that of the other estrogens and distinct from that observed with Selective Estrogen Receptor

Modulators (SERMs) (**30, 31**). So far the physiological function of estetrol is unknown. The possible use of estetrol as a marker for fetal well- being has been studied quite extensively. However, due to the large intra- and inter- individual variation of maternal estetrol plasma levels during pregnancy this appeared not to be feasible (**32**).

Structure of Estetrol [Fig 4.02]

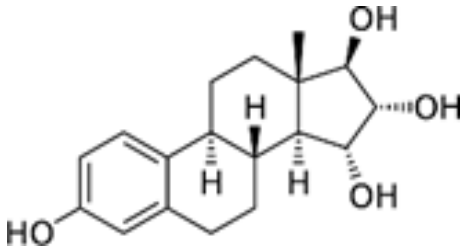

Figure 4.02: Estetrol's chemical structure

4.2.2Name of the IUPAC: (8R,9S,13S,14S,15R,16R,17R)-13-methyl- 6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthrene-3,15,16,17-tetrol

4.2.3Formula molecular: $C_{18}H_{24}O_4$

4.2.4Molecular weight: 304.386 g/mol

4.2.5Category:
A steroid hormone called oestetrol (E4), Estetrol (E4), or oestetrol, is discovered solely in pregnant women. The foetal liver is the only source of this hormone. Estriol (E3), which is likewise a weak oestrogen, is closely linked to esttrol (E2), which is present in high concentrations exclusively during pregnancy.

4.2.6Mechanism of Action:
In pregnancy, a naturally occurring oestrogen, Estetrol, is synthesised and has been shown to be selective for both the oestrogen receptor- and the oestrogen receptor– a (ER–a) receptors. 13 ER alpha and ER beta bind Estetrol with low to moderate affinity, with a preference for the ER alpha. 8 A unique method of action is shown by Estetrol's ability to display oestrogen receptor agonist activity on the uterus, the vagina, and the endometrium, and negative estrogenic activity on breast tissue.

4.2.7Side effects of Estetrol:
Estetrol in women has been shown to have minimal adverse effects. For four weeks, large doses of estetrol (20–40 mg/day) were shown to reduce libido in males by 40% and nipple soreness by 35%. Similar to other estrogens, the medicine increases the risk of endometrial hyperplasia and endometrial cancer in women.

4.2.8Contraindications:
Drug contraindications and black box warnings on the label are covered in this organised database. Concomitant usage and co-administered drugs are both prohibited and restricted under each of the drug's "contraindications." The dangers, contraindications, and side effects detailed in our black box warnings are significant and life-threatening.

4.2.9Absorption:
When taken orally, Estetrol is quickly taken up by the body. According to a pharmacokinetic research, the Cmax of estetrol is 18 ng/mL and the AUC is 36.4 ng•h/mL. Drospirenone may be given in combination with estetrol to raise blood levels to 48.7ng/mL within one to three hours. The combination's bioavailability varies from 76% to 86%. 17 According to one clinical trial, the Tmax may be anywhere between 0.5 and 2 hours long, with a half-life of 4 days or less.

4.2.10 Uses:
Drospirenone and Estetrol are oral contraceptives that include oestrogen. Steroid estrogens have a broad variety of pharmacological purposes, from hormonal contraception to the treatment of menopausal symptoms, whether they are naturally or synthetically generated.

4.2.11 Adult dose: tablet14.2mg.

4.3 LITERATURE RESURVEY

Praveen C*, Ranganath MK and Divakar P, Method Development and Validation for Simultaneous Estimation of Ethinyl Estradiol and Drospirenone and Forced Degradation Behavior by HPLC in Combined Dosage Form: High performance liquid chromatographic (HPLC) methods for the detection of ethinyl estradiol and drospirenone in tablet format have been developed and validated. Thermo

Hypersil BDS C18 Column (4.6250 mm and 5 m) and a flow rate of 1.0 ml/min with a load of 15 l are used in this procedure. In the 30:70 mixture, the mobile phase consisted of acetonitrile and an ammonium acetate buffer. At a wavelength of 258 nm, the sample was detected. In the case of drospirenone, the linearity ranges were 0.06- 0.18 g/ml, and 6-18 g/ml. Retention It was discovered that ethinyl estradiol and drospirenone had a half-life of 1.4 and 5.3 minutes, respectively The ethinyl estradiol and drospirenone percent recovery study findings ranged from 97 to 103%. Acid/base, hydrolytic, photolytic and peroxide stress conditions were applied to the combination product and the stressed samples were evaluated. Ethinyl estradiol and drospirenone dose formulations were satisfactorily quantified using this newly discovered approach. ICH requirements were followed in the validation of this technique, which resulted in its approval for accuracy, precision, linearity, and robustness.

Shrikant Warkad, B. Santhakumari, A. V. Chandewar, Development and validation of a simple and sensitive RP-HPLC method for simultaneous estimation off Drospirenone and Ethynylestradiol in combined tablet dosage form: For the quantification of Drospirenone and Ethinylestradiol in tablet dosage form, a simple, selective, appropriate, quick, exact, and cost-effective Reverse-Phase HPLC technique has been designed and validated. A SHIMADZU UV180 spectrometer was used to measure absorption peaks, while a WATERS HPLC system was used to develop the method and perform the validation. The method was validated using a 2707 Auto- sampler, a WATERS C18 column (250x46x5 mm, 5 m) and an accompanying guard column of the same type, at a flow rate of 1.0 ml/min. At a wavelength of 275 nm, the detection was made. We used 70:30 acetonitrile/formic acid as the mobile phase solvents A and B, respectively. Drospirenone had a retention time of 4.15 minutes, whereas Ethinylestradiol had a retention duration of 2.25 minutes. For a linearity range of 50-150 g/ml, the technique was created and evaluated. In order to ensure its appropriateness, linearity, precision, and sturdiness, the method's developers conducted tests.

Viviane Benevenuti Silva, Angel Arturo Gaona Galdos, Cintia Maria Alves Mothe, Michele Bacchi Pallastrelli, Maria Segunda Aurora Prado, Anil Kumar Singh, Erika Rosa Maria KedorHackmann, Maria Inês Rocha Miritello Santoro: A high-performance liquid chromatographic technique for the simultaneous detection of

ethinyl estradiol and drospirenone in coated tablets has been devised and effectively implemented. With an acetonitrile: water 50:50 (v/v) mobile phase, the HPLC was run on a LiChroCART® 100RP column (125x4 mm i.d., 5 m) at a flow rate of 1.01 mL/min-1. Fluorescence detection of ethinyl estradiol and drospirenone were performed at wavelengths of 200 and 280 nm, respectively. Ethinyl estradiol took 4.0 minutes to elute, whereas drospirenone took 5.7 minutes. USP 34 guidelines were used to verify the procedure. Ethinyl estradiol and

drospirenone quality control tests in coated tablets may benefit from the suggested HPLC approach, which has benefits over previously published methods.

Saravanan Chandran, Xavier Rajarathinam SR and Anandan Kalaiselvan, Simultaneous Quantification of Drospirenone, Ethinyl Estradiol and Levomefolate by Stability Indicating RP-HPLC Method: For the simultaneous measurement of drospirenone, ethinyl estradiol, and levomefolate in bulk and combination tablet dosage form, a novel sensitive, selective, exact, and accurate reverse phase high performance liquid chromatographic technique has been established. On a Waters C18 (5 m, 250 mm 4.6 mm) column, separation and analysis of drospirenone, estradiol ethinyl, and levomefolate were accomplished using 60:20:20 (v/v/v) mobile phase ratios of acetonitrile, methanol, and 0.1 percent H3 PO4 at a temperature of 27°C. One millilitre per minute was the flow rate. With a detector set at 245nm, the effluents were analysed. For the technique validation, the International Conference on Harmonization and the FDA were consulted for guidance. The acceptance requirements are met for all of the validation attributes. Chemical oxidation using hydrogen peroxide, thermal decomposition, and photodecomposition were all used to test the medications under investigation. It was easy to distinguish the peaks of degradation products from the peaks of three other analytes (drospirenone, ethinyl estradiol and levomefolate). Thus, the liquid chromatographic technique devised and verified is capable of determining the drospirenone, ethinyl estradiol, and levomefolate in the presence of degradation products.

Sirajunisa Talath*, Sunil Dhaneshwar, A simple and rapid validated stability indicating HPLC method for the determination of Drospirenone in a pharmaceutical product: Reversed-phase high performance liquid chromatography (RP-HPLC) was used in this study to design and verify a simple technique for the assessment of synthetic progestin drospirenone's stability. Allure C18 (250 mm x 4.6

mm i.d., 3 m particle size) column was used for chromatographic separation in the Shimadzo Prominance model L20 HPLC system with SPD 20A prominence UV-Vis detector. Methanol and water were used as solvents, and UV detection at 247 nm was used for isocratic elution. Validation of the RP-HPLC technique for the analysis of drospirenone was carried out in accordance with the ICH recommendations for specificity and selectivity. Correlation coefficients of 1.0 were observed in the range of 3-18 g/mL for the proposed method's linearity. From 99.06 to 100.62 percent, drospirenone had a high degree of accuracy. Inter-day accuracy had a relative standard deviation less than 2 percent. Tablet dosage form drospirenone test was found to be within acceptable limits. For this study, Drospirenone was exposed to oxidation and photolysis as per the standards of the International Committee for Harmonization (ICH). Drospirenone quality control assays may employ this approach because it successfully separates degradation product peaks from the reference drug peak. Alkaline degradation conditions were shown to breakdown the medication the most (74.27%), followed by oxidative degradation conditions (36.41). A variety of degradation processes failed to break down the medication, including neutral, acidic, and photolytic.

4.4 EXPERIMENTAL

4.4.1Chemical and reagents:

Merck (India) Ltd., Worli, Mumbai, India provided the acetonitrile and formic acid (both UPLC labelled). Drospirenone and Estetrol APIs have their reference standards sourced from Hyderabad-based Spectrum Pharma Research Solutions Pvt. Ltd.

4.4.2Instrumentation:

An Acquity UPLC system with a PDA detector-2998 pump was utilised. The data was processed using Empower 2.0.

4.4.3Standard Solution Preparation:
Add roughly 70 mL of diluence, sonicated for 30 minutes to melt it, and then add diluence up to the mark in a volumetric flask of capacity 100 mL, carefully weighing and transferring 30 mg of Drospirenone and 142 mg of Estetrol. Diluents should be added to the above solution to make up for the prior solution that was diluted to 50 mL.

4.4.4Sample Solution Preparation:
You'll need to weigh out 30 mg of Drospirenone and 142 mg of Estetrol before adding them to a 100-mL flask with 70mL of diluent. A solution containing diluent was used to achieve the desired dilution. Take 5 mL of the solution, dilute it to 50 mL, and do this procedure. Filter your solution using a nylon syringe filter with a 0.45-micron mesh.

4.5 Method Development Analytical method development:
Drospirenone and Estetrol analysis by using UPLC, is a successful effort was undertaken to develop assay method.

4.5.1Method Development Parameters:
Selection of following parameters in method development is very important.

- Mode of chromatography
- Wavelength
- Column
- Mobile phase composition
- Solvent delivery system
- Flow rate
- Injection volume

4.5.1.1 Selection of Mode of Chromatography:

Selected mode of chromatography	:	Reversed phase chromatography Basis of
selection	:	polarity of the molecule
Reason for selection	:	as Drospirenone and Estetrol is polar

molecule it elutes at faster along with mobile phase

4.5.1.2 Detector Wavelength Selection:
The last stage in the analytical procedure is the choosing of the detector's wavelength. PDA detector and wavelength are used to determine precise wavelength of the standard API, which is manufactured and injected into the chromatographic system using PDA detector and wavelength.

Selected wave length	**:**	262 nm
Basis for selection	**:**	Maximum absorbance of analytes and impurities
Reason for selection	**:**	Drospirenone and Estetrol having maximum absorbance

262 nm.

4.5.1.3 Selection of Column:

Column selected: Luna C_{18}column (100X2.6 mm, 1.6 µ)

Basis for selection: On the basis of polarity, and chemical differences among analytical methods

Reason for selection: large variety of bonding chemistries, high mechanical stability, great physiochemical characteristics, and compatibility with a wide range of organic solvents are only some of the advantages of this material.

4.5.1.4 Selection of the mobile phase composition and of the buffer:

As far as peak symmetries and separation are concerned, the buffer and its intensity are critical factors. The ionic form may be altered during chromatography if the injection load on the column is not covered by the proper strength buffers.

Mobile phase preparation:

Solution A: Acetonitrile

Solution B: 0.1% formic acid

4.5.1.5 Selection of the rate of flow:

Even in reverse phase separation for the resolution of tiny molecules, flow rate is cited as a crucial element. Loading sample solution is critical in large-scale inversion phase chromatography, although analytical studies are not dependent on the flow rate. Dynamic binding capacity might vary depending on the flow rate employed during sample loading. It is necessary to assess the dynamic binding capacity while increasing the purification scale before determining the optimal flow rate for loading. Based on flow factor, retention duration, column composition, separation impurity, and peak symmetry, a flow rate of 1 ml/min has been chosen in this system.

4.5.1.6 Selection of injection volume:

For API estimate, a volume of 10 to 20 µl is often advised. Extraction has been a problem thus the test concentration may be kept low and injection volume can be increased to 50 µl. As long as the specified column volume isn't overflowing, it's all ok. Drospirenone and Estetrol are injected at a volume of 10 µl each in this manner.

4.6 RESULTS AND DISCUSSION

4.6.1Trials in optimization of chromatographic condition: Trial-1 [Fig 4.03]

Mobile phase	:	Acetonitrile and 0.1% OPA (80:20)
Column	:	X-Bridge C18 100mmx4.6mm, 3.5µm
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	10 min
Wavelength	:	200-400 nm
Observation	:	Peaks are not separated properly

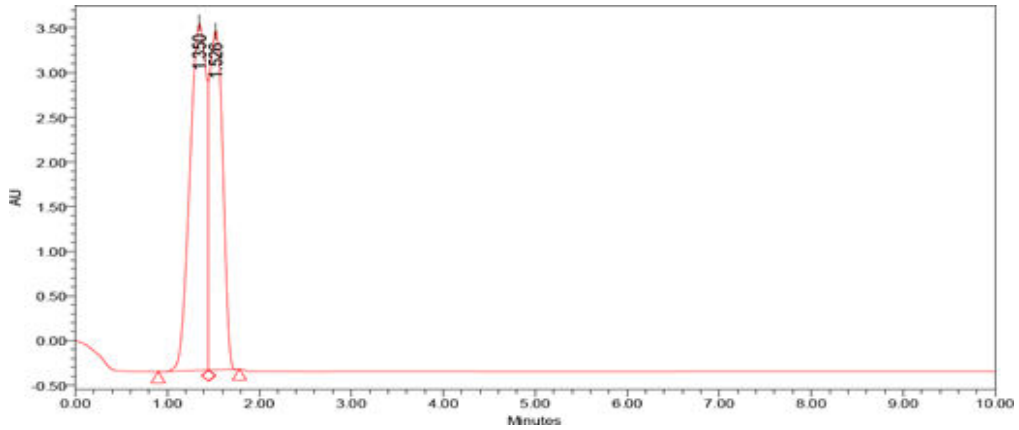

Figure 4.03: chromatogram of trial-1

Trial-2 [Fig 4.04]

Mobile phase	:	Acetonitrile and 0.1% OPA (70:30)
Column	:	X-Bridge C18 100mmx4.6mm, 3.5µm
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	10 min
Wavelength	:	262 nm
Observation	:	Peak tailing is not within the limit

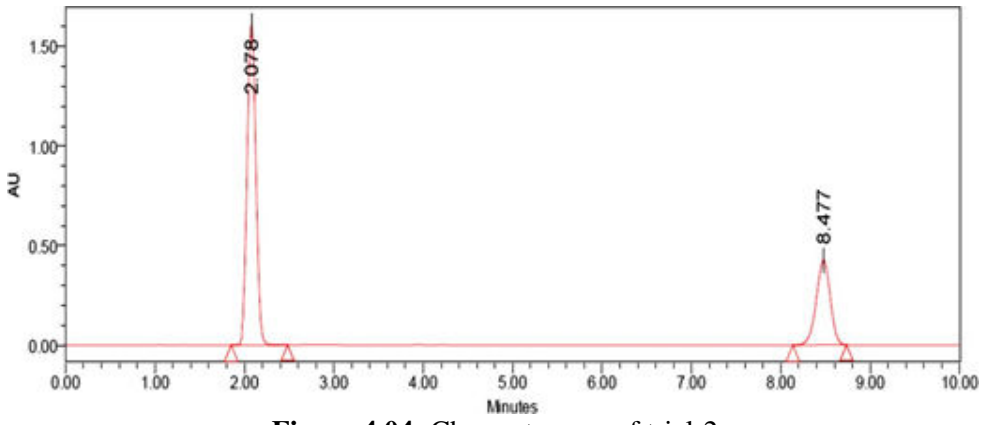

Figure 4.04: Chromatogram of trial-2

Trial-3 [Fig 4.05]

Mobile phase	:	Acetonitrile and 0.1% OPA (75:25)
Column	:	X-Bridge C18 100mmx4.6mm, 3.5µm
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	6 min
Wavelength	:	262 nm
Observation	:	First peak height is very high

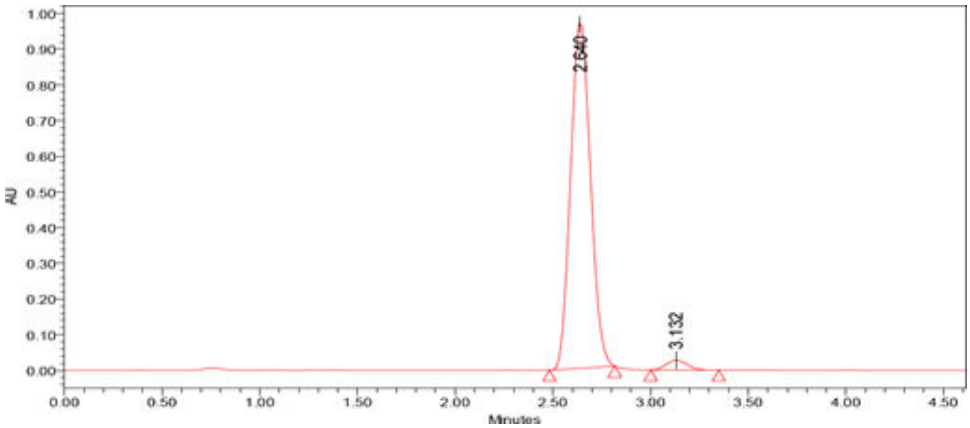

Figure 4.05: Chromatogram of trial-3

Trial-4 [Fig 4.06]

Mobile phase	:	Acetonitrile and 0.1% TEA (50:50)
Column	:	X-Bridge C18 100mmx4.6mm, 3.5µm
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	0 min
Wavelength	:	262 nm
Observation	:	Broad peak is observed

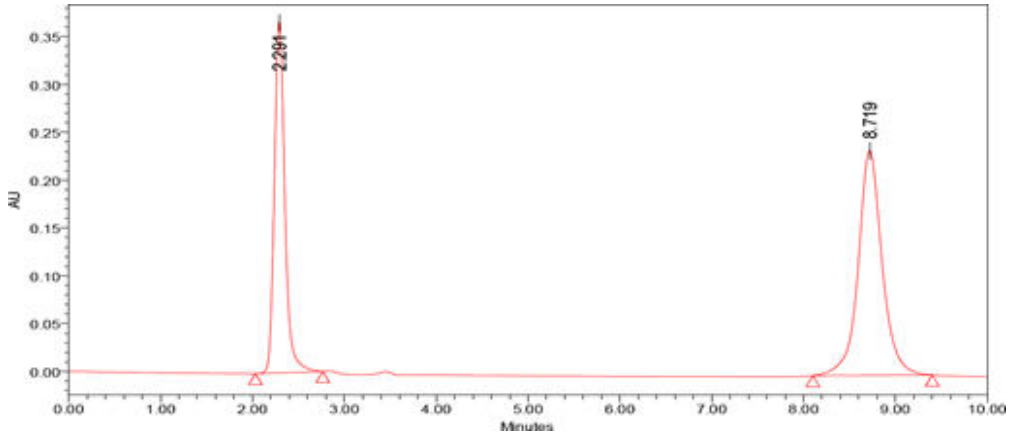

Figure 4.06: Chromatogram of trial-4

Trial-5 [Fig 4.07]

Mobile phase	:	Acetonitrile and 0.1% TEA (60:40)
Column	:	X-Bridge C18 100mmx4.6mm, 3.5µm
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	9 min
Wavelength	:	262 nm
Observation	:	Height is not within the limit

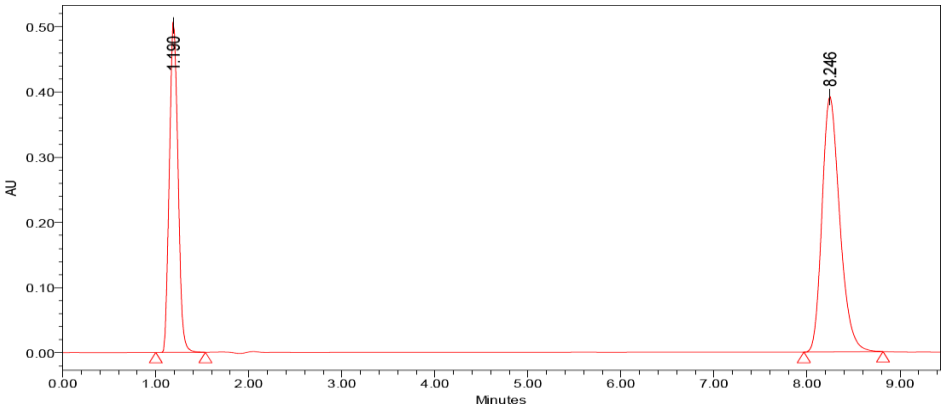

Figure 4.07: Chromatogram of trial-5

Trial-6 [Fig 4.08]

Mobile phase	:	Acetonitrile and 0.1% TEA (70:30)
Column	:	Luna C_{18}column (100X2.6 mm, 1.6 µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	8 min
Wavelength	:	262 nm
Observation	:	Two peaks plate count is not within the limit.

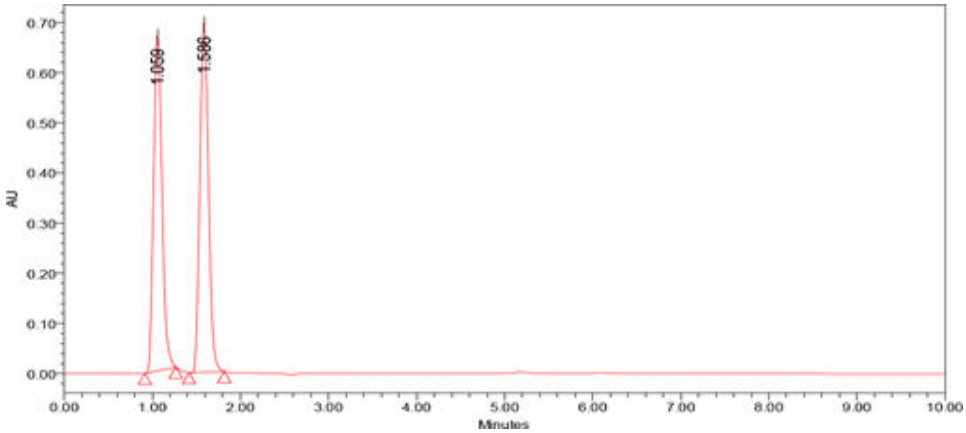

Figure 4.08: Chromatogram of trial-6

Trial-7 [Fig 4.09]

Mobile phase	:	Acetonitrile and 0.1% formic acid (50:50)
Column	:	Luna C_{18}column (100X2.6 mm, 1.6 µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	8 min
Wavelength	:	262 nm
Observation	:	System suitability conditions are not within the limit

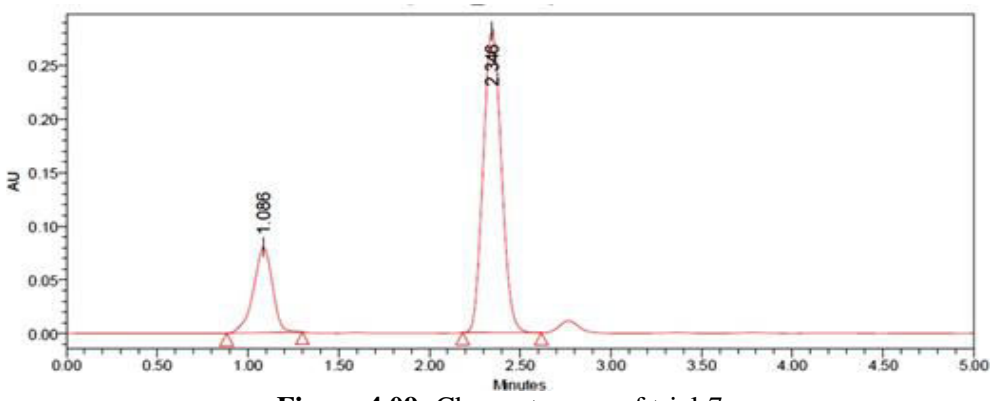

Figure 4.09: Chromatogram of trial-7

Trial-8 [Fig 4.10]

Mobile phase	:	Acetonitrile and 0.1% formic acid (60:40)
Column	:	Luna C_{18}column (100X2.6 mm, 1.6 µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	6min
Wavelength	:	262 nm
Observation	:	Extra peak is formed

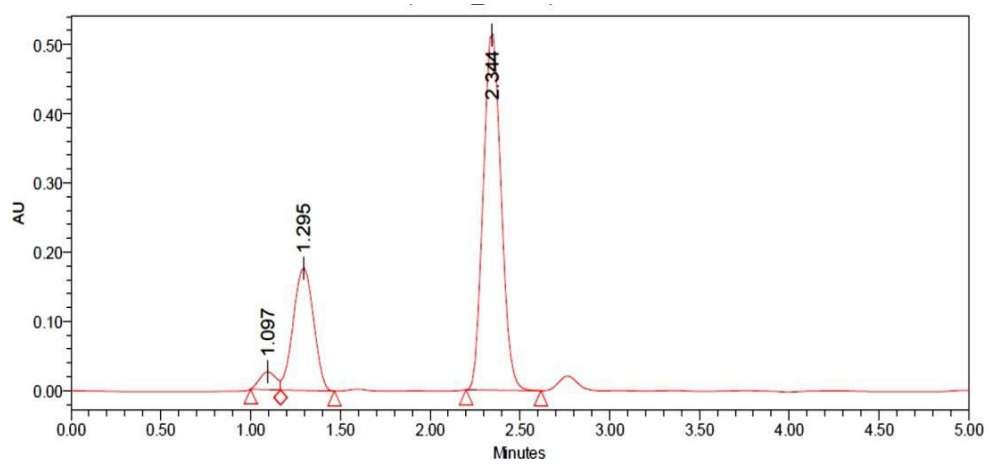

Figure 4.10: Chromatogram of trial-8

Trial-9 [Fig 4.11]

Mobile phase	:	Acetonitrile and 0.1% formic acid (65:35)
Column	:	Luna C_{18}column (100X2.6 mm, 1.6 µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	4 min
Wavelength	:	262nm
Observation	:	Resolution and plate count are not within the limit

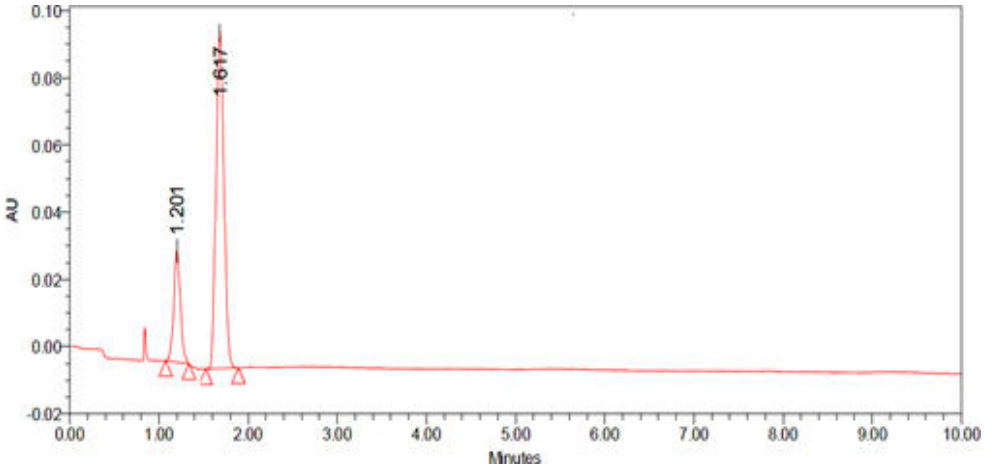

Figure 4.11: Chromatogram of trial-9

Trial-10 [Fig 4.12]

Mobile phase	:	Acetonitrile and 0.1% formic acid (70:30)
Column	:	Luna C_{18}column (100X2.6 mm, 1.6 µ)
Rate of flow	:	1 ml/min
Volume of injection	:	10µl
Period of run	:	3 min
Wavelength	:	262 nm
Observation	:	suitable for validation

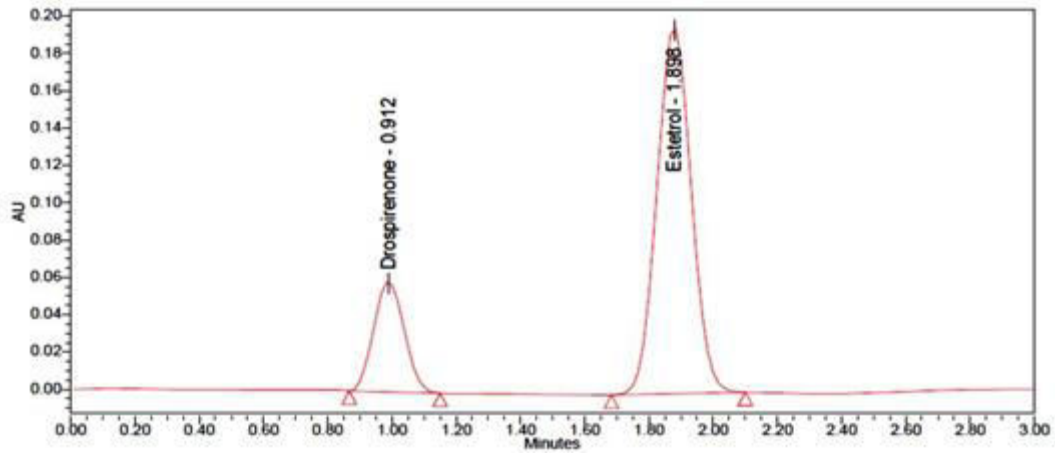

Figure 4.12: Chromatogram of trial-10

The chromatographic results of all trials were shown in **table 4.01**.

Trial No.	RT	Area	% Area	USP Resolution	USP Tailing	USP Plate count
1	1.350	683589	54.25		1.05	2536
	1.526	272341	45.75	1.09	1.23	3597
2	2.078	11134006	70.53		1.05	2064
	8.477	4652013	29.47	26.82	2.02	13933
	2.640	6789154	38.65		1.24	4572

3	3.132	245788	61.25	4.65	0.99	3568
	2.291	1140016	72.68		1.12	1534
4	8.719	1882801	28.32	5.68	1.75	3189
	1.190	7854412	76.47		1.05	1489
5	8.246	3458787	23.53	5.46	1.65	2657
	1.059	578945	68.54		1.02	986
6	1.586	246567	32.66	4.56	1.45	1532
	1.086	95610	13.59		2.56	1388
7	2.346	2824564	86.41	1.59	1.48	5410
	1.097	8487	14.65		1.02	1220
	1.295	148766	35.35	0.73	1.06	2537
8	2.344	98745633	51.38	0.87	1.01	5863
	1.201	25641	23.53		0.76	1339
9	1.617	8549611	76.47	1.13	1.90	1604
	0.912	553184	37.46		1.24	2934
10	1.898	2352679	63.54	4.36	0.89	5175

Table 4.01: Chromatographic results of all trials

4.6.2 Optimized Method [Fig 4.13 and Table 4.02]

S.NO	Parameter	Chromatographic condition
1	Mobile phase	Acetonitrile: 0.1% formic acid (70:30)
2	Column	Luna C_{18}column (100X2.6 mm, 1.6 µ)
3	Rate of flow	1ml/min
4	Column temperature	Room temperature
6	Wavelength	262 nm
7	Volume of injection	10µl
8	Period of run	3min
9	Retention time	Drospirenone Retention time-0.912 Estetrol retention time-1.898

Table 4.02: Optimized method chromatographic conditions

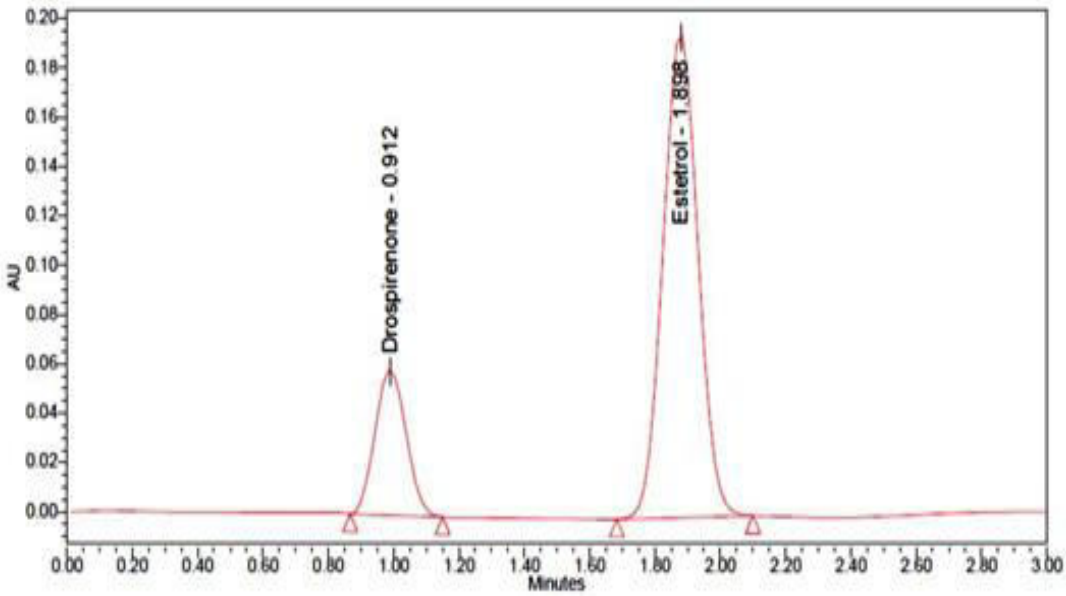

Figure 4.13: Chromatogram of standard

4.7 Validation of Method

ICH Q2 (R1) guidelines were used to evaluate the analytical procedure for characteristics such as device compatibility, accuracy, specificity and precision as well as linearity and robustness as well as limits of detection and limits of quantification (LOD and LOQ).

4.7.1 Specificity:

Certainty is the ability to test for certain components such as contaminants and excitements that could be presumed to be present in the sample solution and norm solution. Samples laced with Drospirenone and Estetrol were used to test it. Fig 4.14 shows blank chromatogram.

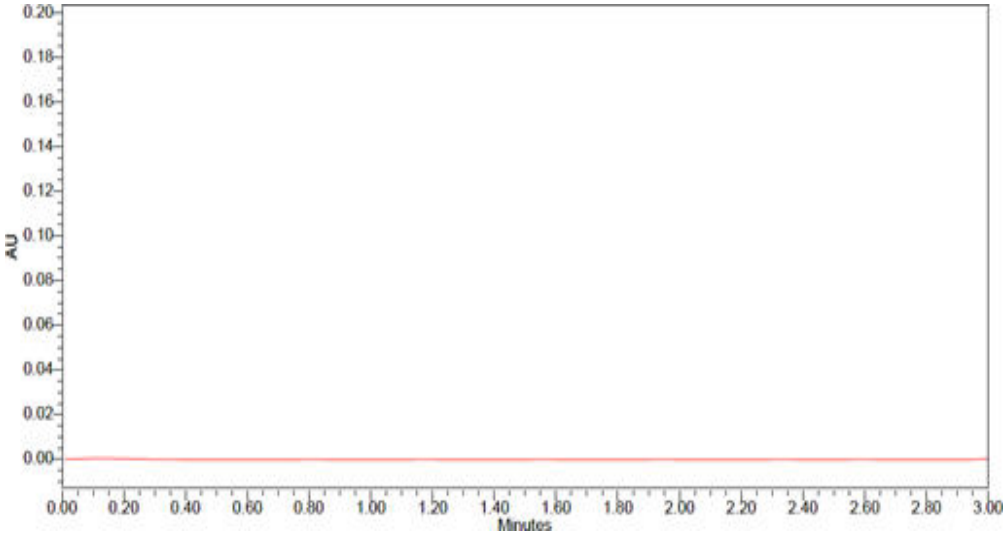

Figure 4.14: Chromatogram of blank

4.7.2 Linearity:

A calibration curve was used to plot the peak area vs its concentration and assess the linearity of the peak area. For Drospirenone and Estetrol, the curve was shown to be linear between 3.0 and 45.0 micrograms per millilitre (mg/mL).

Linearity stock solution preparation:

In a 100ml volumetric flask, add 70ml of diluents and weigh and transfer 30mg of Drospirenone and 142mg of Estetrol, and mix. For 15 minutes, sonicate the diluents to dissolve.

10 percent solution preparation: (3ppm of Drospirenone and 14.2ppm of Estetrol): Dilution of the stock solution to the specified concentration, in another 50 ml volumetric flask, was carried out using the diluents.

25 percent solution preparation: (7.5ppm of Drospirenone and 35.5ppm of Estetrol):

One of the stock solutions was diluted in a 50 ml volumetric flask with diluents to the mark in another 50 ml volumetric flask.

50 percent solution preparation: (15ppm of Drospirenone and 71ppm of Estetrol): An additional 50 ml volumetric flask was used to dilute 2.5ml of the aforementioned stock solution up to the mark.

75 percent solution preparation: (22.5ppm of Drospirenone and 106.5ppm of Estetrol):

Dilution of 3.75 ml of the above specified stock solution in another 50ml volumetric flask was done using the diluents to the mark.

100 percent solution preparation: (30ppm of Drospirenone and 142ppm of Estetrol):
Diluted to the desired concentration, 5 ml of the above-mentioned stock solution was added to a new 50 ml volumetric flask.

125 percent solution preparation: (37.5ppm of Drospirenone and 177.5ppm of Estetrol):
6.25% of the stock solution was diluted with the diluents in a 50 ml volumetric flask to the mark.

150 percent solution preparation: (45ppm of Drospirenone and 213ppm of Estetrol):
Dilution of the stock solution in a new 50 ml volumetric flask was carried out in the same manner.

Procedure:
Using a chromatographic technique, measure the peak area for each degree. Making a correlation coefficient graph by plotting the peak area vs concentration (X axis concentration, Y-axis peak area).

Range:
The gap between the upper and lower analytical stages, which has been proved to be trustworthy, accurate, and linear, is known as the range of analytical techniques.

Acceptance Criteria:
The correlation coefficient should not be less than 0.999.

Table 4.03 gives linearity table and **figures** from **4.15-4.23** gives linearity chromatograms.

S.No	Drospirenone		Estetrol	
	Conc. (µg/ml)	**Area**	**Conc. (µg/ml)**	**Area**
1	3.00	56014	14.20	234633
2	7.50	129150	35.50	564156
3	15.00	261298	71.00	1132584
4	22.50	435298	106.50	1726321
5	30.00	550851	142.00	2326321
6	37.50	688077	177.50	2862896
7	45.00	809562	213.00	3424821
CC	0.99922		0.99992	
Slope	18252.64		16149.69	
Intercept	87.60		351.33	

Table 4.03: Results of linearity

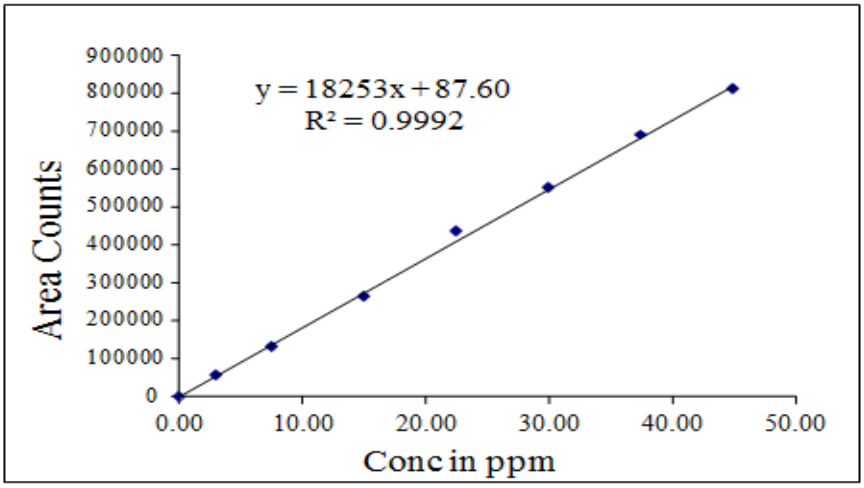

Figure 4.15: Calibration plot of Drospirenone

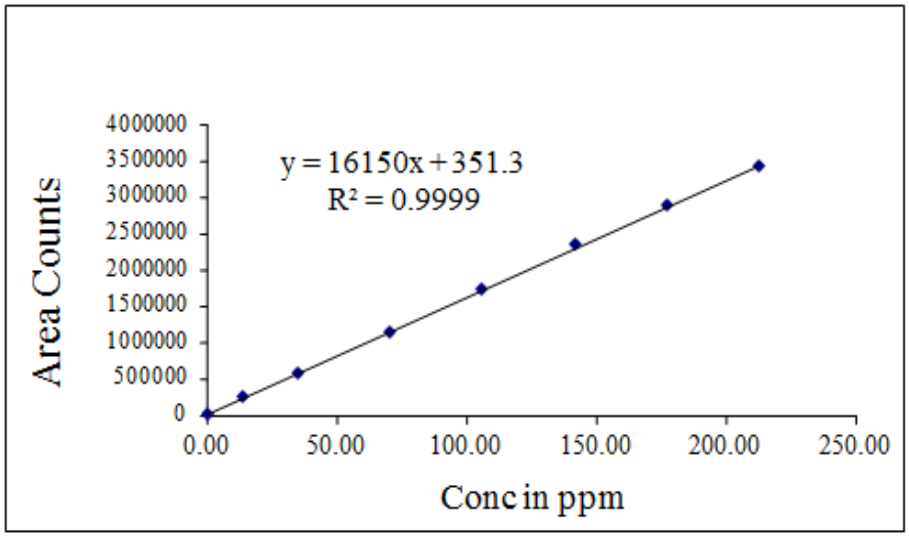

Figure 4.16: Calibration plot of Estrol

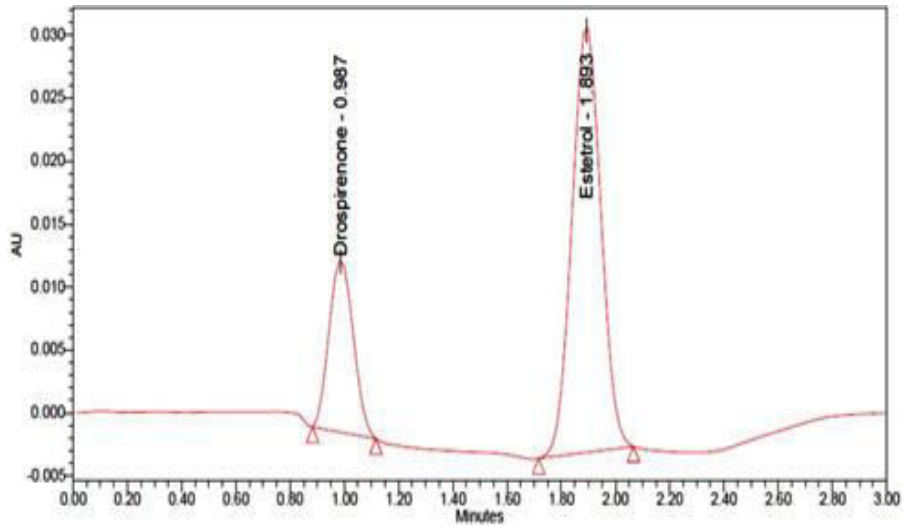

Figure 4.17: 10 percent linearity chromatogram

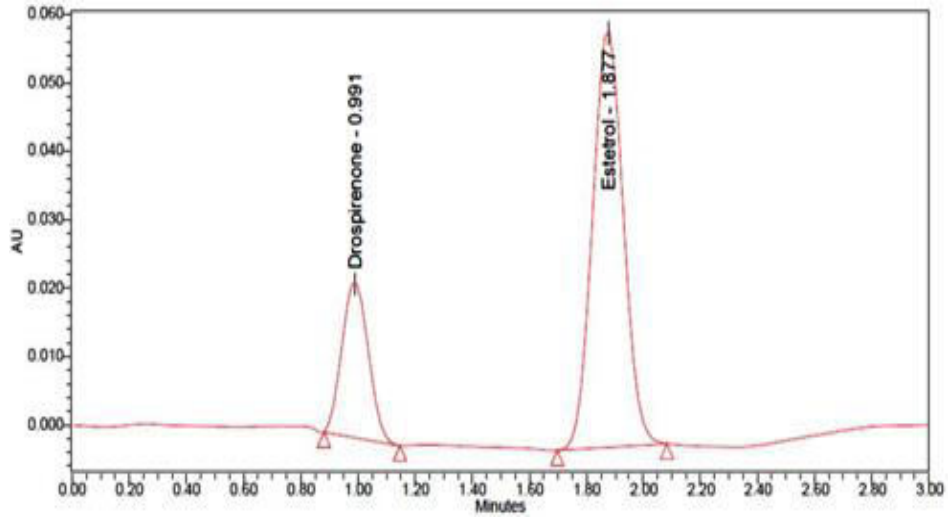

Figure 4.18: 25 percent linearity chromatogram

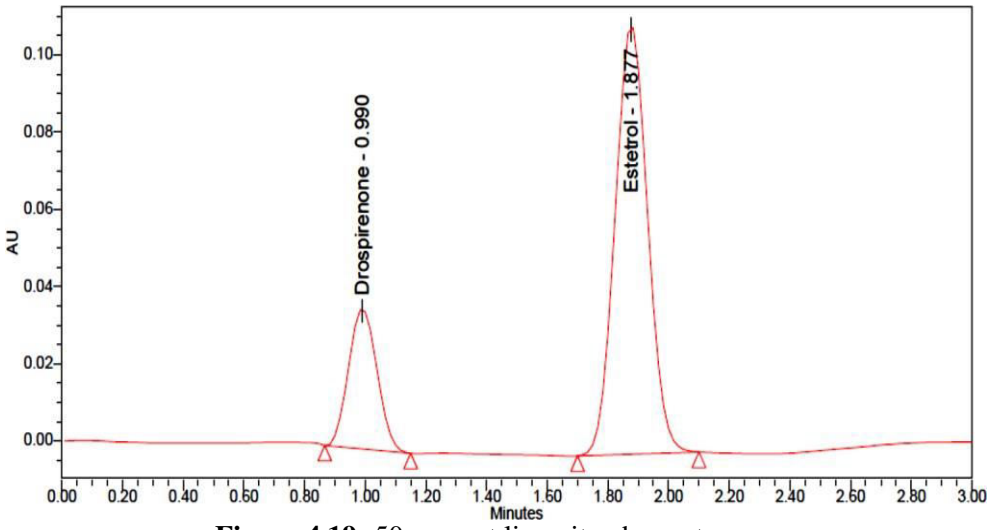

Figure 4.19: 50 percent linearity chromatogram

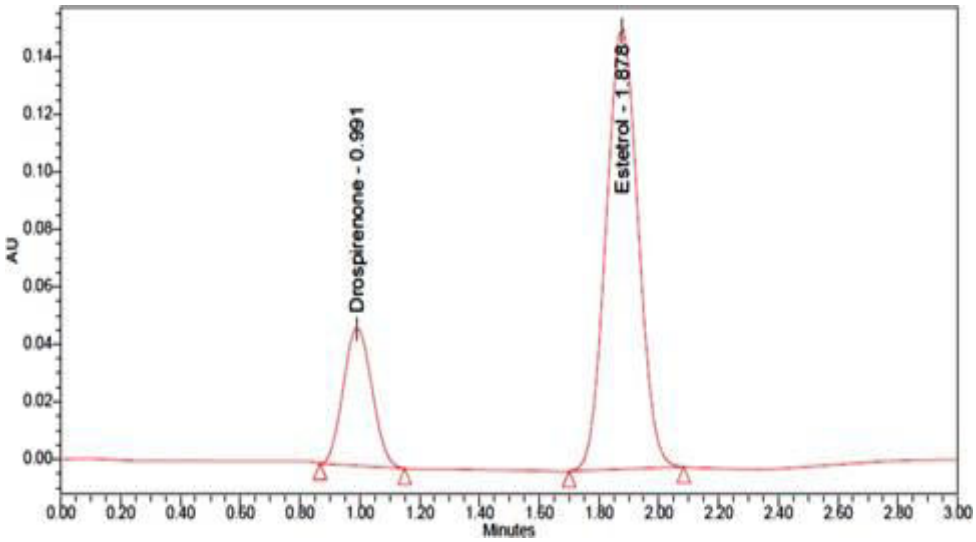

Figure 4.20: 75 percent linearity chromatogram

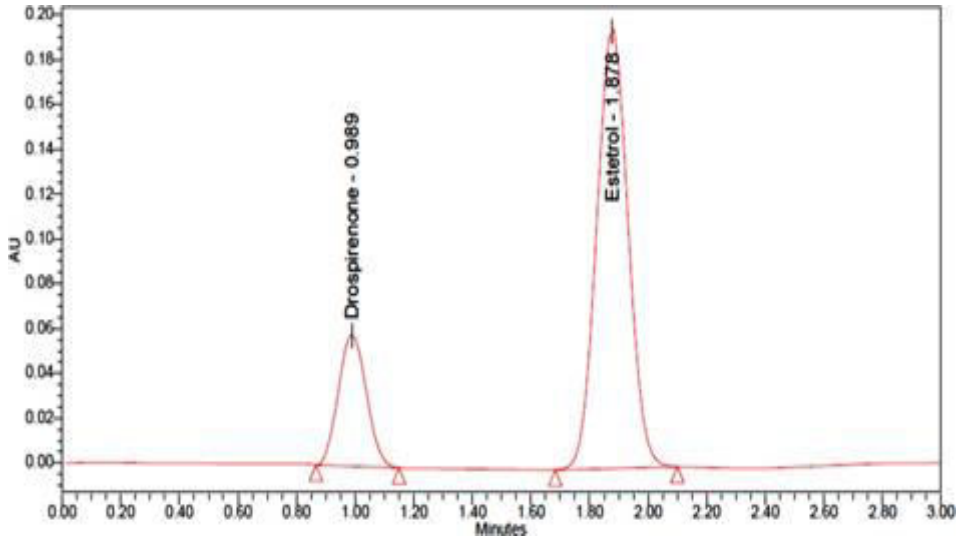

Figure 4.21: 100 percent linearity chromatogram

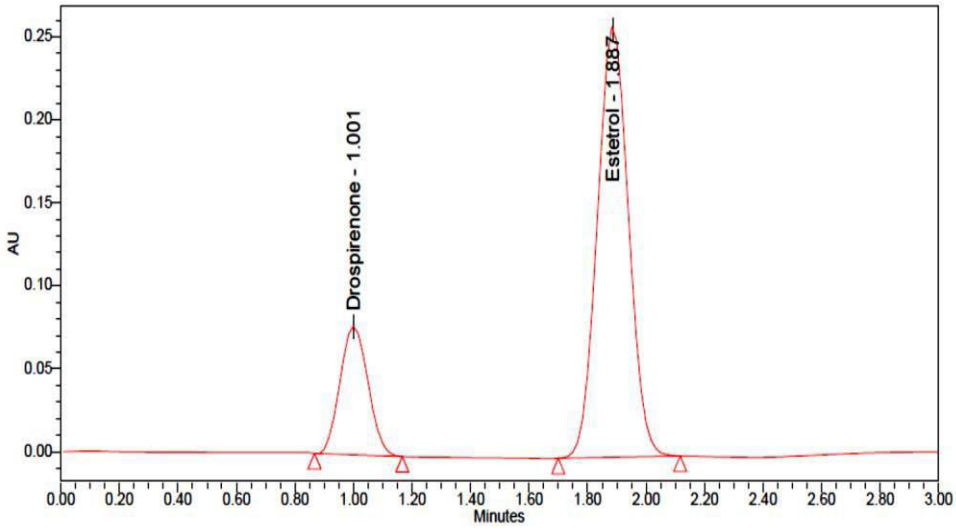

Figure 4.22: 125 percent linearity chromatogram

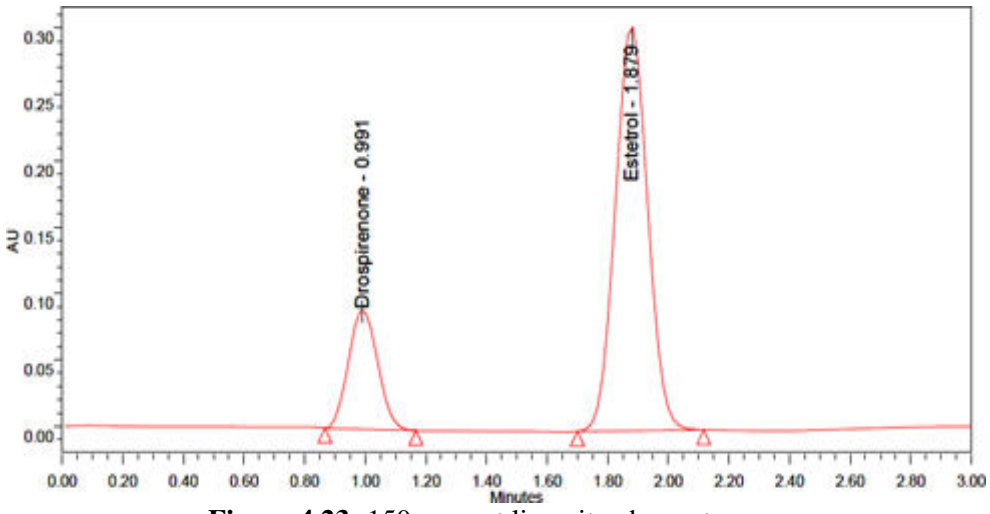

Figure 4.23: 150 percent linearity chromatogram

4.7.3 ACCURACY:

50 percent solution preparation (with respect to the concentration of the target assay):
Apply diluents and sonicate to dissolve Drospirenone and Estetrol in a 100ml clean and dry volumetric flask. Sonicate till the diluents level is reached.

Prepare a 50-ml volumetric flask and fill it halfway with the aforementioned solution, then add diluents to dilute it to the desired concentration. Drospirenone (15 ppm) and Estetrol (71 ppm) were used.

100 percent solution preparation (with respect to target assay concentration):
Apply the diluents and sonicate to dissolve the Drospirenone and Estetrol in a clean and dry volumetric flask to bring up to the diluents level.

In a 50ml volumetric flask, pipette 5ml of the aforementioned solution and dilute it with diluents to the desired volume. Drospirenone (30 ppm) and Estetrol (142 ppm) were used.

150 percent solution preparation (with respect to target assay concentration):
Apply diluents and sonicate to dissolve completely 45 mg of Drospirenone and 213 mg of Estetrol in a 100 ml clean and dry volumetric flask.

Take 5ml of the aforesaid stock solution and dilute it to your desired concentration in a 50ml volumetric flask using diluents. (213mg Estetrol and 45ppm Drospirenone))

Procedure:
The standard solution, 50 percent accuracy, 100 percent accuracy, and 150 percent accuracy solutions should be injected at the same time.

Acceptance Criteria:
The rate of recovery for each stage should be between 98-102 percent.

Table 4.04 gives accuracy results and **figures** from **4.24-4.26** gives accuracy chromatograms.

Accuracy	Amount of Drospirenone	% Recovery	Amount of Estetrol	% Recovery
50*	15	99.5	71	98.8
100*	30	99.8	142	99.1
150*	45	99.9	213	99.8

Table 4.04: Accuracy results of Drospirenone and Estetrol

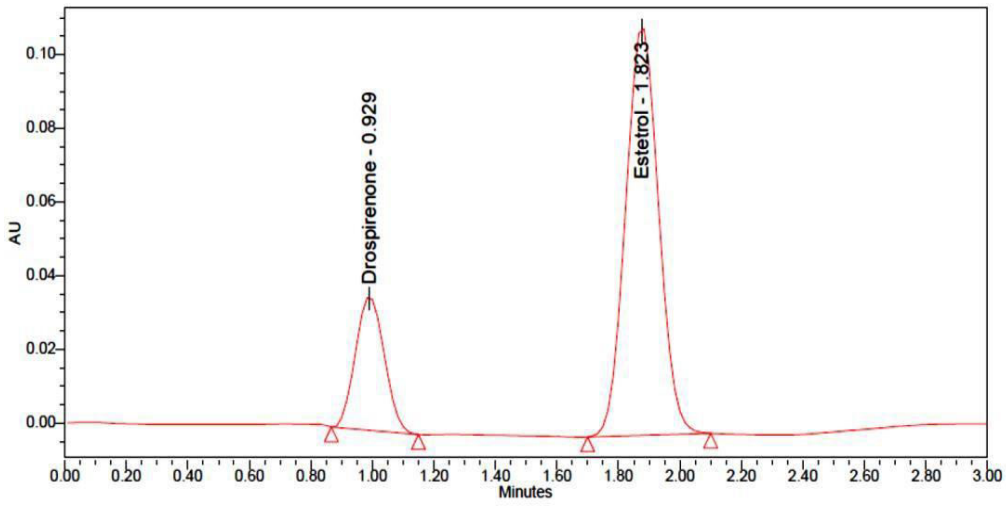

Figure 4.24: 50 percent accuracy chromatogram

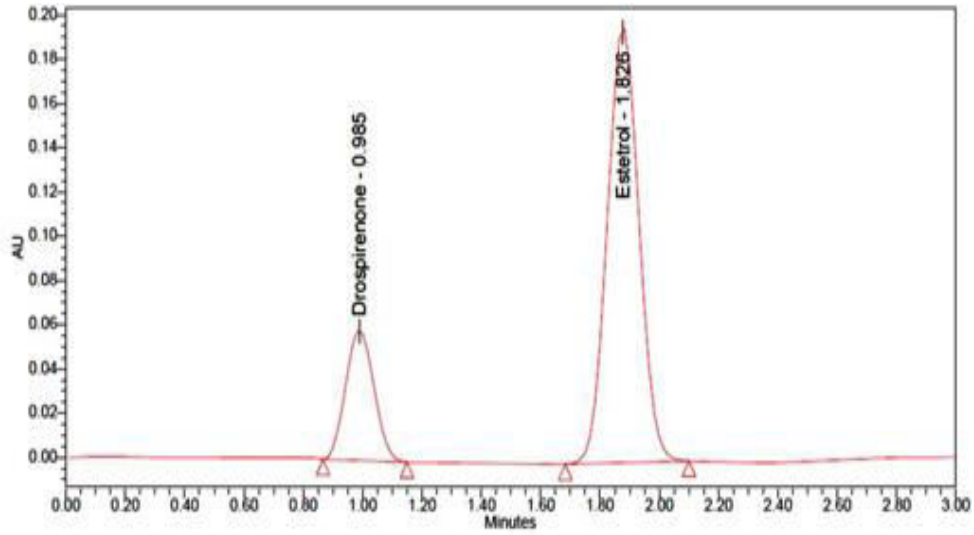

Figure 4.25: 100 percent accuracy chromatogram

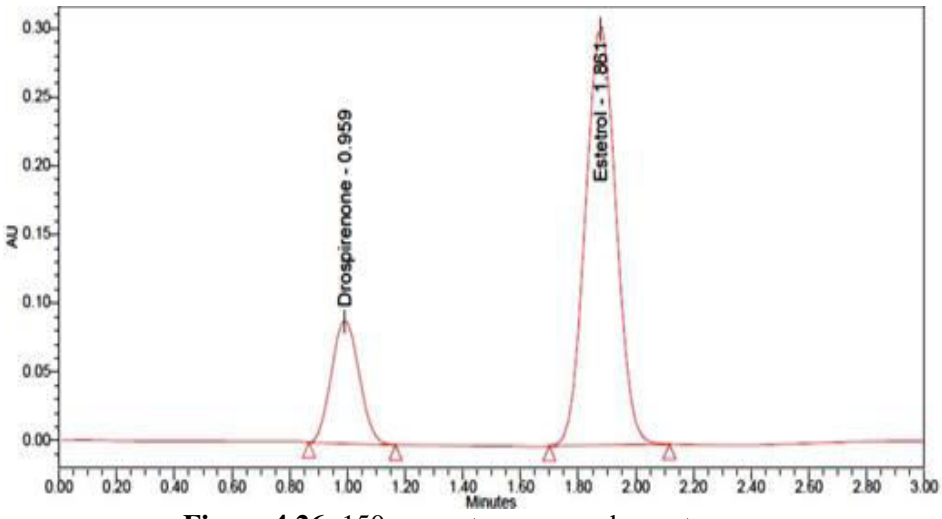

Figure 4.26: 150 percent accuracy chromatogram

4.7.4 Precision:

The rate of closeness of a set of measurements obtained from multiple homogeneous samplings is the accuracy of an analytical technique. Six individual injection determinations of Drospirenone (30ppm) and Estetrol (142ppm) were spiked for the accuracy of the process of related substances by injection. **Table 4.05** gives suitability results. **Figures** from **4.27-4.32** gives suitability chromatograms.

S.NO	Conc. of Drospirenone (µg/ml)	Area of Drospirenone	Conc. of Estetrol (µg/ml)	Area of Estetrol
1	30	559393	142	2360981
2	30	558271	142	2357098
3	30	555449	142	2351049
4	30	554183	142	2342301
5	30	557998	142	2360385
6	30	556959	142	2383672
Mean	557042		2359248	

Std dev	1932.36	13855.5
% RSD	0.347	0.587

Table 4.05: Results of system precision

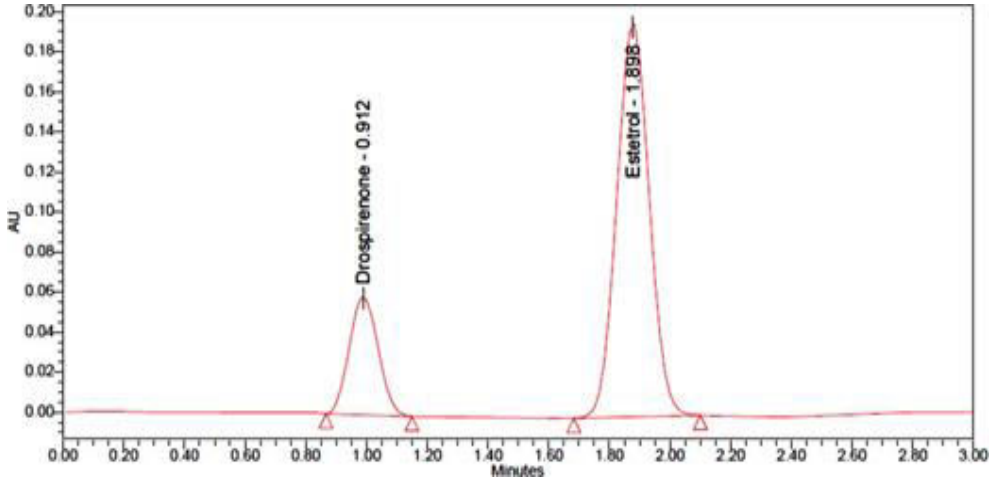

Figure 4.27: Chromatogram of system precision-1

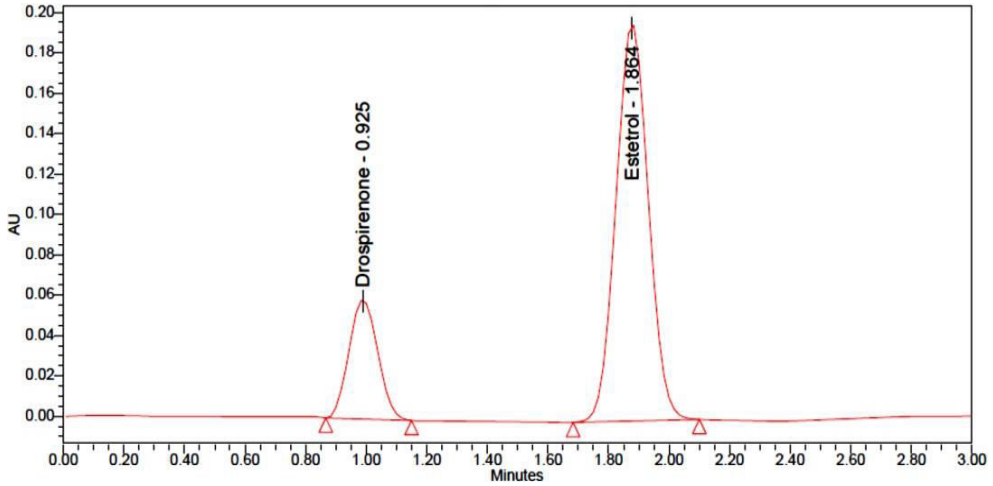

Figure 4.28: Chromatogram of system precision-2

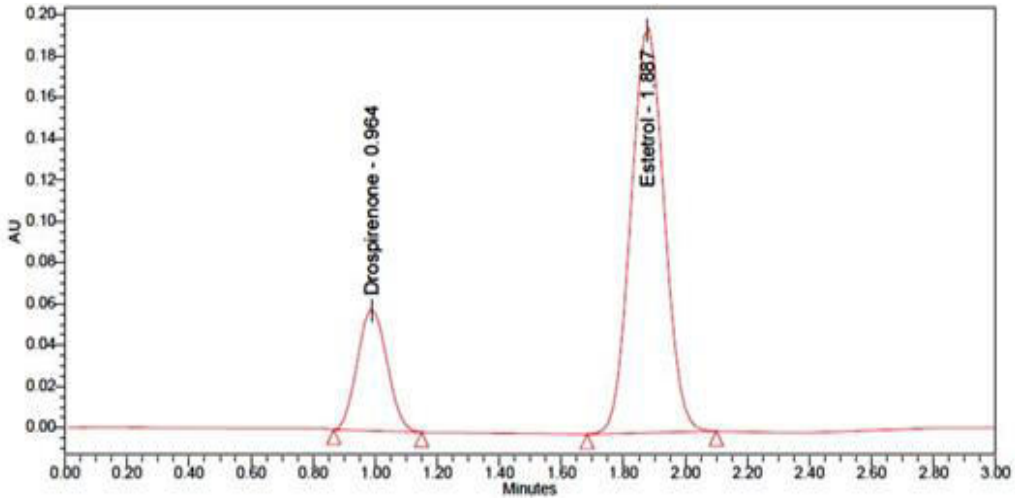

Figure 4.29: Chromatogram of system precision-3

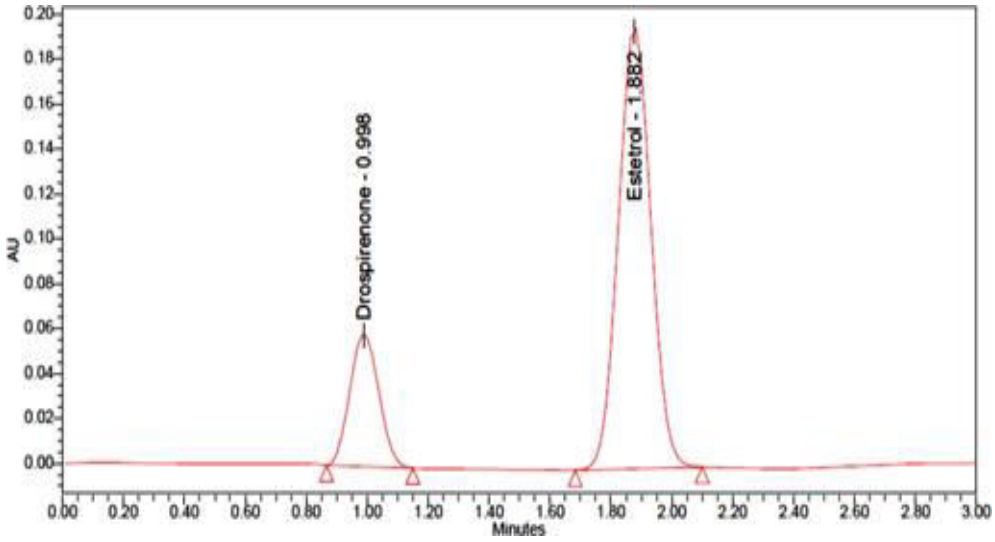

Figure 4.30: Chromatogram of system precision-4

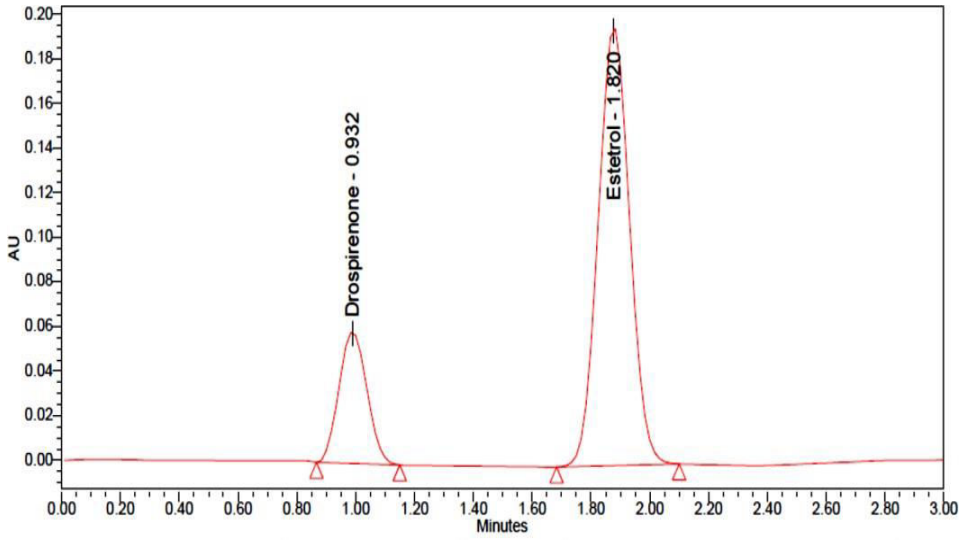

Figure 4.31: Chromatogram of system precision-5

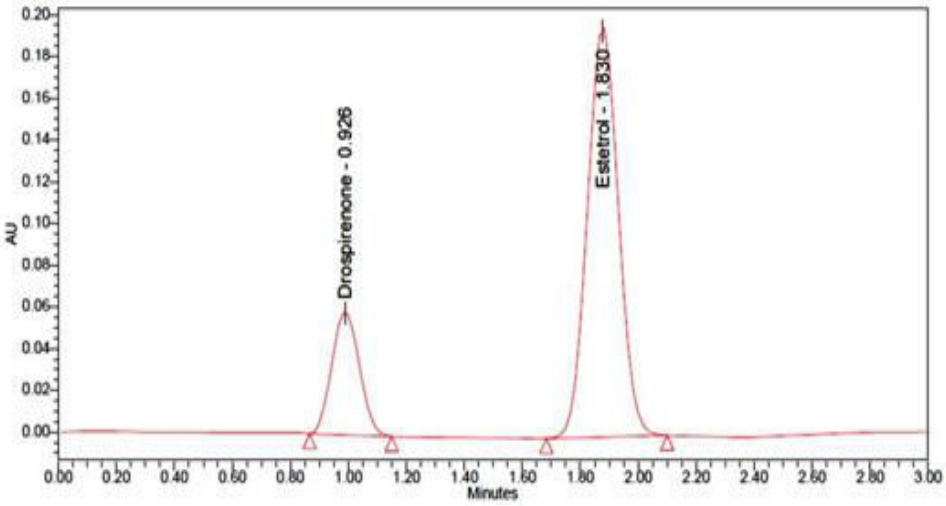

Figure 4.32: Chromatogram of system precision-6 Method precision

[Table 4.06 and Fig 4.33]

S.NO	Drospirenone area	Estetrol area
1	562417	2345012
2	501269	2350234
3	542815	2345025
4	546251	2345873
5	536215	2302125
6	524864	2353641
Mean	563972	2340318
Std dev	2101.592	19024.101
% RSD	0.37	0.81

Table 4.06: Results of method precision

Acceptance Criteria: The RSD percent for the area six standard injection results should be more than 2 %.

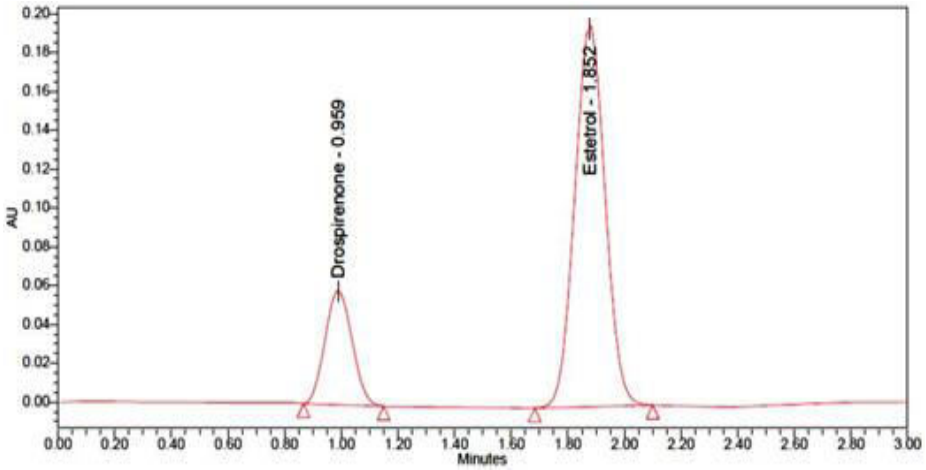

Figure 4.33: Chromatogram of method precision Intermediate precision (Day-Day precision)

[Table 4.07 and Fig 4.34]

S.No.	Area of Drospirenone	Relative standard Deviation	Area of Estetrol	Relative standard Deviation
1	562012	0.57	2302568	0.78
2	561365		2345278	
3	567215		2335624	
4	565626		2335265	
5	568692		2355869	
6	568576		2343206	

Table 4.07: Results of intermediate precision

Acceptance criteria: The RSD percentage for the six normal injection results should not be more than 2%.

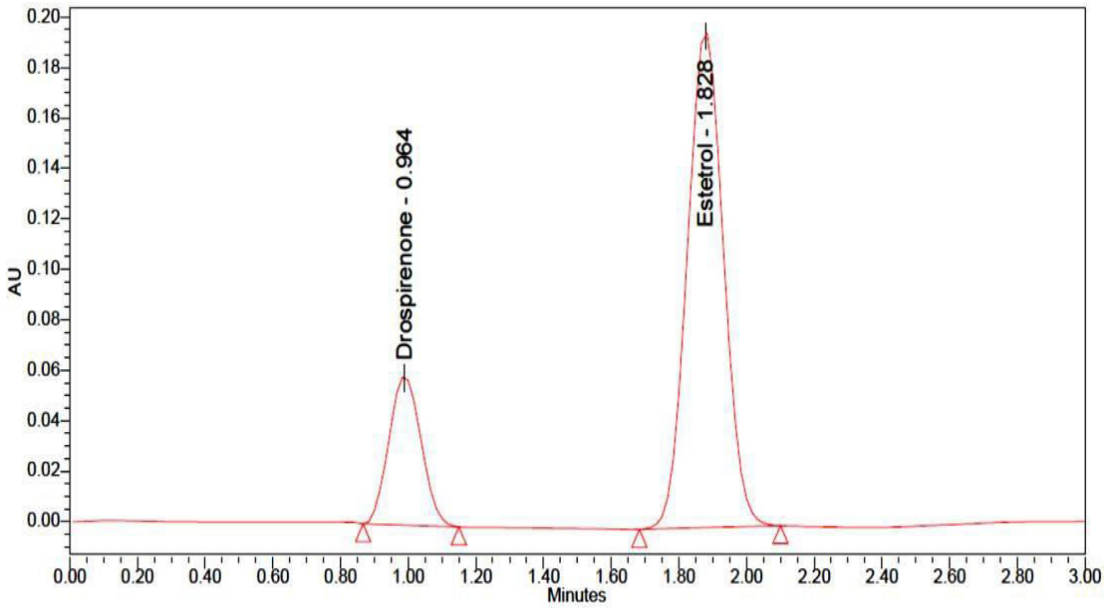

Figure 4.34: Chromatogram of intermediate precision

4.7.5 Limit of Detection (LOD) and Limit of Quantification (LOQ):

LOD and LOQ were calculated separately by the calibration curve method. LOD and LOQ of the compound were determined by injection of progressively lower concentrations of standard solution using developed UPLC method. The LOD concentration for Drospirenone is 0.03µg/ml and s/n value is 7 and Estetrol 0.142µg/ml and s/n value is 5. The LOQ concentration for Drospirenone is 0.099µg/ml and their s/n values are 24 and Estetrol 0.4686µg/ml and s/n value is 22. **Table 4.08** shows sensitivity results and Figures **4.35 and 4.36** gives LOD and LOQ chromatograms.

Drug name	LOD (µg/ml)	LOQ (µg/ml)
Drospirenone	0.03	0.099
Estetrol	0.142	0.4686

Table 4.08: Sensitivity parameter values

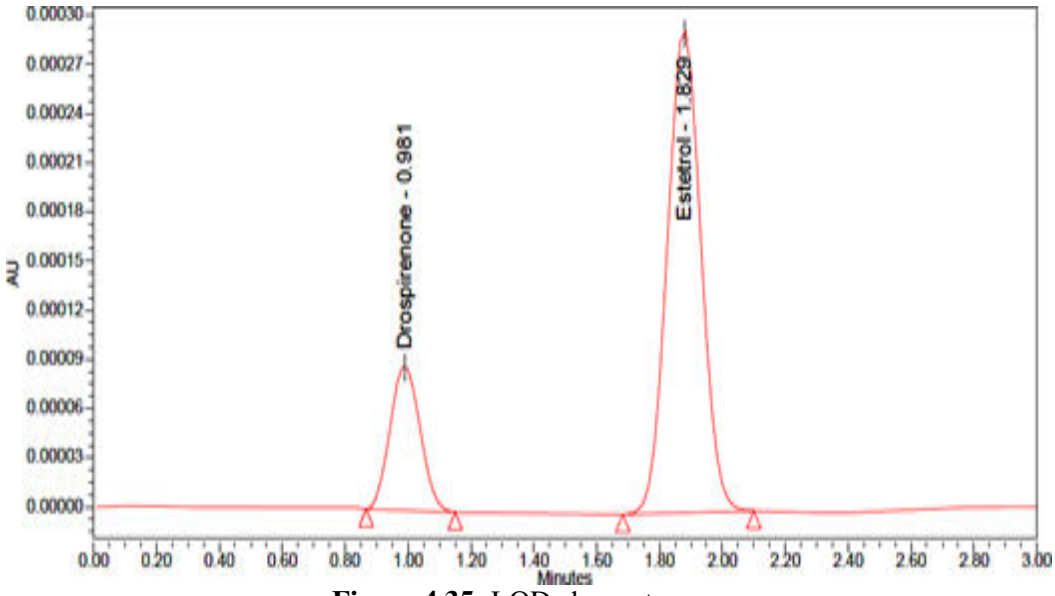

Figure 4.35: LOD chromatogram

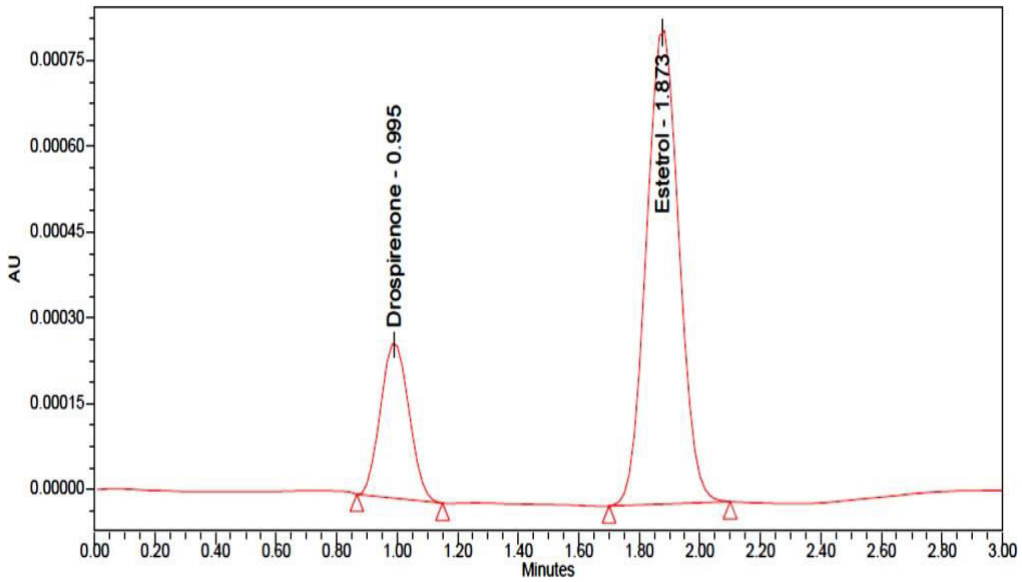

Figure 4.36: LOQ chromatogram

4.7.6 Robustness [Table 4.09 and Fig 4.37-4.40]

The experiment was set up to assess the resilience of an established system whose parameters had been deliberately adjusted, such as the flow rate and the mobile phase's organic content. Retention duration, plate count, and tailing factor were not substantially impacted by the addition of contaminants to the pharma components. That's why it worked.

Parameter	% RSD of Drospirenone	% RSD of Estetrol
Flow (0.8 mL/min)	0.55	0.29
Flow (1.2 mL/min)	0.82	0.89
Organic phase (77:23)	1.41	0.97
Organic phase (63:37)	0.66	0.46

Table 4.09: Robustness results of Drospirenone and Estetrol

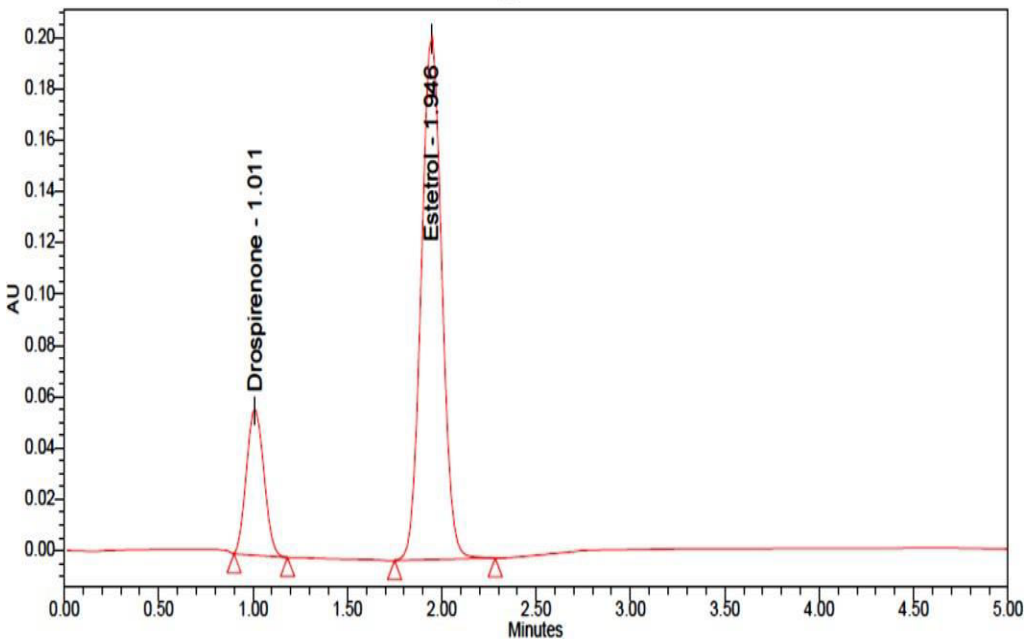

Figure 4.37: Less flow rate chromatogram (0.8ml/min)

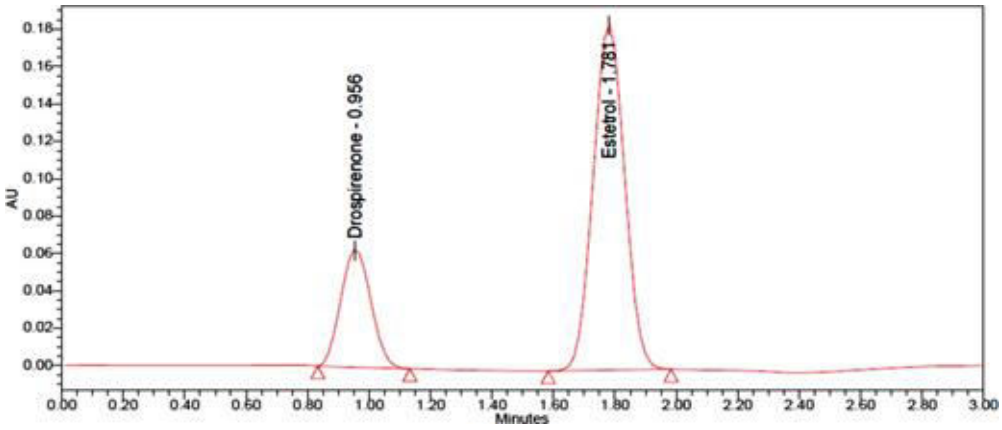

Figure 4.38: More flow rate chromatogram (1.2ml/min)

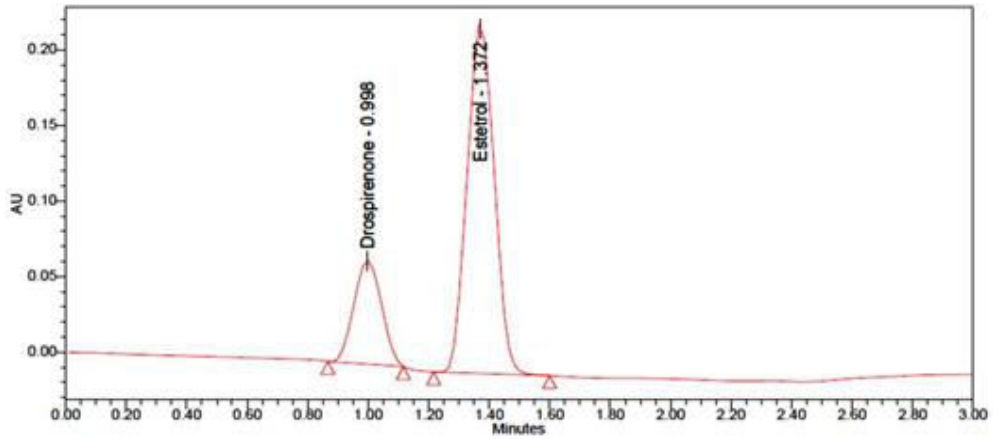

Figure 4.39: Less organic chromatogram (63:37)

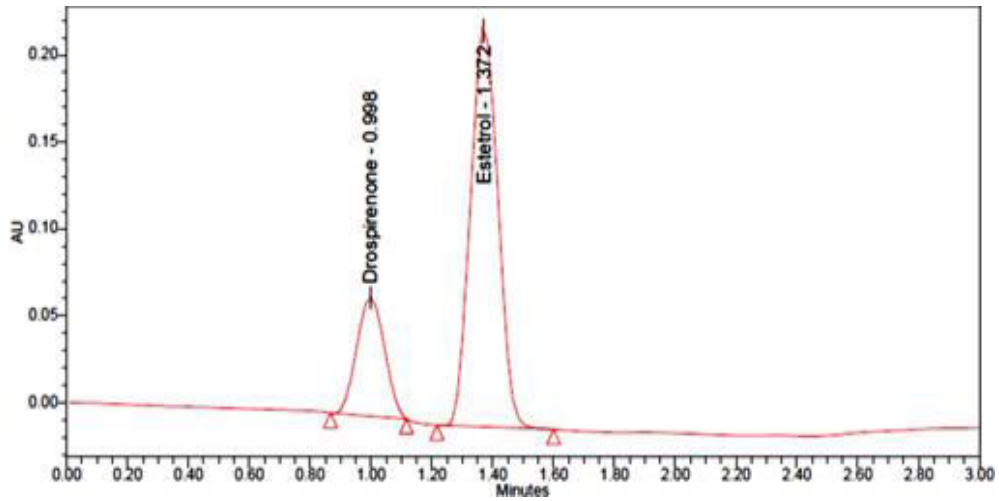

Figure 4.40: More organic chromatogram (77:23)

4.7.7 Forced degradation studies:

In order to partly decompose the chemical, Drospirenone and Estetrol samples were treated to various degradation conditions. Forced degradation studies have been conducted to determine that the procedure may be used to degrade materials. For this reason, the research includes information on what conditions could lead to instability in order to guarantee the formulation process is always safe.

Stock Solution Preparation:
Diluents are carefully weighed and added to the volumetric flask with 30 mg of Drospirenone and 142 mg of Estetrol. Sonication is used to dissolve for 30 minutes to make up the diluents mark.

Acid Degradation:
For 15 minutes, add 1 ml of 1N Hcl to 5 ml of sample solution in a volumetric flask of 50 ml. A solution of 1N NaOH should be added to the solution after 15 minutes.

Alkali Degradation:
1 ml of 1N NaOH is added to a 50ml volumetric flask containing 5 ml of sample stock solution and left for 15 minutes. Adding 1 ml of 1N Hcl and diluting it with diluents was done after 15 minutes.

Peroxide Degradation:
Five millilitres of stock solution were put into a 50-milliliter volumetric flask, and 0.3 millilitres of 30 percent hydrogen peroxide were added.

Reduction Degradation:
Five millilitres of the sample stock solution were transferred to a 50 millilitre volumetric flask, and one millilitre of a 30 percent sodium bi sulphate solution was added and the diluents level was reached.

Thermal Degradation:
The sample solution was set at 105º in the oven for 6 hours. The resultant solution was injected into UPLC.

Forced degradation results was given by **table 4.10** and **figures** from **4.41-4.46** gives FD chromatograms.

Stress Parameter	% of Degradation	
	Drospirenone	**Estetrol**
Acid degradation (1N HCl)	14.5	14.1
Alkali degradation (1N NaOH)	14.2	13.9
Peroxide degradation (30% Peroxide)	15.7	14.8
Reduction degradation (30% sodium bi sulphate)	13.3	12.1
Thermal (sample, 70°C, 6 Hrs)	12.4	11.3

Table 4.10: Forced degradation results

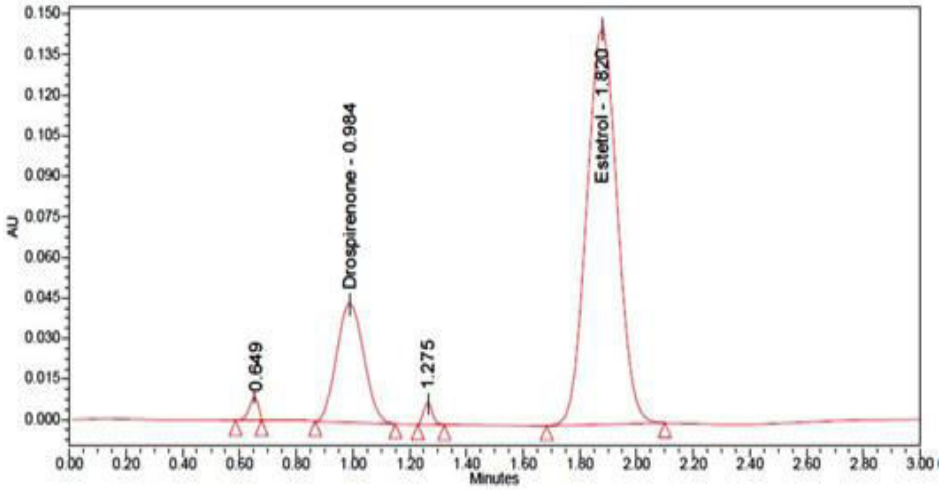

Figure 4.41: Chromatogram of acid degradation

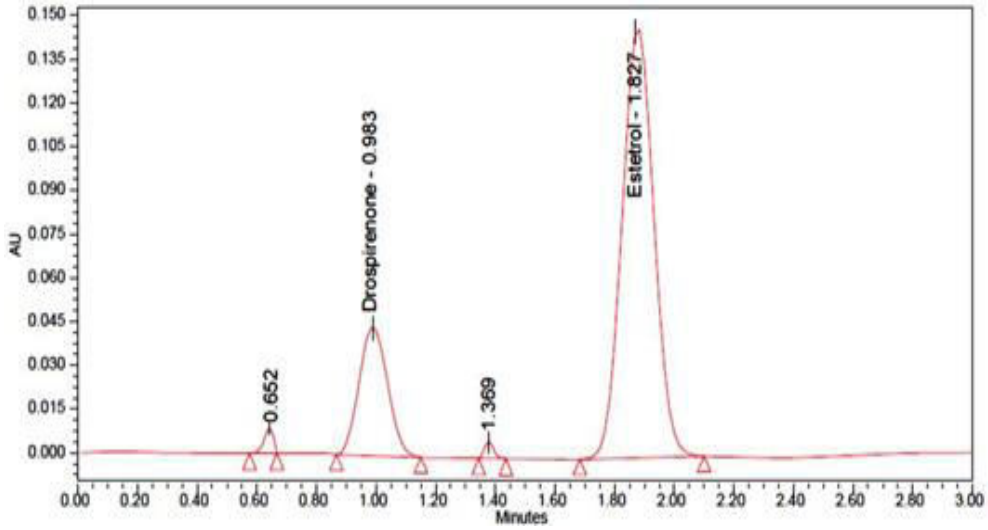

Figure 4.42: Chromatogram of alkali degradation

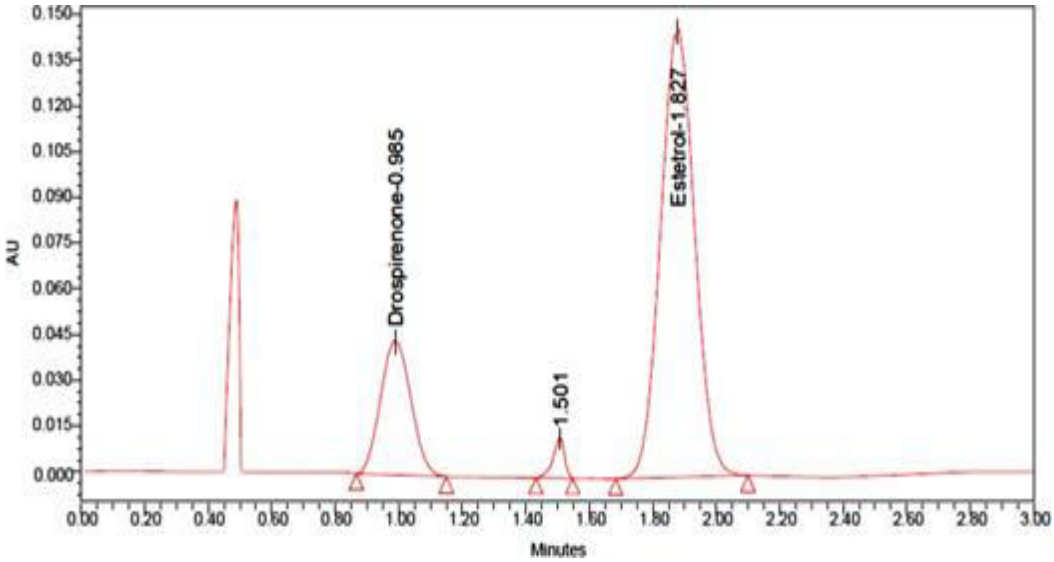

Figure 4.43: Chromatogram of peroxide degradation

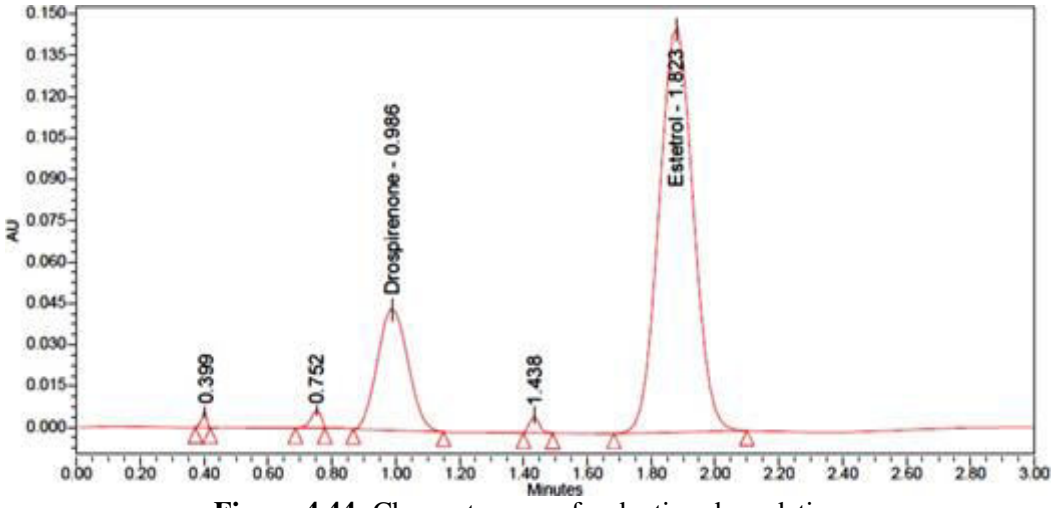

Figure 4.44: Chromatogram of reduction degradation

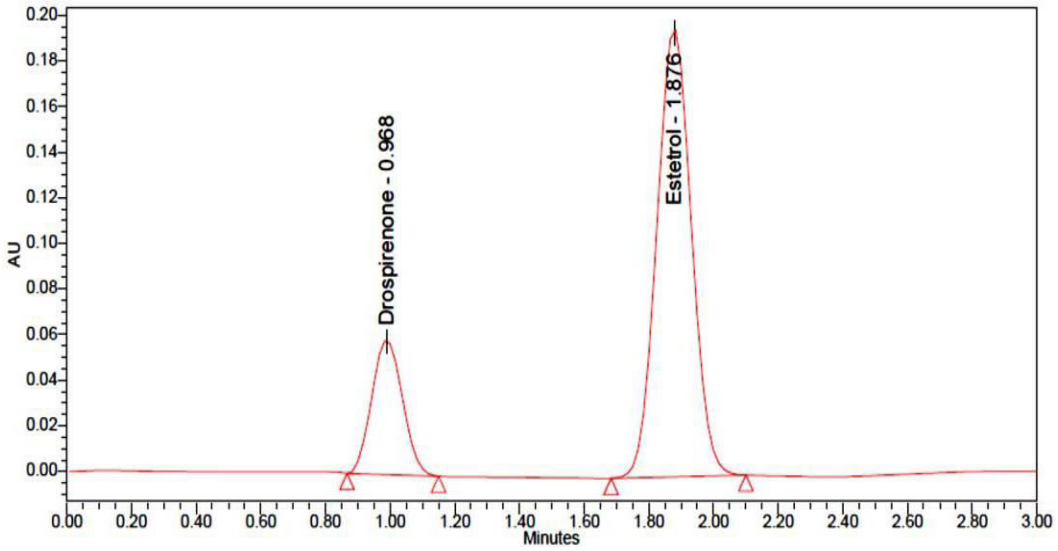

Figure 4.45: Chromatogram of thermal degradation

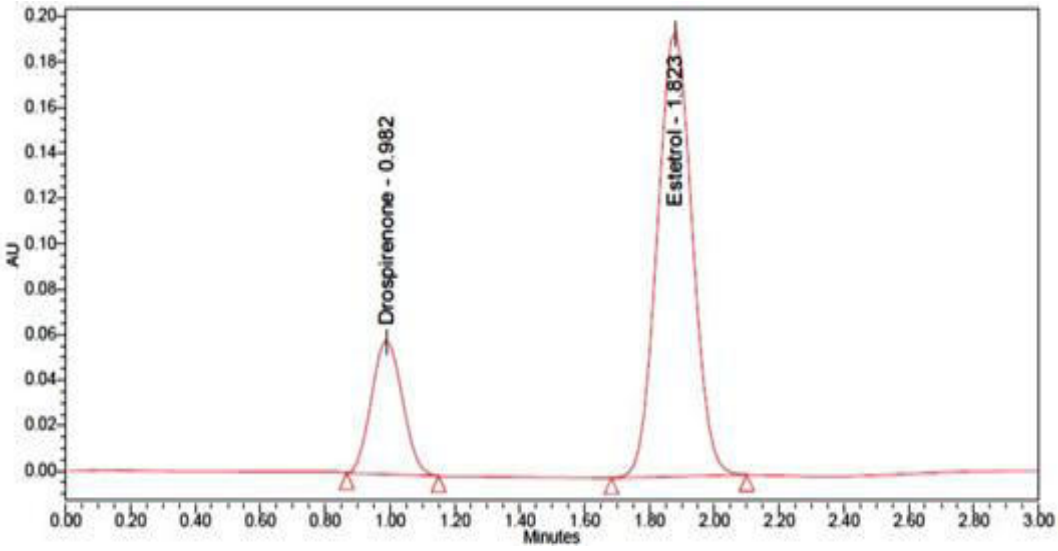

Figure 4.46: Chromatogram of control degradation

4.7.8 Stability:

At room temperature and between 2-8°C for up to 24 hours, the normal and sample solutions were stored. These solutions were then poured into the apparatus, and the percentage of divergence from the initial to 24 hours was calculated. Stable answers were found up to 24 hours into the study with no noticeable deviations. **Table 4.11** gives stability results and **figures** from **4.47-4.51** gives stability chromatograms.

Stability	Drospirenone		Estetrol	
	Purity	% of deviation	Purity	% of deviation
Initial	100	0	100	0
6 Hrs	99.6	-0.40	99.8	-0.20
12 Hrs	99.4	-0.60	99.6	-0.40
18 Hrs	99	-1.00	99.3	-0.70
24 Hrs	98.9	-1.10	99	-1.00

Table 4.11: Results of stability

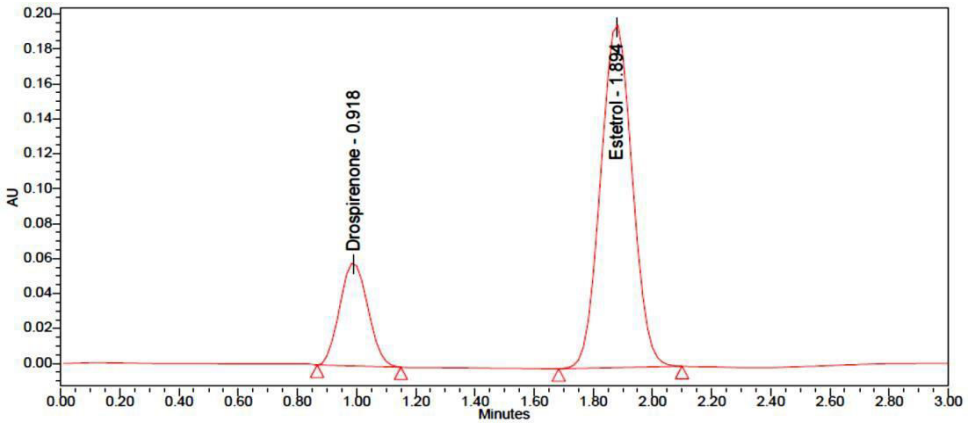

Figure 4.47: Chromatogram of stability initial RT

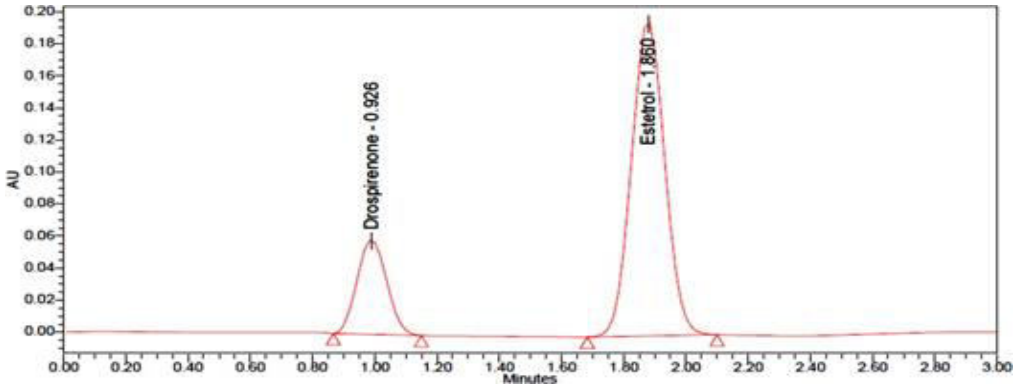

Figure 4.48: Chromatogram of stability 6 Hrs RT

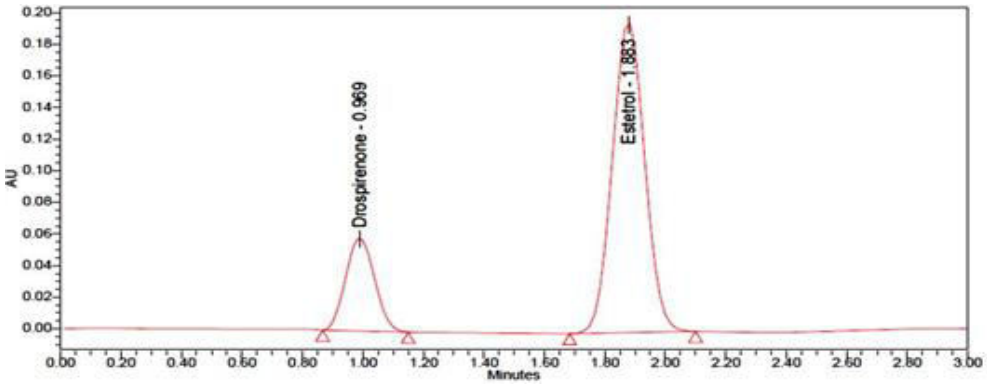

Figure 4.49: Chromatogram of stability 12 Hrs RT

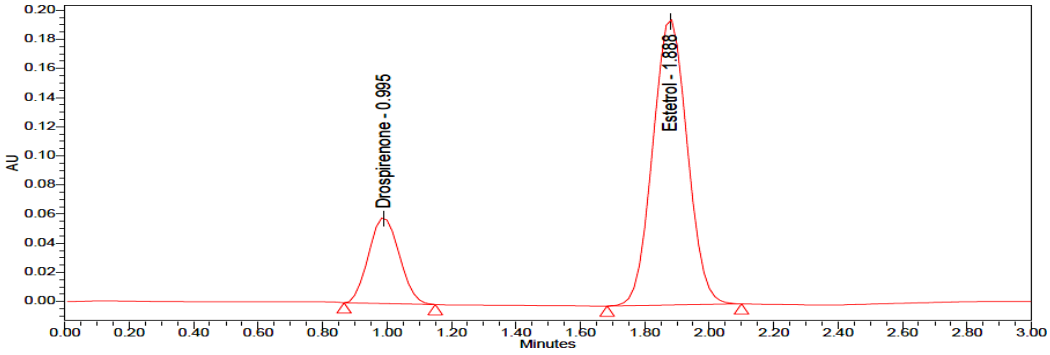

Figure 4.50: Chromatogram of stability 18 Hrs RT

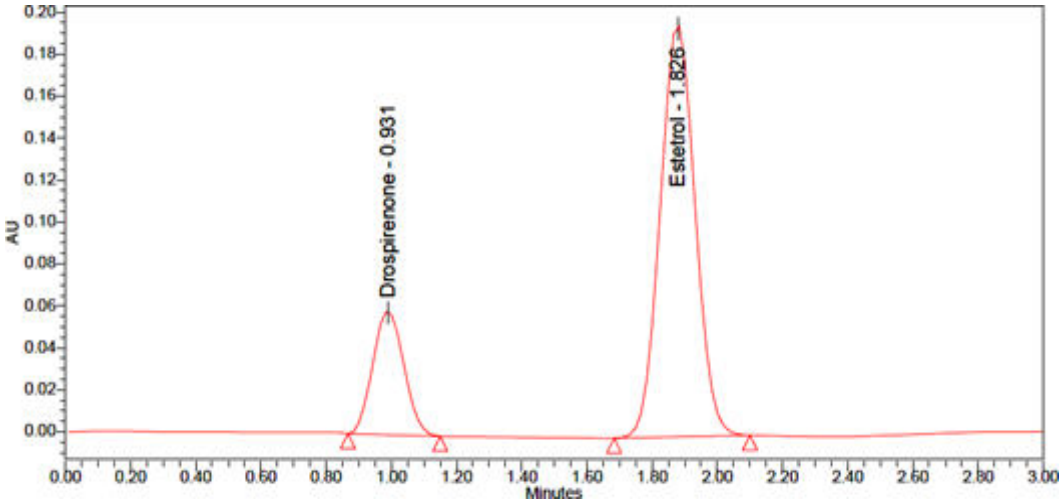

Figure 4.51: Chromatogram of stability 24 Hrs RT

4.8 CONCLUSION:

Rapid, inexpensive, sensitive, and readily accessible, UPLC was used to measure drospirenone and estetrol in both the API and pharmaceutical dose form. A benefit of this approach is that there seems to be no UPLC method described. Cheap running expenses, a fast start-up time, a low price tag and broad availability are just a few of the other advantages. As more samples are needed to be analysed, these features become more important. There were no issues with any of the parameters evaluated. If you look at what we've learned so far, we can say that this method works as expected. With no prior separation phase, Drospirenone and Estetrol might be used in standard quality control labs and pharmaceutical formulations without difficulty.

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5.

NEW VALIDATED METHOD FOR THE ESTIMATION OF PIOGLITAZONE AND ROSIGLITAZONE USING RP-HPLC

5.1PIOGLITAZONE

5.1.1Drug profile of Pioglitazone

Pioglitazone, marketed with a trade name Actos, among others, is an anti- diabetic drug used to treat type 2 diabetes (**1, 2**). It can be utilized with metformin, sulfonylurea (**3**) or insulin (**4**). Use of exercise and diet is advised. It is not recommended for type 1 diabetes (**5**). It's taken by the mouth. Usual side reactions include headaches, muscle pain (**6**), inflammation (**7**) of the throat, and swelling. Severe side reactions can include cancer of the bladder (**8, 9**), hypoglycemia (**10**), heart failure (**11**) and osteoporosis (**12**). Usage not recommended during pregnancy or breast- feeding. It is in the class of thiazolidinedione (TZD) (**13**) and functions by bettering the sensitivity of tissues to insulin.

Structure of Pioglitazone [Fig 5.01]

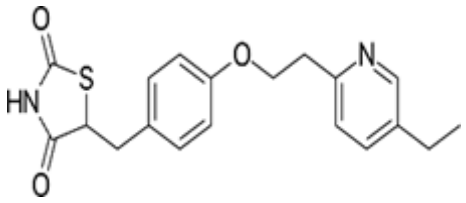

Figure 5.01: Pioglitazone's chemical structure

5.1.2Name of the IUPAC:

5-[[4-[2-(5-ethylpyridin-2-yl)ethoxy]phenyl]methyl]-1,3-thiazolidine-2,4-dione

5.1.3Molecularformula: $C_{19}H_{20}N_2O_3S$

5.1.4Molecular weight: 356.44 $g \cdot mol^{-1}$

5.1.5Category:

One of the thiazolidinediones in the pioglitazone category is pioglitazone. An increase in insulin sensitivity may assist regulate blood sugar levels in the body.

5.1.6Mechanism of Action:

As an insulin agonist, pioglitazone binds to the peroxisome proliferator activator-gamma (PPAR-gamma) receptors in adipose and skeletal muscle as well as the liver. PPAR activation enhances the transcription of insulin-responsive genes that govern the synthesis, transport, and utilisation of glucose and lipids in the body. Pioglitazone improves insulin resistance associated with type 2 diabetes mellitus without an increase in insulin secretion from pancreatic beta cells by enhancing tissue sensitivity to insulin and reducing hepatic glucose synthesis (i.e. gluconeogenesis).

5.1.7Side effects of Pioglitazone:

- When used with sulfonylurea or insulin, edoema (swelling) might occur.
- Hypoglycemic symptom (hypoglycemia)
- Respiratory infection of the upper respiratory tract.
- Headache.
- Acute myocardial infarction.

- Acute sinusitis.
- Fracture of the skeleton.
- Throat pain (pharyngitis)

5.1.8Contraindications:

- A disease.
- There is a risk of bladder cancer.
- Blood sugar levels are too low.
- Heart failure that goes on for a long time.
- Acute coronary syndrome.
- Heart failure symptoms that are severe and unexpected are known as sudden decompensated heart failure.
- Liver disease.
- Urine stained with blood

5.1.9Absorption:

Glucose sensitivity in target tissues may be improved with pioglitazone (thiazolidinedione). A mean absolute bioavailability of 83% is achieved, and the

maximal concentrations are reached in 1.5 hours or less. It is broken down by the cytochrome P450 enzyme system in the liver.

5.1.10 Uses:

To treat type 2 diabetes, pioglitazone may be used. When the body doesn't produce enough insulin or the insulin it produces doesn't act effectively, it has Type 2 diabetes. This has the potential to lead to an increase in blood sugar levels (hyperglycaemia).

5.1.11 Adult dose:

Tablet

- 15mg
- 30mg
- 45mg

5.2ROSIGLITAZONE

5.2.1Drug Profile of Rosiglitazone:

Rosiglitazone (trade name Avandia) is an anti-diabetic drug in the thiazolidinedione class (**14**). It acts as an insulin sensitizer, binding to PPAR in fat cells and producing the cells more sensitive to insulin. It is sold by the pharmaceutical firm GlaxoSmithKline (GSK) as a stand-alone drug or for use in conjunction with metformin or glimepiride. However, following a meta-analysis in 2007, which related drug utilized to rised the risk of heart attack (**15, 16**).

5.2.1 Structure of Rosiglitazone [Fig 5.02]

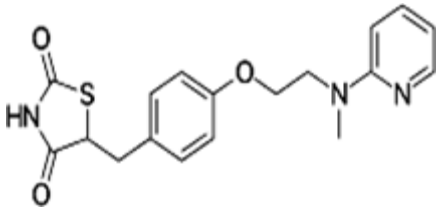

Figure 5.02: The molecular structure of Rosiglitazone

5.2.2 Name of the IUPAC: 5-[[4-[2-[methyl(pyridin-2- yl)amino]ethoxy]phenyl]methyl]-1,3-thiazolidine-2,4-dione

5.2.3 Formula molecular: $C_{18}H_{19}N_3O_3S$

5.2.4 Molecular weight: 357.4g/mol

5.2.5 Category:
rosiglitazone (brand name Avandia) is an anti-diabetic medication in the family of thiazolidinediones. As an insulin sensitizer, it binds to the PPAR receptor in fat cells and enhances insulin sensitivity in the cells.

5.2.6 Mechanism of action:
The peroxisome proliferator-activated receptors (PPARs) in tissues such as adipose tissue, skeletal muscle, and the liver are specifically and potently agonists of rosiglitazone. In the regulation of glucose synthesis, transport, and utilisation, PPAR- gamma receptor activation affects the transcription of insulin-responsive genes. Rosiglitazone improves insulin sensitivity in this manner.

5.2.7 Side Effects of Rosiglitazone:

- The pulse is quick or thumping,
- Modifications to the menstruation cycle,
- In the event of a fracture,
- Urine with a bluish tint,
- Skin or eye discoloration,
- Vomiting or nausea that does not go away,
- Pain in the stomach or abdomen.
- The alteration of the state of one's eyesight (e.g., colour or night vision problems).

5.2.8 Contraindications:
Patients with active liver illness, hypersensitivity, Type 1 diabetes, diabetic ketoacidosis, and hyperosmolar hyperglycemic condition should not use rosiglitazone.

5.2.9 Absorption:
Oral tablet dosage yielded an absolute bioavailability of 99 percent, whereas oral solution dosing yielded a 95 percent absolute bioavailability, and clearance was predominantly metabolic in nature.

5.2.10 Uses:
To treat type 2 diabetes, rosiglitazone is often used with a diet and exercise regimen and one or more additional drugs (condition in which the body does not use insulin normally and therefore cannot control the amount of sugar in the blood).

5.2.11 Adult dose: Tablet

- 2mg
- 4mg

5.3 LITERATURE RESURVEY

Nighat Shafi M. Saeed Arayne, **Najma Sultana, Azhar Hussain**, **Farhan Ahmed Siddiqui, Validation of New Assay Method for the Simultaneous Analysis of Diltiazem, Rosiglitazone, Pioglitazone and Metformin by RP-HPLC and its Applications in Pharmaceuticals and Human Serum**: It has been developed and validated for the simultaneous measurement of diltiazem, metformin, pioglitazone hydrochloride and rosiglitazone hydrochloride in raw materials, their pharmaceutical formulations, and human blood via HPLC technique. Using acetonitrile-methanol-water (30:20:50, v/v, pH 2.59 0.02) as the mobile phase in HPLC, all of the aforementioned medicines were chromatographed at an ambient temperature flow rate of 1.0 mL/min. A Hiber, 250–4.6 RP-18 column fitted with a UV-vis detector at 230 nm is used for the separation. All of the anti-diabetic medications eluted at various retention times, but all demonstrated satisfactory resolution from diltiazem. There is no Chromatographic interference from the tablet excipients when the approach is used to pharmaceuticals. Detection and quantification limits are determined to be apposite for the approach. Validation in accordance with ICH recommendations demonstrates the method's suitability for quantitative determination of the medicines. The International Conference on Harmonization (ICH). This method's dependability was proved by the validation findings and statistical treatment of the data.

T. Rajesh, K. S. Lakshmi, RP-HPLC technique for simultaneous measurement of glipizide, rosiglitazone, pioglitazone, glibenclamide, and glimepiride in pharmaceutical dosage forms and human plasma has been developed and validated: Pharmaceutical dosage forms and human plasma may now be tested for all of these

drugs at the same time using an easy-to-use high-performance liquid chromatographic technique. Triethylamine (pH-3.5, corrected with orthophosphoric acid), acetonitrile, and methanol were used in the elution mixture in the ratio of 55:15:30 at a flow rate of 1 ml min-1 over a 150 x 4.6 mm Phenomenex C18 column at ambient temperature for the extraction. A wavelength of 248 nm was used to monitor the medications, and they were separated within 20 minutes. Suitable dilutions of formulations were made and plasma samples were extracted using acetonitrile. Percentage RSD for intra- and inter- day studies was determined to be less than 4.34 for all the specified concentrations of the medicines. It was also determined to be within the permissible range of findings after validation according to ICH recommendations, as well. Aside from ordinary quality control, the suggested technique may also be employed in pharmacokinetic investigations.

M. Saeed Arayne, Agha Zeeshan Mirza, Najma Sultana, Develop and validate an HPLC technique to study the in vitro influence pioglitazone has on the availability of H1 receptor antagonists: Pioglitazone and H1-receptor antagonists (fexofenadine, cetirizine, and levocetirizine) may be detected simultaneously in formulations and human blood using this approach. Pioglitazone's in vitro effects on H1-receptor antagonists were studied using HPLC methods. UV spectrophotometric analysis was used to confirm the accuracy of the obtained data. Drug concentrations were plotted using the first-derivative values vs concentration and found to be in agreement with HPLC results. All three antihistamines had no effect on pioglitazone's availability, regardless of whether they were present or absent. Pioglitazone and H1-receptor antagonists may be safely combined in vitro, and this study serves as a platform for future in-vivo investigations.

Nuran Özaltın, Ceren Yardımcı, Development and validation of capillary electrophoresis methods for simultaneous measurement of rosiglitazone and metformin in pharmaceuticals: For the simultaneous measurement of rosiglitazone and metformin, a new and easy capillary zone electrophoresis technique was devised and validated. With an 80.5 cm i.d. fused-silica capillary column and 25 mM acetate buffer at pH 4.0, these analytes were separated in less than 9 minutes at 25 °C with an effective length of 72.0 cm. At 50 mbar and +25.0 kV, the samples were injected hydrodynamically for three seconds at 50 mbar. The detection wavelength was set at

203 nm. It was utilised as a benchmark in the company's internal testing. The technique was thoroughly tested in terms of specificity, linearity, limit of detection and quantitation, accuracy, precision, and robustness, as well as its ability to handle a variety of samples. Both metformin and rosiglitazone had a detection and quantitation limit of 0.01 and 0.01 ug mL-1, respectively. Rosiglitazone and metformin were effectively analysed using the approach proposed by the researchers. It was determined that the findings obtained with this approach were statistically equivalent to those obtained using an existing liquid chromatographic method described in the scientific literature.

Bappaditya Chatterjee, Pinaki Sengupta, BapiGorain, Uttam Kumar Mandal, Tapan Kumar Pal, Research on the simultaneous measurement of pioglitazone and telmisartan in rat plasma, using an ultra-high-throughput LC– MS/MS technology, as well as its application in the field of pharmacokinetics: The fact that diabetes and cardiovascular risk factors often coexist necessitates a multifaceted approach to their management. Cardiovascular complications associated with diabetes may be effectively managed when pioglitazone (PIO) and telmisartan (TLM) are used together (in combination). A high-throughput LC–MS/MS technique for simultaneous PIO and TLM quantification in rat plasma was developed and validated in this study. While prior techniques were capable of measuring individual analytes, this new approach is more sensitive and can do so in a shorter amount of time. As a result, there is currently no bioanalytical approach that can concurrently measure both PIO and TLM in a single experiment. The bioanalytical approach was verified in accordance with USFDA requirements. Analytes were found to respond linearly in the range of 0.005–10 g/mL with acceptable precision and accuracies. Four layers of quality control ensured an accuracy of 94.27–106.10 percent. Both the intra- and inter- day precisions varied from 2.32 per cent to 10.14 per cent and from 5.0 per cent to 8.12 per cent, respectively A preclinical pharmacokinetic investigation used rat plasma samples to quantify PIO and TLM using this approach. It is believed that the PIO-TLM combination will prove valuable in the future because of its potential therapeutic applications.

Sayeeda Sultana, N. Balaji, HPLC-based detection of Pioglitazone Hydrochloride related compounds: Pioglitazone hydrochloride drug substance related substance measurement has been made easier with the development and validation of a high performance liquid chromatographic technique. Methods: Pioglitazone hydrochloride may be tested for the presence of three related compounds using this technique. The pH of the mobile phase A is adjusted to 2.5 using dilute phosphoric acid and 0.1 percent w/v triethylamine in water. Premixed and degassed acetonitrile and methanol mixtures make up mobile phase B. It was running at a rate of 1 ml/min. Gradient mode elution was utilised. To perform the study, a symmetrical C18 HPLC column, with a 250-millimeter length and 4.6-mm internal diameter, was employed. It was found to be linear with a coefficient of correlation of 0.99 across the 0.006-250 percent scale. The relative standard deviation as a percentage of the mean was found to be within the allowable range by the precision study. Pioglitazone hydrochloride hydrochloride test concentration of 2000 mg/ml has a detection and quantitation limit of less than 0.002% and 0.006%, respectively. Q2 This procedure has been approved in accordance

with ICH criteria (R1). Conclusion: A robust HPLC technique for the quantitative measurement of pioglitazone hydrochloride drug substance-related chemicals was successfully developed.

Gurupadayya BM, Anandkimar R. Tengli, Vishwanathan B and Neeraj Soni, Method Development and Validation of Tablet Dosage Forms of Metformine, Pioglitazone, and Glibenclamide by RP-HPLC: Glibenclamide has been established as an internal standard for the simultaneous determination of metformine, pioglitazone and glibenclamide in tablet dose using a reverse phase high performance liquid chromatographic technique. low gradient mode was obtained with the use of phenomenex luna 100R 2504.60 (mm) column and the mobile phase of acetonitrile (60:20:20) water and buffer (0.5 percent potassium dihydrogen phosphate) pH 2.5 adjusted with orthophosphoic acid in the ratio of 60 to 20 to 20 percent. The eluent was measured at 230 nm using a UV detector at a flow rate of 1 mL min-1. The retention times of Metformine, pioglitazone, and Glibenclamide were 2.2, 2.8, and 5.8 minutes, respectively, using the chromatographic conditions employed. There is a linearity range for metformine, pioglitazone and glibenclamide between 50-300 mL-1, 1.5-9.0 mL-1 and 0.5-3 mL-1. Accuracy, simplicity, specificity and reproducibility were discovered

in the devised approach. Anti-diabetic medications in combination dose forms may also be tested using this method.

Dharmendra L and Freddy H. Havaldar. Vairal, Analyses for the tablets dosage form of Metformin Hydrochloride, Rosiglitazone Hydrochloride, and Pioglitazone Hydrochloride: Methods for the detection of metformin hydrochloride, rosiglitazone hydrochloride, and pioglitazone were developed and confirmed using an isocratic reversed phase liquid chromatographic technique. In order to achieve separation, we used an ammonium dihydrogen phosphate buffer, which was adjusted to pH 3.0 by using orthophosphoric acid diluted in acetonitrile (65:35 v/v) as eluent while running the sample through the column at a constant rate of 0.7 ml per minute on a Zorbax C8 column measuring 1504.6 mm in diameter. At a wavelength of 215 nm, the UV signal was detected. At a concentration of 50 mg/mL, pioglitazone hydrochloride was shown to be stable in the blood for around 6 minutes after administration. Analyzing antidiabetic substances in pharmaceutical preparations may be as easy, fast, and precise as this procedure. In pharmaceutical formulations, it's an easy way to separate and measure metformin hydrochloride, rosiglitazone, and pioglitazone hydrochloride all at once.

A Anil Kumar, CH Ravikanth, S Prashanth, V Uday Kiran, B Madhu, Y Narsimha Reddy, Accurate and Fast HPLC Method for Rat Serum Pioglitazone Determination: An HPLC-UV technique for the detection of Pioglitazone in rat serum has been developed. As an internal standard, rosiglitazone was used. Ethyl acetate is used to extract Pioglitazone and Rosiglitazone from serum using a liquid–liquid extraction process. Phenomenex C18 (250 mm x 4.6 mm; 5 m) reversed-phase column is used for the isocratic separation of Pioglitazone and Rosiglitazone in a 60:40 (v/v) ratio, using UV detection at 269nm to measure the concentrations of the two drugs. The analysis was completed in just ten minutes. 97.12 percent of the injected 0.1-10 ng/ml samples were recovered on average. There was a strong linear correlation in the results of the experiment. It was possible to accurately and precisely measure Pioglitazone concentrations ranging from 0.1 to 10 micrograms per litre. Pioglitazone may be routinely monitored in the clinic using this approach.

Omnia Ismaiel, Hisham Elrefay, Abdalla Shalaby, Wafaa S Hassan. Work on an HPLC-UV approach for the measurement of pioglitazone and metformin hydrochloride in bulk and as combination dosage forms that indicates stability: This technique was developed and validated for the measurement of pioglitazone hydrochloride and metformin hydrochloride in bulk medication and pharmaceutical dosage form using high performance liquid

chromatography (HPLC). On a Kromasil C18 4.6 x 250 mm, 5 m 100. column, we were able to separate and quantify the samples. At a flow rate of 1.5 ml/min, the mobile phase was (50:50) methanol: phosphate buffer, pH 6.5 containing 0.01 M sodium dodecyl sulphate, v/v. For the purposes of this experiment, the wavelength of detection was set at 270 nanometers. Precision, accuracy, durability, and recovery were all tested in the method's validation process. Acidic, basic, and oxidative stress conditions were used to test Pioglitazone and Metformin's effects on the stressed samples. A linear correlation coefficient of 0.995 was found in the concentration range of 50% to 150% of the target concentration. Both analytes had intra- and inter-day precision of less than 2%. This method's ability to identify target analytes in the presence of degradants may be shown by analysing the chromatograms of stressed samples.

5.4 EXPERIMENTAL STUDY

5.4.1Chemical and reagents:

Merck India Ltd. in Mumbai, India provided the acetonitrile, HPLC-grade formic acid, and water needed for this experiment. Glenmark in Mumbai provided the APIs for the Pioglitazone and Rosiglitazone standards.

5.4.2Instrumentation:

For this work, we employed a Waters Alliance liquid chromatography (model e- 2695) that was monitored by the empower 2.0 data management system and a light diode array detector (model 2998).

5.4.3Standard stock solution preparation:

For normal stock solution preparation, add 70ml of diluents to 30mg of Pioglitazone and 10mg of Rosiglitazone taken in a 100 ml volumetric flask and sonicate for 10 minutes to thoroughly dissolve the contents and then bring up to the mark with diluents.

Preparation of Standard solution:

A 50ml volumetric flask is filled with 5 ml of the aforesaid normal stock solution and diluted to the desired level.

5.5 METHOD DEVELOPMENT ANALYTICAL METHOD DEVELOPMENT:

Pioglitazone and Rosiglitazone analysis by RP-HPLC has been attempted successfully in the suggested study.

5.5.1Method development parameters:

When developing a new technique, selecting the following parameters is critical.

- The chromatographic technique
- Wavelength
- Column
- Composition of the mobile phase
- A mechanism for delivering the solvents
- Flow speed
- Volume of injection

5.5.1.1 Selection of Mode of Chromatography:

Reversed phase chromatography is the method of chromatography that was chosen. Rasis of selection : Polarity of the molecule

Reason for selection : Pioglitazone and Rosiglitazone are polar

molecules, which mean that they elute at a quicker rate with the mobile phase.

5.5.1.2 Detector Wavelength Selection:

The choice of a detector wavelength is critical to completing the analytical procedure. When using a PDA detector and a certain wavelength, the standard API is manufactured and injected into the chromatographic system to determine the precise wavelength..

Selected wave length **:** 261 nm

Basis for selection **:** Maximum analyte and impurity absorption

Reason for selection **:** Having a maximum absorption of 261 nm, Pioglitazone and Rosiglitazone.

5.5.1.3 Selection of column:

Column selected **:** Inertsil ODS (150x4.6mm, 3.5 µ)

Basis for selection **:** Analytical differences based on polarity and chemical composition

Reason for selection **:** Excellent physiochemical surface characteristics and compatibility with a wide variety of organic solvents are only some of the advantages of these materials.

5.5.1.4 Selection of the mobile phase composition and of the buffer:

Peak symmetries and separation are heavily influenced by the buffer and its intensity. There are a number of factors that must be taken into consideration when selecting the proper buffer strength for a chromatographic injection load.

Mobile phase preparation:

Solution A **:** Acetonitrile

Solution B **:** 0.1percent formic acid

5.5.1.5 Selection of the rate of flow:

Even in reverse phase separation for the resolution of tiny molecules, flow rate is a crucial element. However, in large-scale inverted phase chromatography, adding the sample solution at a high flow rate has a significant impact on the analytical results. The dynamic binding capacity of a sample might vary depending on the flow rate employed for sample loading. When scaling up the purification process, the dynamic binding capacity must be estimated to determine the optimal flow rate for loading. In this system, the flow rate is set to 1 ml/min and is dependent on factors such as flow factor, retention duration, column composition, separation impurity, and peak symmetrical symmetry.

5.5.1.6 Selection of injection volume:

For API estimate, a volume of 10 to 20 µl is often advised. Extraction proved problematic, thus the test concentration may be reduced and the injection volume increased to 50 µl in the end. As long as the specified column volume isn't overflowing, it's all ok. This approach uses a 10 µl injection volume for Pioglitazone and Rosiglitazone.

5.6 RESULTS AND DISCUSSION

5.6.1Trials in optimization of chromatographic condition:

Trial-1 [Fig 5.03]

Buffer	:	Ammonium acetate pH-4 adjusted with OPA
Mobile phase	:	Acetonitrile and Buffer (80:20)
Column	:	Agilent eclipse XDB (250x 4.6mm, 5µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	10 min
Wavelength	:	200-400 nm
Observation	:	First peak is splitted into two peaks

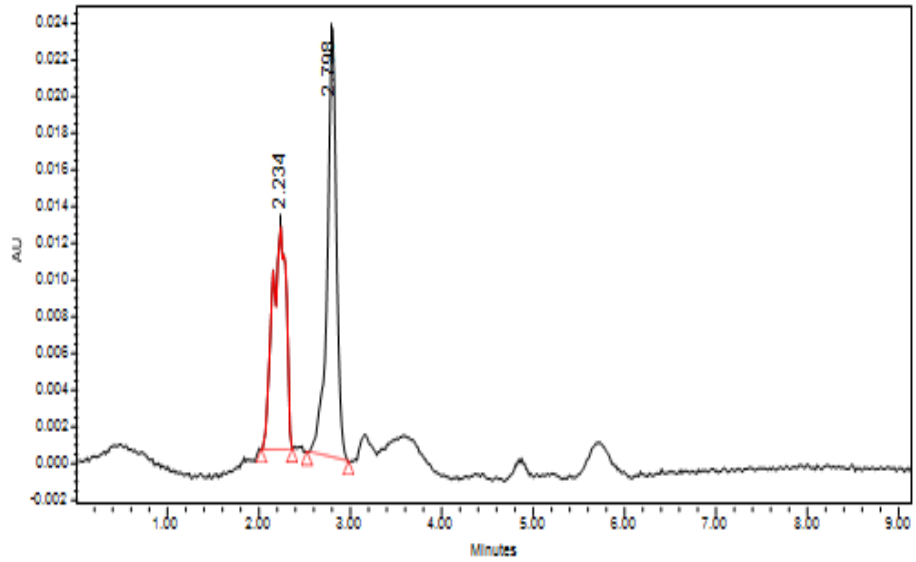

Figure 5.03: chromatogram of trial-1

Trial-2 [Fig 5.04]

Buffer	:	Ammonium acetate pH-4 adjusted with OPA
Mobile phase	:	Acetonitrile and Buffer (70:30)
Column	:	Agilent eclipse XDB (250x 4.6mm, 5µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	30 min
Wavelength	:	261 nm
Observation	:	System suitability conditions are not within the limit

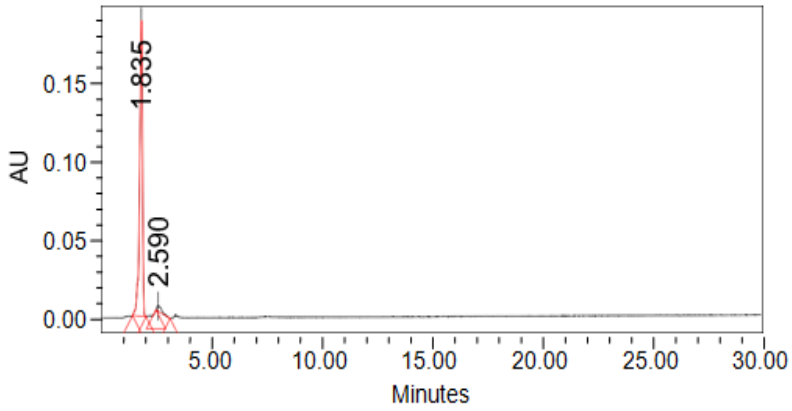

Figure 5.04: Chromatogram of trial-2

Trial-3 [Fig 5.05]

Buffer	:	Ammonium acetate pH-4 adjusted with OPA
Mobile phase	:	Acetonitrile and Buffer (60:40)
Column	:	Agilent eclipse XDB (250x 4.6mm, 5µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	10min
Wavelength	:	261 nm
Observation	:	Base line is not sufficient

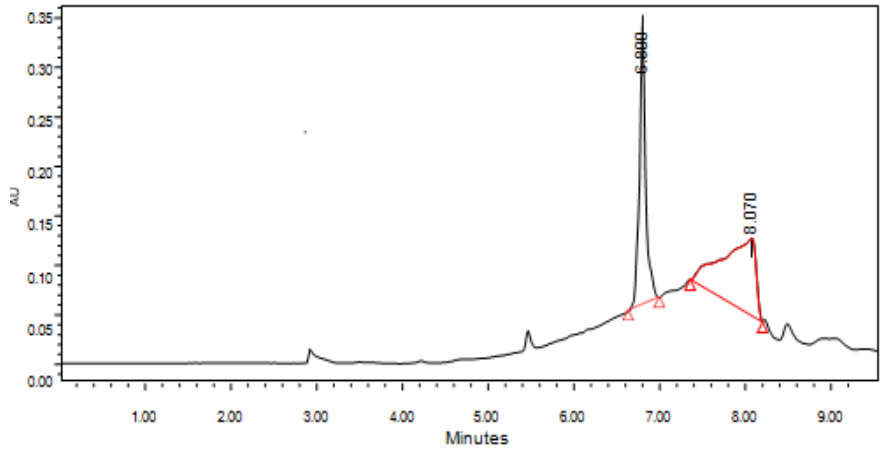

Figure 5.05: Chromatogram of trial-3

Trial-4 [Fig 5.06]

Mobile phase	:	Acetonitrile and 0.1% TEA (35:65)
Column	:	Agilent eclipse XDB (250x 4.6mm, 5µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl

Period of run	:	9min
Wavelength	:	261 nm
Observation	:	Peak height is not within the limit

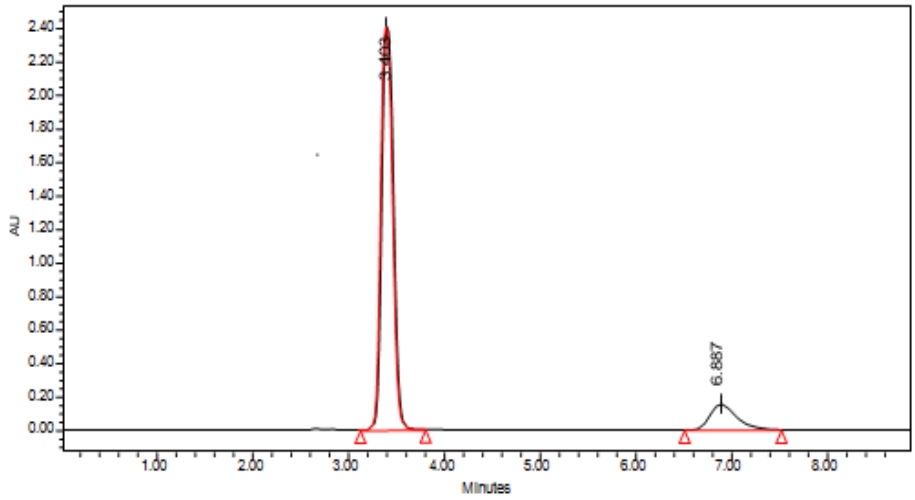

Figure 5.06: Chromatogram of trial-4

Trial-5 [Fig 5.07]

Mobile phase	:	Acetonitrile and 0.1% TEA (40:60)
Column	:	Agilent eclipse XDB (250x 4.6mm, 5µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	15min
Wavelength	:	261 nm
Observation	:	Resolution is not within the limit

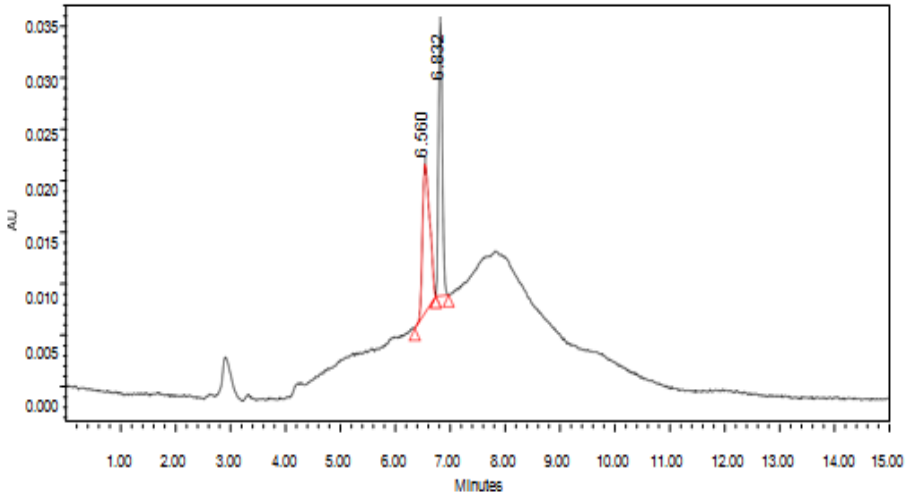

Figure 5.07: Chromatogram of trial-5

Trial-6 [Fig 5.08]

Mobile phase	:	Acetonitrile and 0.1% TEA (45:55)
Column	:	Inertsil ODS (150x4.6mm, 3.5 µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	4min
Wavelength	:	261 nm
Observation	:	Broad peaks are observed.

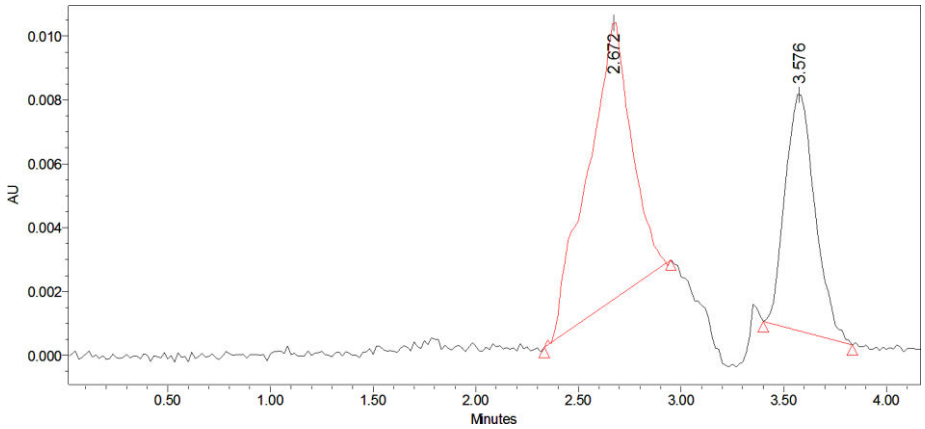

Figure 5.08: Chromatogram of trial-6

Trial-7 [Fig 5.09]

Mobile phase	:	Acetonitrile: 0.1% formic acid (50:50)
Column	:	Inertsil ODS (150x4.6mm, 3.5 µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	9 min
Wavelength	:	261 nm
Observation	:	Peak shapes are not good.

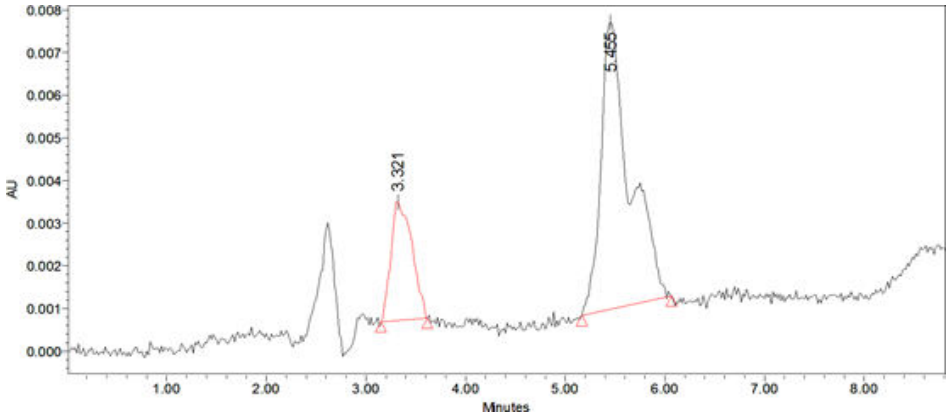

Figure 5.09: Chromatogram of trial-7

Trial-8 [Fig 5.10]

Mobile phase	:	Acetonitrile: 0.1% formic acid (65:35)
Column	:	Inertsil ODS (150x4.6mm, 3.5 µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	9min
Wavelength	:	261 nm
Observation	:	Height is not within the limit and Unknown peak is formed

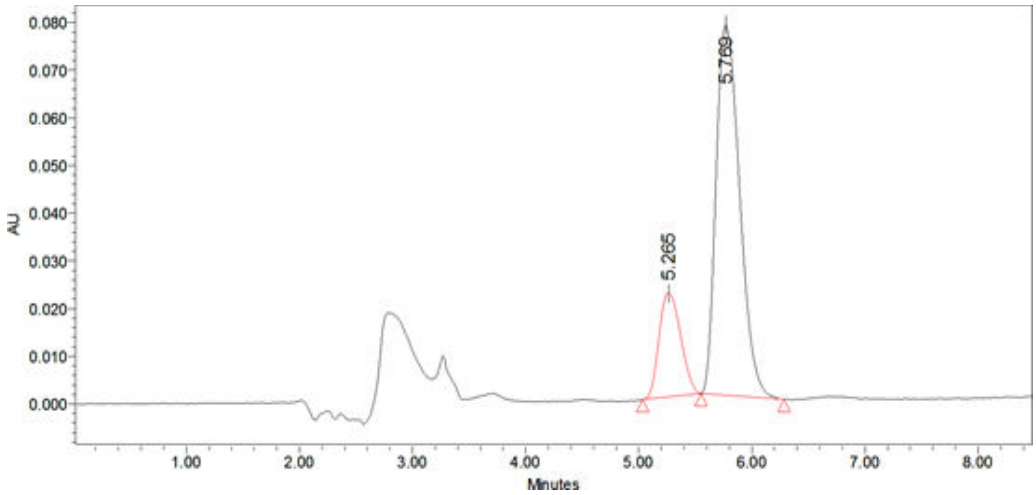

Figure 5.10: Chromatogram of trial-8

Trial-9 [Fig 5.11]

Mobile phase	:	Acetonitrile: 0.1% formic acid (60:40)
Column	:	Inertsil ODS (150x4.6mm, 3.5 µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	5min
Wavelength	:	261nm
Observation	:	Broad peak is observed

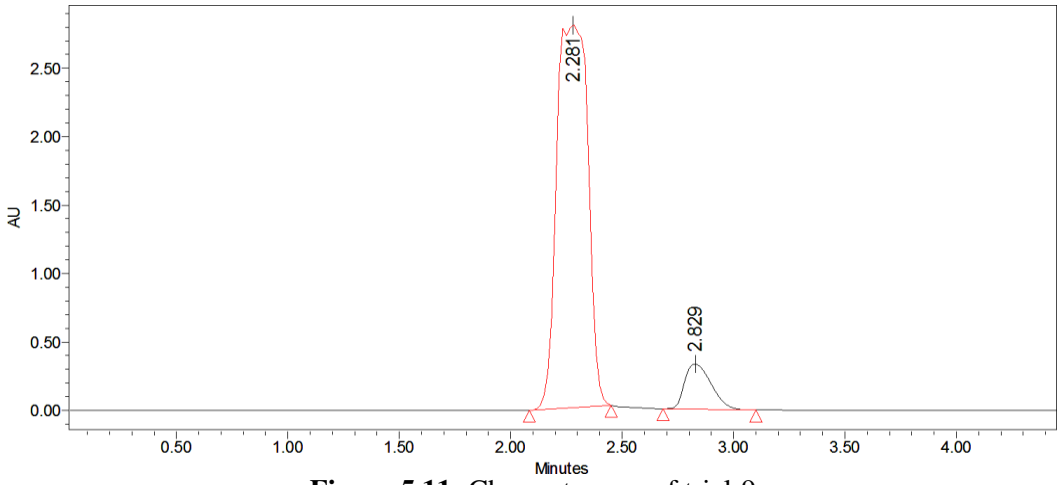

Figure 5.11: Chromatogram of trial-9

Trial-10 [Fig 5.12]

Mobile phase	:	Acetonitrile: 0.1% formic acid (70:30)
Column	:	Inertsil ODS (150x4.6mm, 3.5 µ)
Rate of flow	:	1 ml/min
Volume of injection	:	10µl
Period of run	:	8 min
Wavelength	:	261 nm
Observation	:	This method is suitable for validation

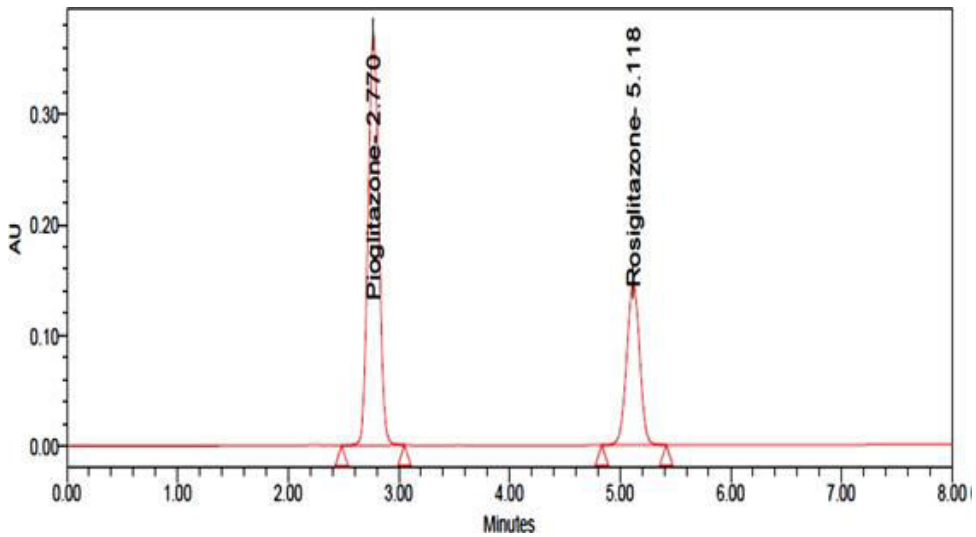

Figure 5.12: chromatogram of trial-10

The chromatographic results of all trials were shown in **table 5.01**.

Trial No.	RT	Area	% Area	USP Resolution	USP Tailing	USP Plate count
1	2.234	126008	43.47		0.81	1163
	2.798	163862	56.53	2.55	0.84	4306
	1.835	1666451	96.67		0.74	986

2	2.590	57480	3.33	2.38	3.34	737
3	6.800	1427640	41.52		1.16	56820
	8.070	2010957	58.48	2.46	0.58	2219
4	3.403	20555811	87.42		1.12	4164
	6.887	2957198	12.58	9.71	2.45	3112
5	6.560	131075	50.57		1.19	8734
	6.832	128116	49.43	1.29	1.15	44525
6	2.672	128829	63.09		0.85	491
	3.576	75372	36.91	2.47	1.26	2535
7	3.321	40819	23.10		1.41	1393
	5.455	135875	76.90	5.29	1.51	3338
8	5.265	288770	20.42		1.17	3569
	5.769	1125197	79.58	1.37	1.36	3723
9	2.281	27298835	90.91		0.97	1877
	2.829	2731214	9.09	2.55	1.41	2811
10	2.770	3620286	71.26		1.10	4132
	5.118	1415540	28.74	11.23	1.07	7065

Table 5.01: Chromatographic data of all trials

5.6.2 Optimized method [Table 5.02 and Fig 5.13]

S.NO	Parameter	Chromatographic condition
1	Mobile phase	Acetonitrile: 0.1% formic acid (70:30)
2	Column	Inertsil ODS (150x4.6mm, 3.5 µ)
3	Rate of flow	1ml/min
4	Column temperature	Ambient temperature
5	Sample temperature	Ambient temperature
6	Wavelength	261 nm
7	Volume of injection	10µl
8	Period of run	8 min
9	Retention time	Pioglitazone Retention time-2.770 Rosiglitazone retention time-5.118

Table 5.02: Optimized method chromatographic conditions

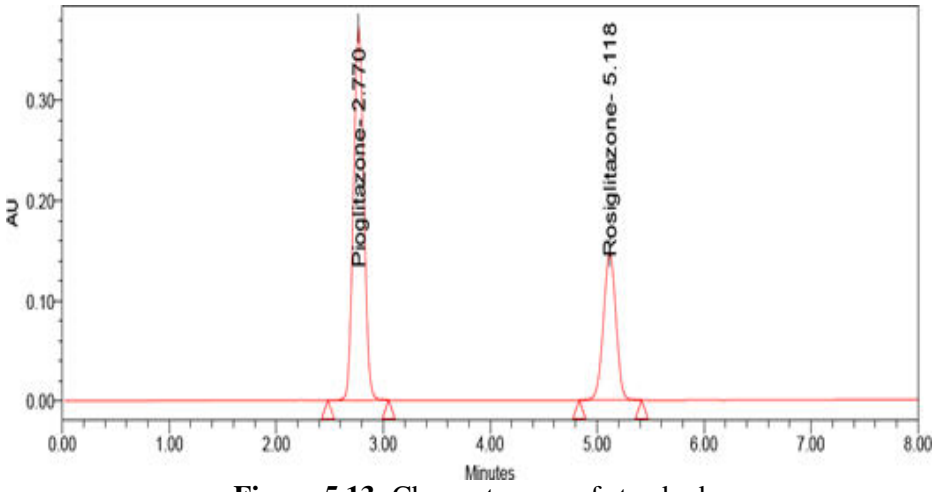

Figure 5.13: Chromatogram of standard

5.7 VALIDATION OF METHOD

It has been shown to be accurate, specific and precise in compliance with the ICH Q2 (R1) requirements for device appropriateness, as well as robustness, LOD, LOQ and stability.

5.7.1 Specificity:

Specificity is the ability to test the analyte in the presence of other components, such as contaminants or excitements, that may be presumed to be present in the sample solution and the norm solution, without any interference. The samples were spiked with Pioglitazone and Rosiglitazone for the tests. **Figure 5.14** gives blank chromatogram.

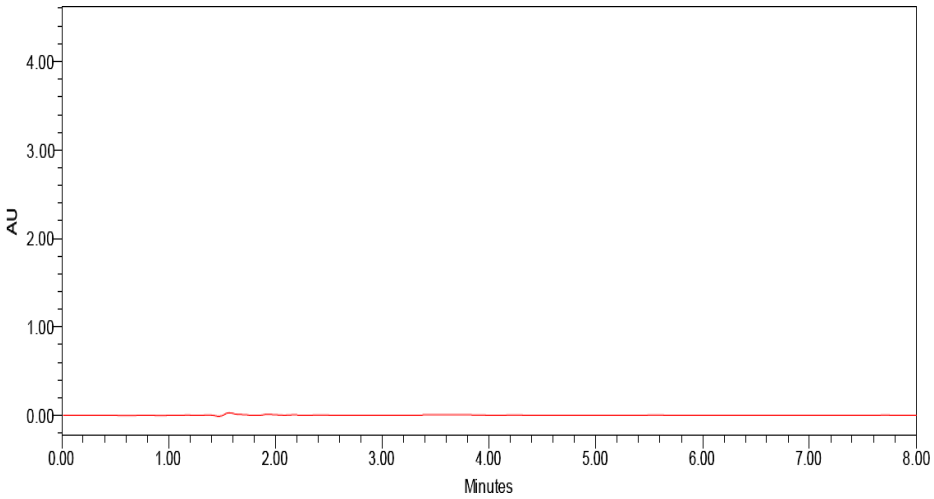

Figure 5.14: Chromatogram of blank

5.7.2 Linearity:

As a consequence of its capacity to deliver findings within a predetermined context, the empirical technique is linear. It was determined that the peak area correlated with the concentration of analytes in the sample: six standard solutions were used. The peak area was displayed on the calibration curve and the regression equations were derived using the normal solution concentration as a reference point. The least squares technique of least squares was used to calculate the slope, intercept, and correlation coefficient.

Linearity stock solution preparation:

Dilution: Weigh 30mg of Pioglitazone and 10mg of Rosiglitazone and add 70ml of volumetric flask diluents. For 15 minutes, sonicate the diluents to dissolve.

10 percent solution preparation: (3ppm of Pioglitazone and 1ppm of Rosiglitazone)

Dilution of the stock solution in a new 50 ml volumetric flask was carried out using the diluents stated above.

25 percent solution preparation: (7.5ppm of Pioglitazone and 2.5ppm of Rosiglitazone)

One of the stock solutions was diluted in a 50 ml volumetric flask with diluents to the mark in another 50 ml volumetric flask.

50 percent solution preparation: (15ppm of Pioglitazone and 5ppm of Rosiglitazone)

2.5 ml of the aforesaid stock solution was diluted with the diluents up to the mark in a separate 50 ml volumetric flask.

75 percent solution preparation: (22.5ppm of Pioglitazone and 7.5ppm of Rosiglitazone)

Stock solution was diluted in a 50ml volumetric flask with diluents up to the mark in a separate 50ml volumetric flask.

100 percent solution preparation: (30ppm of Pioglitazone and 10ppm of Rosiglitazone)
Stock solution was diluted in a 50-ml volumetric flask with the diluents up to the mark in a separate 50-ml volumetric flask.

125 percent solution preparation: (37.5ppm of Pioglitazone and 12.5ppm of Rosiglitazone)
6.25 ml of the above-mentioned stock solution was diluted with the diluents to the appropriate concentration in a separate 50 ml volumetric flask.

150 percent solution preparation: (45ppm of Pioglitazone and 15ppm of Rosiglitazone)
7.5ml of the above-mentioned stock solution was diluted with the diluents to the appropriate concentration in another 50 ml volumetric flask.

Procedure:
Using a chromatographic technique, measure the peak area for each degree. Plotting the peak area against the concentration (on the X-axis) and then calculating the correlation coefficient is what this method entails.

Range:
To put it another way: The range of analytic approaches encompasses the gap between the top and lower levels of analysis.

Acceptance Criteria:
A correlation coefficient of less than or equal to 0.999 is unacceptable.

Table 5.03 gives linearity results and **figures** from **5.15-5.24** gives linearity chromatograms.

S.NO	Pioglitazone		Rosiglitazone	
	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area
1	3.00	356890	1.00	177169
2	7.50	921356	2.50	418184
3	15.00	1805500	5.00	788226
4	22.50	2611265	7.50	1133741
5	30.00	3616456	10.00	1455465
6	37.50	4371404	12.50	1860828
7	45.00	5325525	15.00	2141815
Slope	117695.97		142819.95	
Intercept	14774.06		41820.07	
CC	0.99976		0.99922	

Table 5.03: Results of linearity

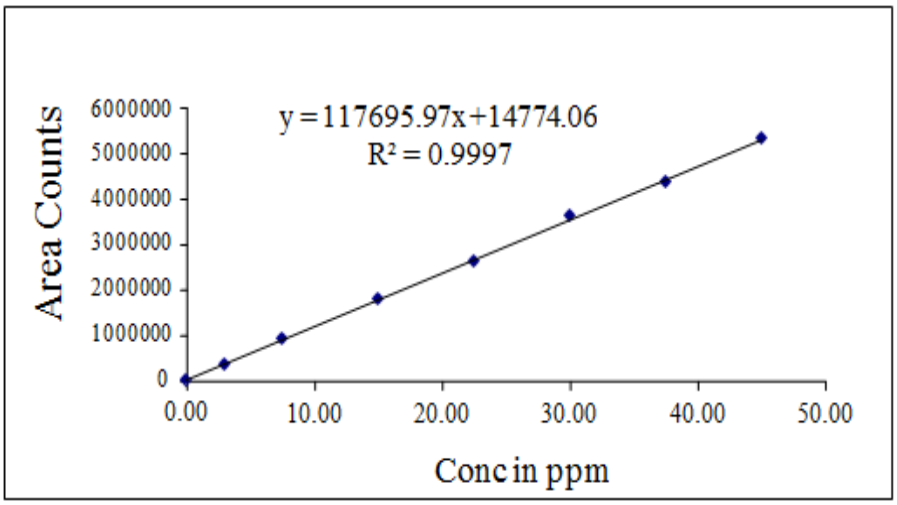

Figure 5.15: This is a Pioglitazone calibration figure

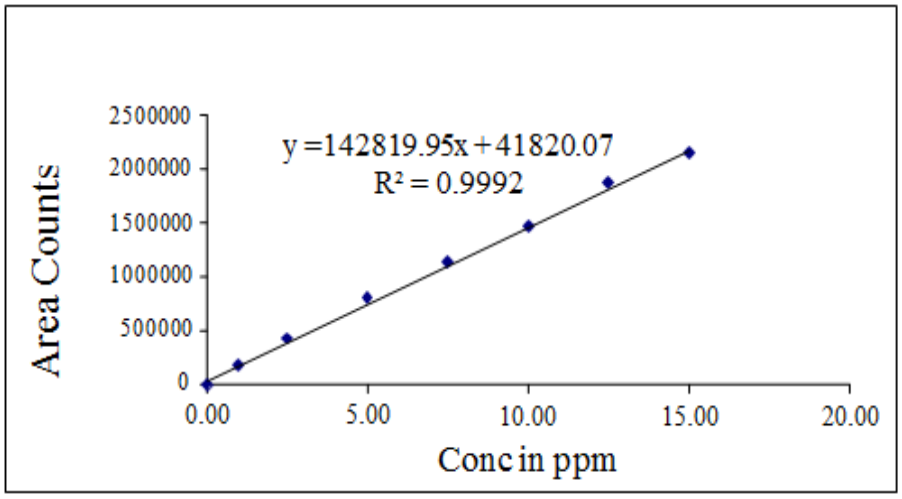

Figure 5.16: Plot of Rosiglitazone's calibration curve

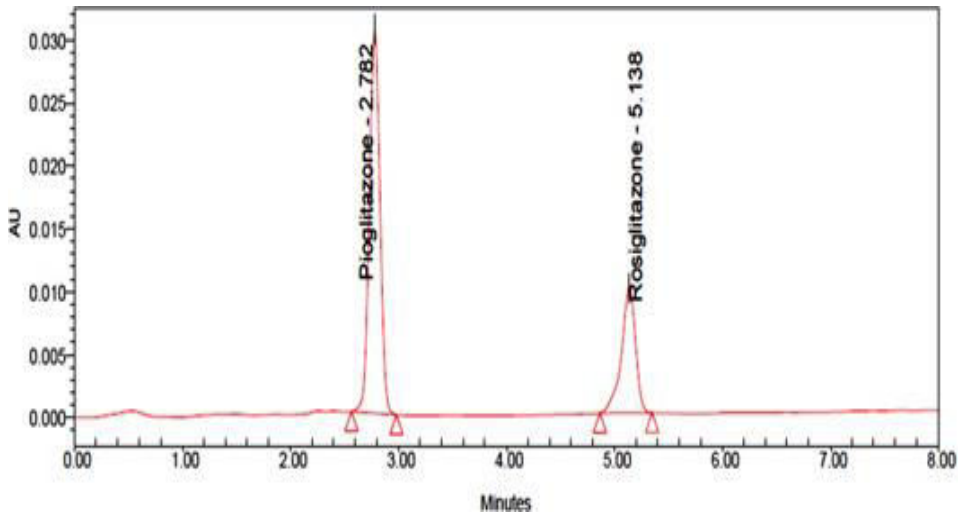

Figure 5.17: Chromatogram with 10% linearity

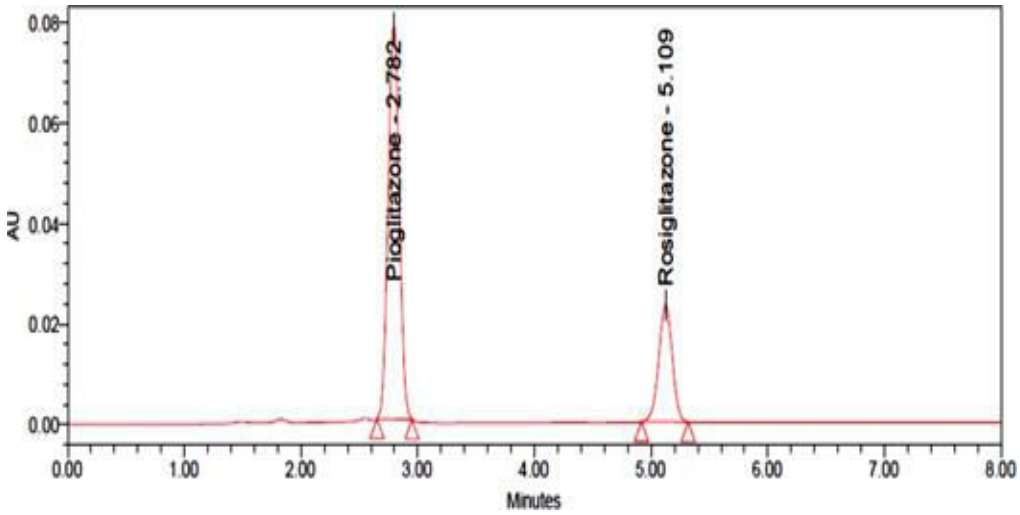

Figure 5.18: Linearity of chromatogram at 25%

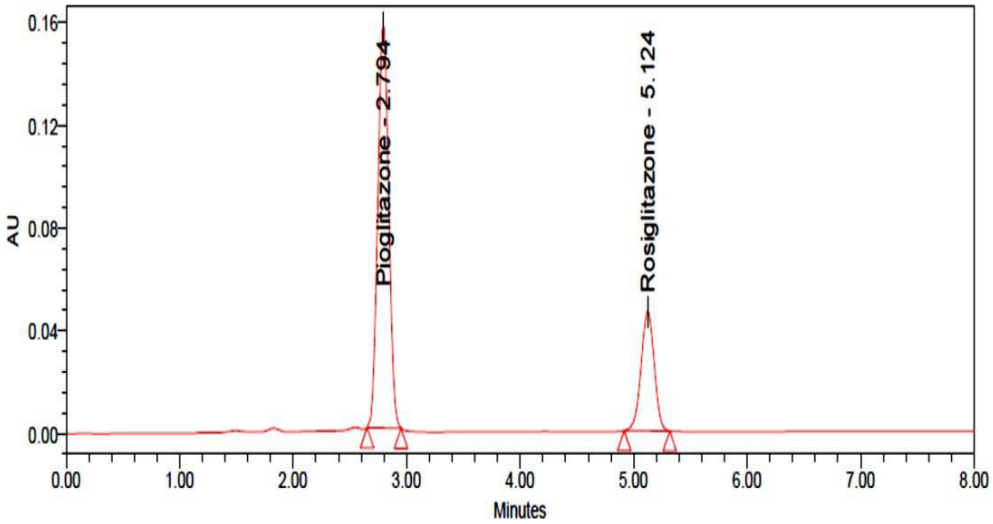

Figure 5.19: Chromatogram with a linearity of 50%

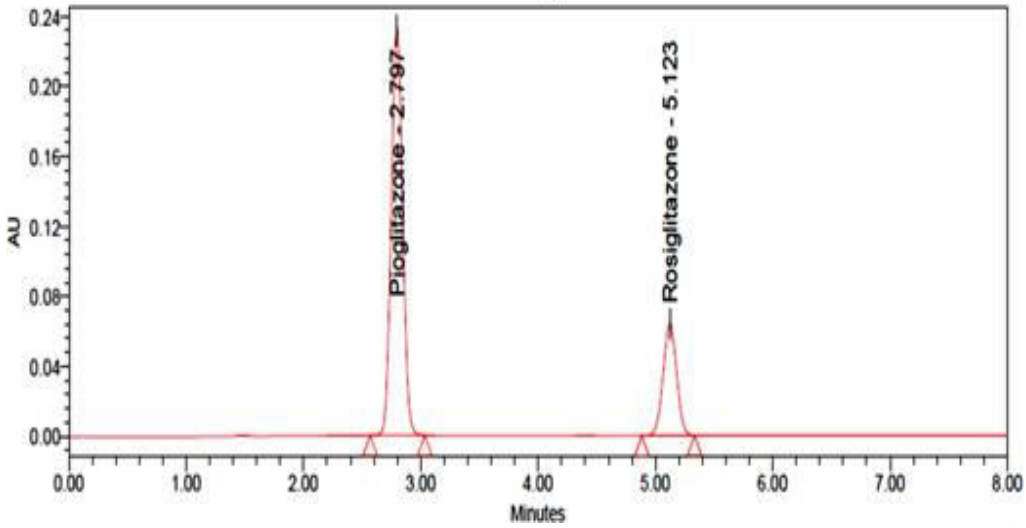

Figure 5.20: The chromatogram has a 75% linearity

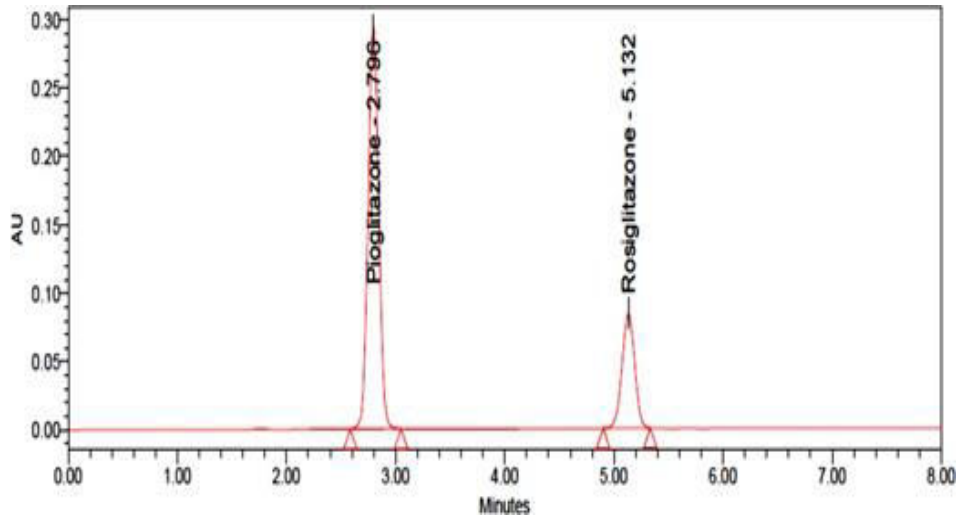

Figure 5.21: Chromatogram with perfect linearity

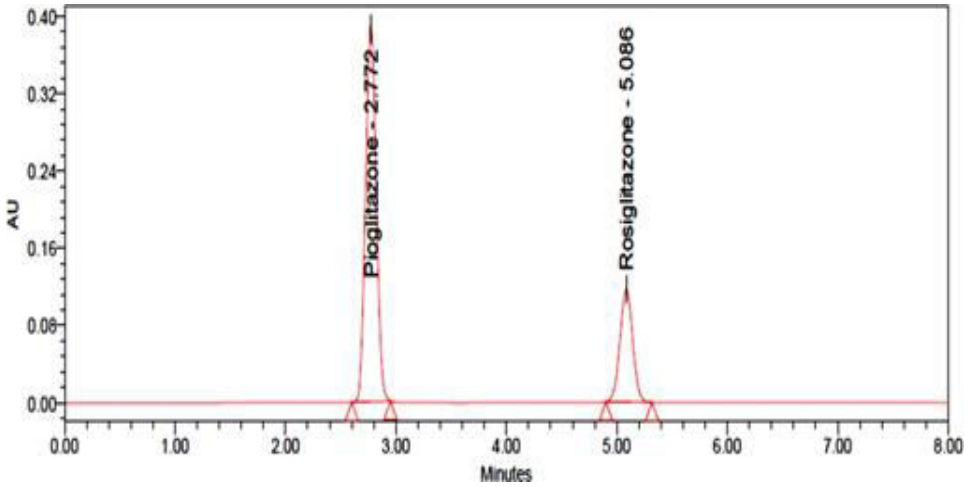

Figure 5.22: A chromatogram with a linearity of 125 percent

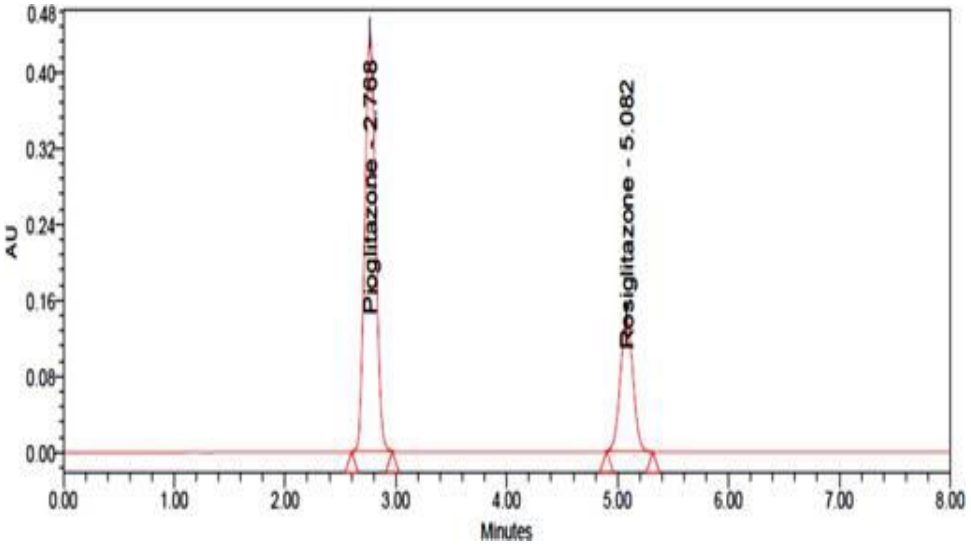

Figure 5.23: Chromatogram with a linearity of 150 percent

5.7.3 ACCURACY:

50 percent solution preparation (with respect to the concentration of the target assay):
In a clean and dry volumetric flask, accurately weigh and transfer 15 mg of Pioglitazone and 5 mg of Rosiglitazone, add diluents, and sonicate to dissolve entirely and bring the diluents level up to the volumetric flask.

When the volumetric flask has been filled to the desired level, pipette 5 ml of the aforesaid solution and dilute it with diluents to the desired concentration. A combination of Pioglitazone (15ppm) and Rosiglitazone (5ppm) is prescribed.

100 percent solution preparation (with respect to target assay concentration):
Add 30 mg of Pioglitazone and 10 mg of Rosiglitazone to a clean and dry volumetric flask, then sonicate to dissolve thoroughly and bring the diluent level up with the addition of diluents, if needed.

In a 50ml volumetric flask, pipette 5ml of the aforementioned solution and dilute it with diluents to the desired volume. A combination of 30ppm Pioglitazone and 10ppm Rosiglitazone was used.

150 percent solution preparation (with respect to target assay concentration):
In a clean and dry volumetric flask, accurately weigh and transfer 45 mg of Pioglitazone and 15 mg of Rosiglitazone, add diluents and sonicate to dissolve entirely and make up to the diluents level.

Take 5ml of the aforesaid stock solution and dilute it to your desired concentration in a 50ml volumetric flask using diluents. There are 45 ppm of Pioglitazone and 15 ppm of Rosiglitazone.

Procedure:
Inject the standard solution, 50 percent accuracy, 100 percent accuracy, 150 percent accuracy solutions.

Acceptance Criteria:
The rate of recovery for each stage should be between 98-102 percent.

Table 5.04 gives accuracy results and **figures** from **5.25-5.27** gives accuracy chromatograms.

S. No.	% Level	Pioglitazone % Recovery	Rosiglitazone % Recovery
1	50	99.63	99.56
2	100	99.78	99.23
3	150	98.69	100.45

Table 5.04: Results of Pioglitazone and Rosiglitazone in terms of accuracy

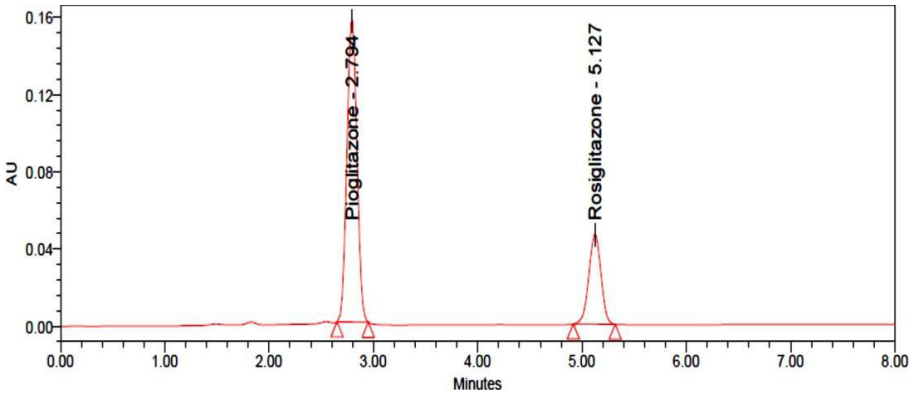

Figure 5.25: 50 percent accuracy chromatogram

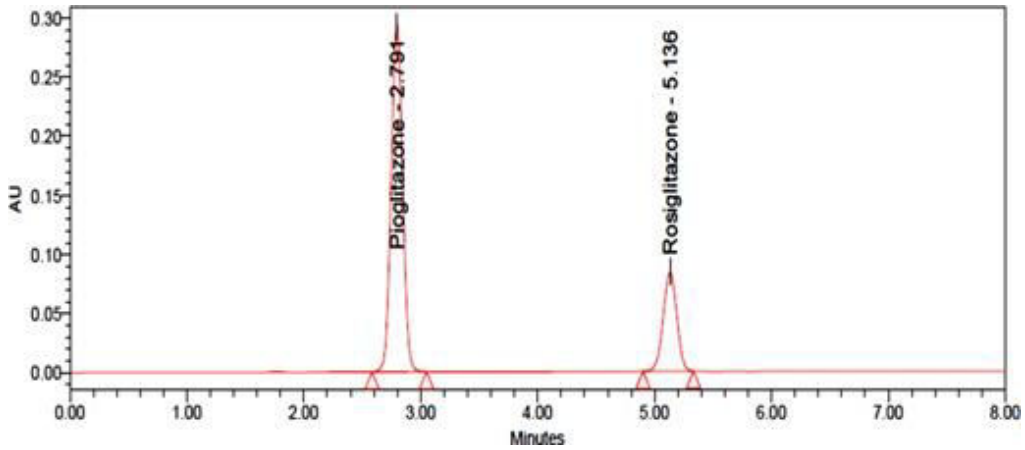

Figure 5.26: 100 percent accuracy chromatogram

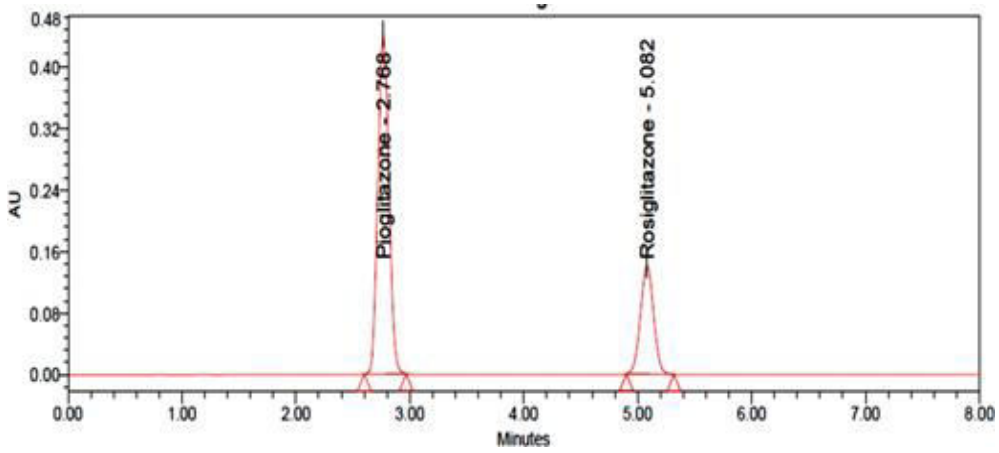

Figure 5.27: 150 percent accuracy chromatogram

5.7.4 Precision:

In an analytical procedure, the rate at which repeated homogenous samplings provide similar results is a measure of accuracy. Pioglitazone (30ppm) and Rosiglitazone (10ppm) were spiked into six different injection determinations for testing accuracy. **Table 5.05** gives suitability results and **figures** from **5.28-5.33** gives suitability chromatograms.

S.NO	Conc. of Pioglitazone (µg/ml)	Area of Pioglitazone	Conc. of Rosiglitazone (µg/ml)	Area of Rosiglitazone
1	30	3620286	10	1415540
2	30	3613277	10	1425272
3	30	3620624	10	1408471
4	30	3658056	10	1443280
5	30	3611947	10	1438098
6	30	3638119	10	1427283
Mean	3627052		1426324	
Std dev	17825.47		13139.13	
% RSD	0.491		0.921	

Table 5.05: Results of system precision

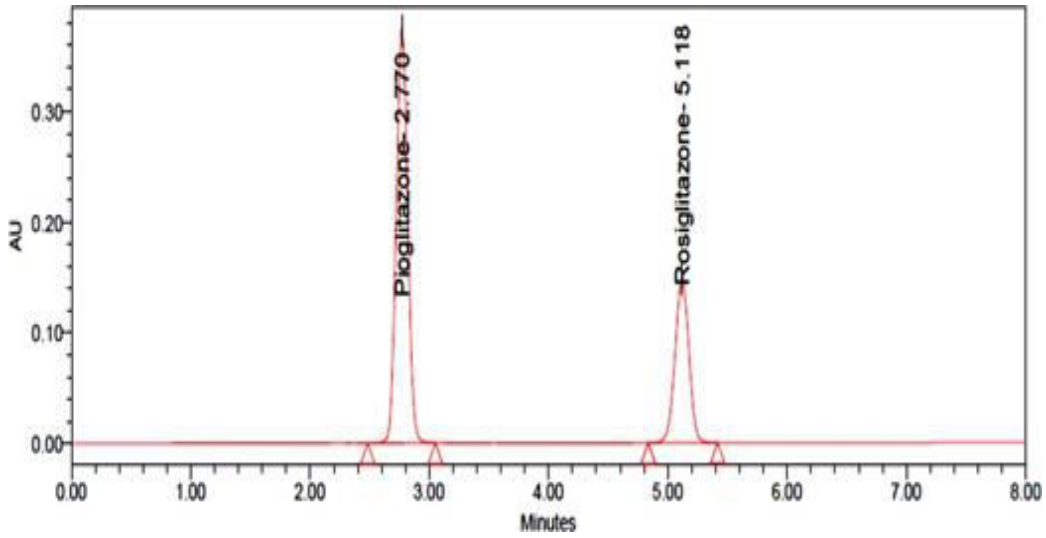

Figure 5.28: Chromatogram of system precision-1

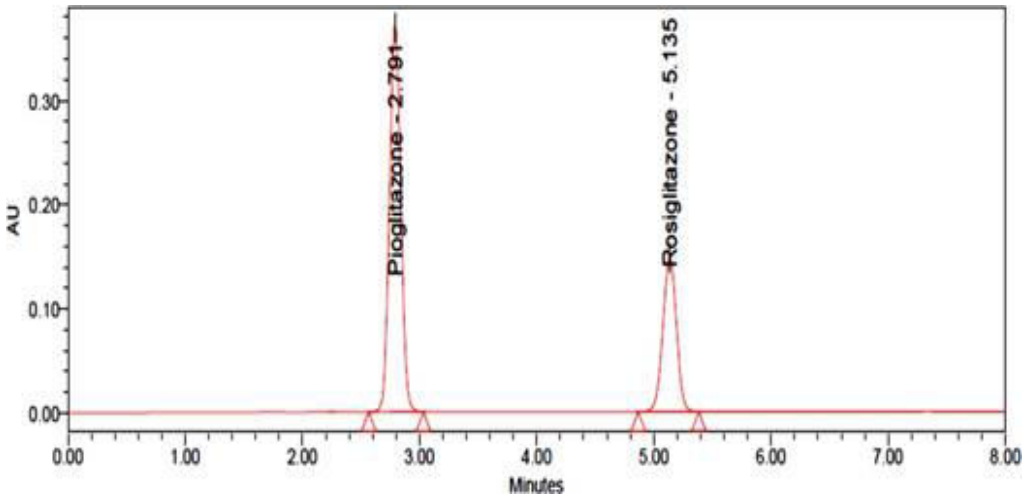

Figure 5.29: Chromatogram of system precision-2

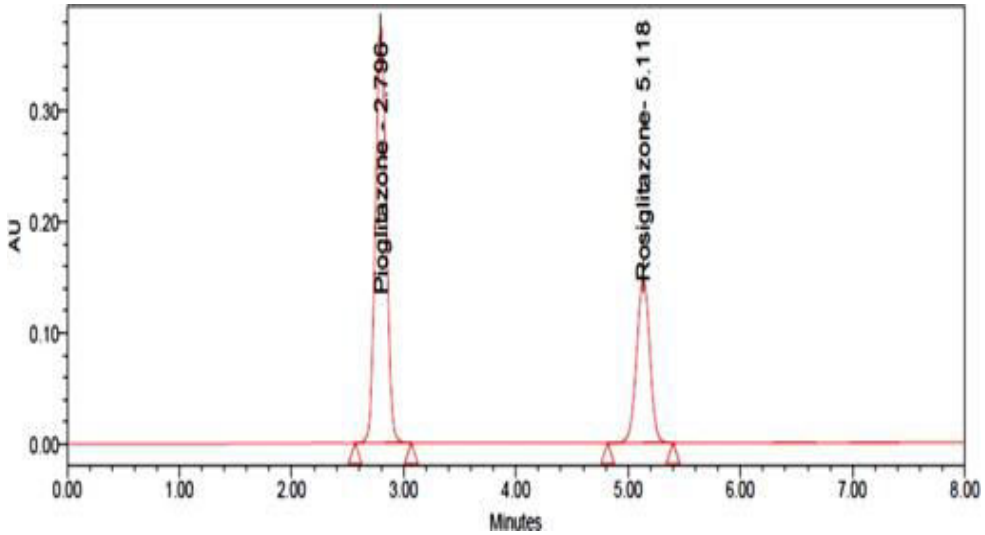

Figure 5.30: Chromatogram of system precision-3

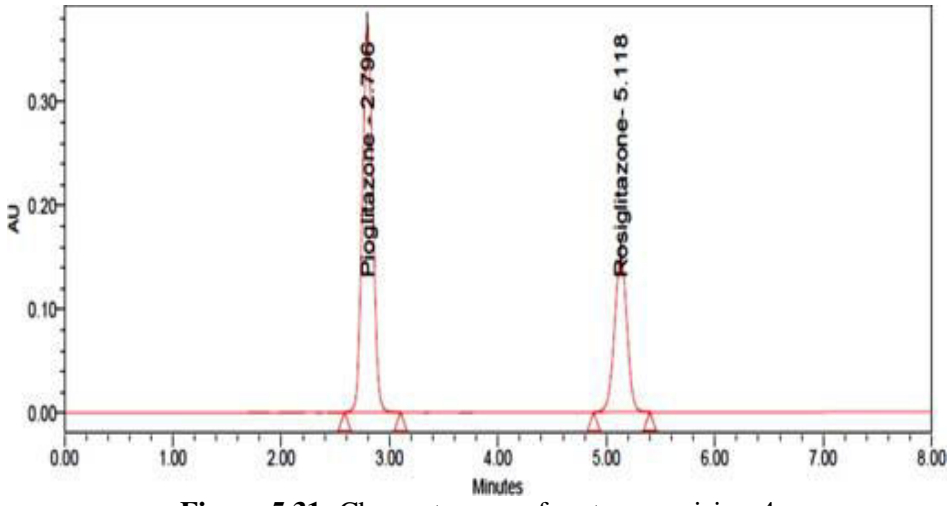

Figure 5.31: Chromatogram of system precision-4

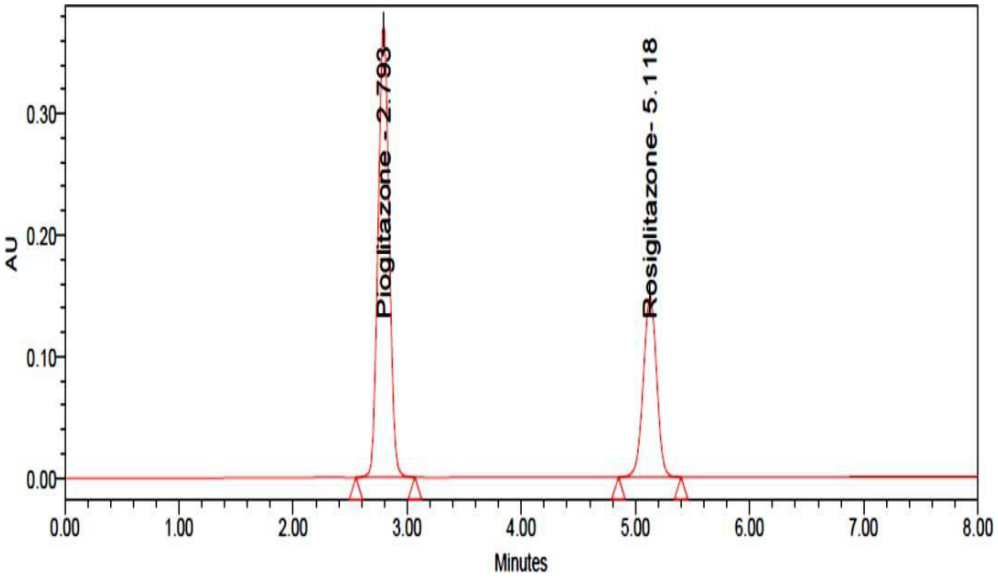

Figure 5.32: Chromatogram of system precision-5

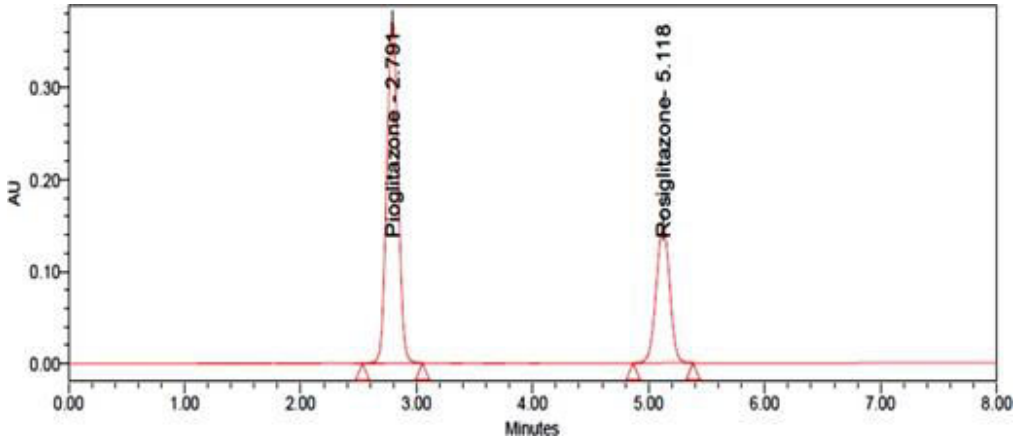

Figure 5.33: Chromatogram of system precision-6 Method precision

[Table 5.06 and Fig 5.34]

S.NO	Pioglitazone area	Rosiglitazone area
1	3652487	1436527
2	3625148	1442359
3	3695284	1425863
4	3687452	1476324
5	3602541	1452638
6	3636326	1475842
Mean	3649873	1451592
Std dev	36100.79	20856.71
% RSD	0.99	1.44

Table 5.06: Results of method precision

Acceptance Criteria: There should be no more than 2% RSD in the area six standard injection results.

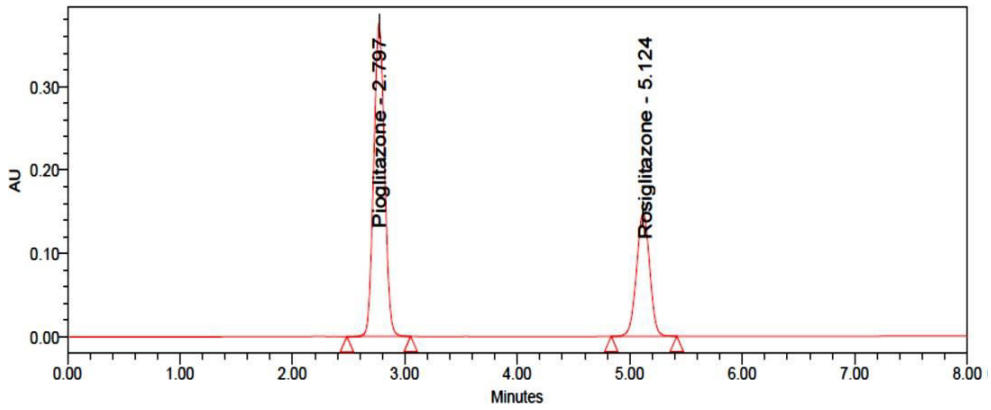

Figure 5.34: Chromatogram of method precision Intermediate precision (Day-Day precision)

[Table 5.07 and Fig 5.35]

S. No.	Pioglitazone			Rosiglitazone		
	Conc. (µg/ml)	Area	% Assay	Conc. (µg/ml)	Area	% Assay
1	30	3652415	100.02	10	1475821	99.68
2		3626352	100.14		1462345	99.23
3		3642157	100.23		1478549	100.14
4		3639568	99.78		1478562	100.42
5		3675423	100.43		1436521	99.15
6		3621232	100.05		1432658	99.36
%CV	0.54			1.45		

Table 5.07: Results of intermediate precision

Acceptance criteria: There should be no more than 2% RSD in the six normal injection outcomes.

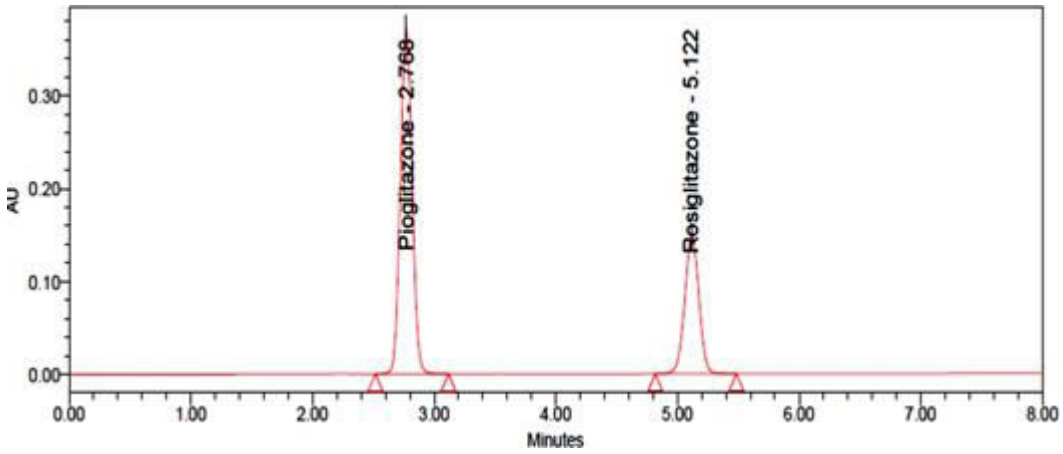

Figure 5.35: Chromatogram of intermediate precision

5.7.5 Limit of detection (LOD) and limit of quantification (LOQ):

Calibration curves were used to calculate LOD and LOQ. The compound's LOD and LOQ were determined by injecting decreasing quantities of the standard solution through the RP-HPLC technique that was developed. For Pioglitazone and Rosiglitazone, the following table shows the LOD and the LOQ concentrations and their s/n ratios. **Table 5.08** gives sensitivity results and **figure 5.36 and 5.37** gives LOD and LOQ chromatograms.

Pioglitazone				**Rosiglitazone**			
LOD		**LOQ**		**LOD**		**LOQ**	
Conc. (µg/ml)	s/n	Conc. (µg/ml)	s/n	Conc. (µg/ml)	s/n	Conc. (µg/ml)	s/n
0.038	6	0.124	26	0.013	3	0.041	24

Table 5.08: Sensitivity parameter values

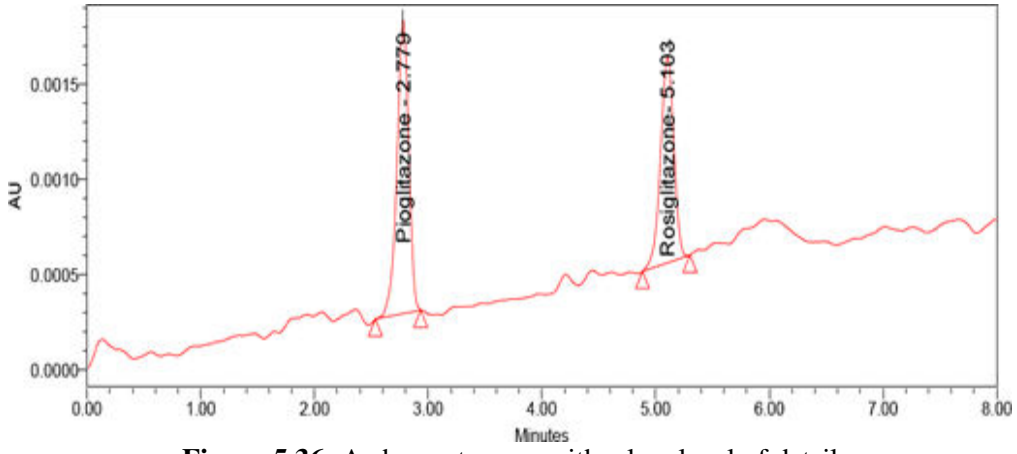

Figure 5.36: A chromatogram with a low level of detail

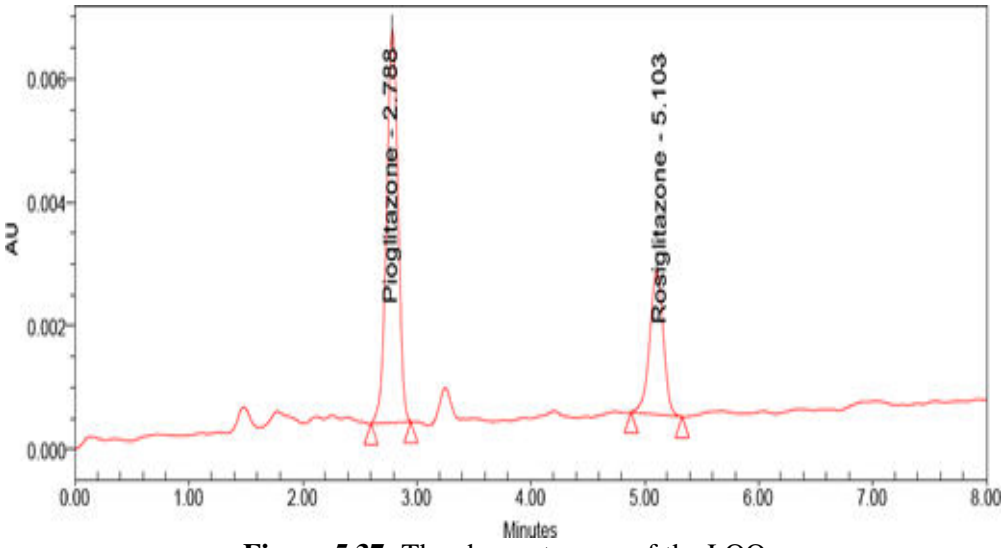

Figure 5.37: The chromatogram of the LOQ

5.7.6 Robustness:

Tests were carried out on a variety of variables that were purposely adjusted, such as the flow rate and the amount of organic material in the mobile phase. The separation of active pharmaceutical substances from contaminants was not considerably altered, and the retention duration, plate count, and tailing factor were not changed either. Thus, this strategy proved to be dependable. **Table 5.09, 5.10** gives robustness results. **Figures** from **5.38-5.41**gives robustness chromatograms.

Parameter	Pioglitazone					
	Condition	RT (min)	Peak area	Resolution	Tailing	Plate count
Flow rate	Less flow (0.8 ml/min)	3.445	3946127		1.06	5173
	More flow (1.2 ml/min)	2.312	3253840		1.03	5730
Organic phase	Less organic (54:46)	3.252	3710539		1.05	4834
	More organic (66:34)	2.463	3431928		1.06	5037

Table 5.09: Test results for Pioglitazone's robustness

Parameter	Rosiglitazone					
	Condition	RT (min)	Peak area	Resolution	Tailing	Plate count
Flow rate	Less flow (0.8ml /min)	6.359	1862417	13.44	1.00	11264
	More flow (1.2 ml/min)	4.235	1187014	10.17	1.04	7153
Organic phase	Less organic (54:46)	7.379	1544863	17.96	0.98	7713
	More organic (66:34)	3.856	1215486	7.43	1.02	6446

Table 5.10: For Rosiglitazone, the robustness findings show

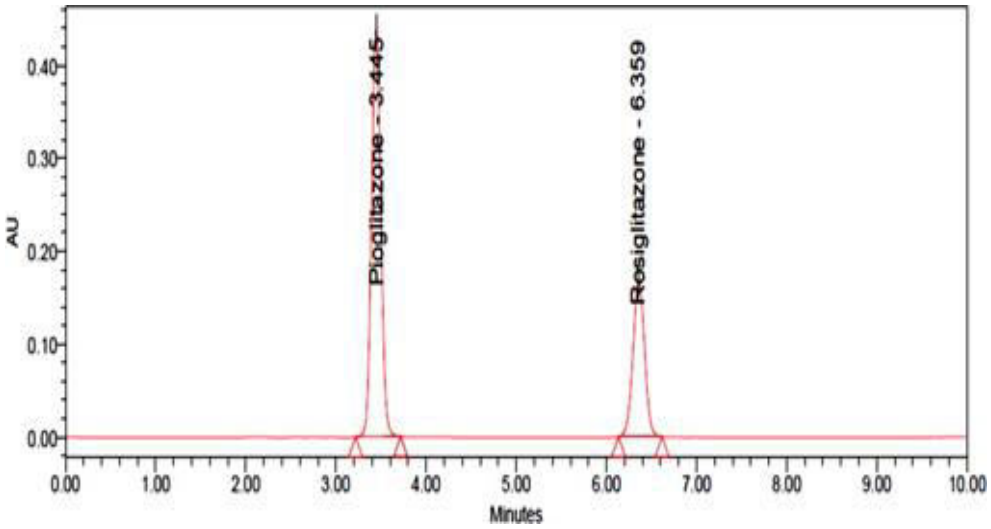

Figure 5.38: Chromatograms with a lower flow rate (0.8ml/min)

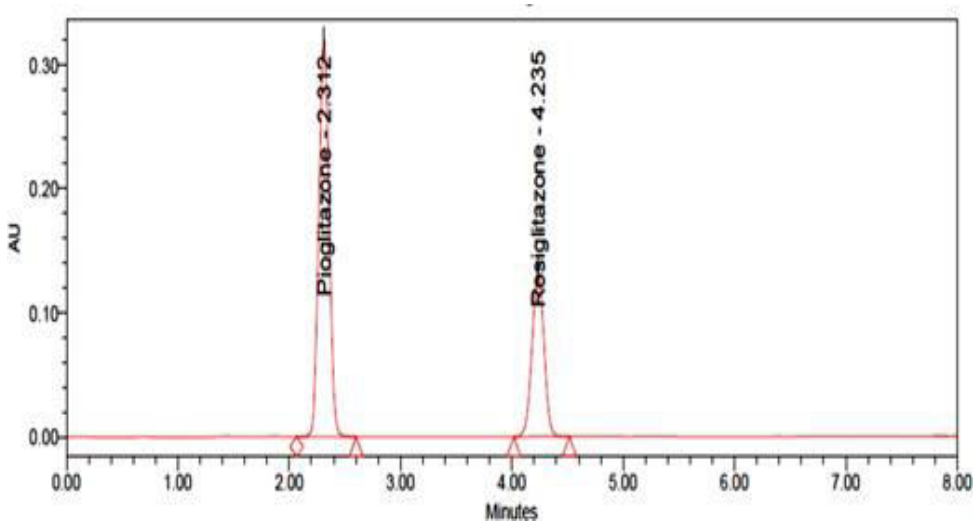

Figure 5.39: Chromatograms with a higher flow rate (1.2ml/min)

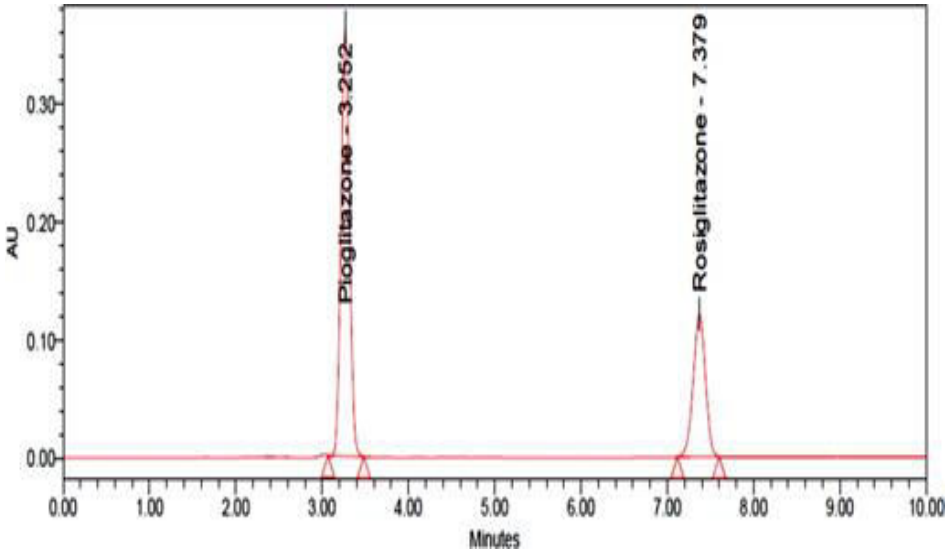

Figure 5.40: Chromatograms with a higher flow rate (54:46)

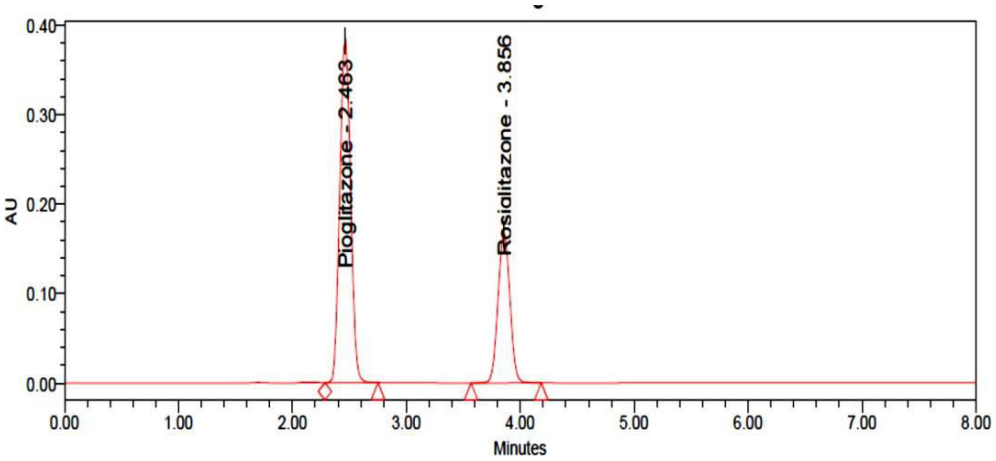

Figure 5.41: A more organic chromatogram (66:34)

5.7.7 Forced Degradation Studies:

To partly breakdown the molecule, the Pioglitazone and Rosiglitazone samples were each submitted to a separate set of forced degradation conditions. Experiments with degrading materials have shown that the technique is suitable for use. For this reason, the research includes information on what conditions could lead to instability in order to guarantee the formulation process is always safe.

Stock Solution Preparation:

It is necessary to precisely weigh out 30 mg Pioglitazone and 10 mg Rosiglitazone, transfer them to a 100 ml volumetric flask, add 70 ml diluent, then sonicate the mixture for 30 minutes to dissolve the diluents and build up the diluent mark.

Acid Degradation:

For 15 minutes, add 1 ml of 1N Hcl to 1 ml of the sample stock solution in a volumetric flask with a capacity of 10 ml. Add 1ml of 1N NaOH after 15 minutes and dilute to the desired strength.

Alkali Degradation:

For 15 minutes, 1 ml of sample stock solution is transferred to a 10 ml volumetric flask and 1 millilitre of 1N NaOH is added. After 15 minutes, add 1 ml of 1N Hcl and diluents to get the solution to the desired volume.

Peroxide Degradation:

Transferring 1ml of the sample stock solution to a 10ml volumetric flask, a 30 percent hydrogen peroxide solution was added, and the volume was brought up to capacity using various diluents.

Reduction Degradation:

It was transferred to a 10-ml volumetric flask, where it was diluted with 30 percent sodium bi sulphate solution and brought up to the diluents mark.

Thermal Degradation:

In the oven for six hours, the sample solution was kept at 105oC. HPLC was used to purify the final product.

Hydrolysis Degradation:

Sample stock was transferred to a volumetric flask of 10ml and diluted with 1ml of water to get the desired amount of water dilution.

Table 5.11 gives FD results and **figures** from **5.42-5.48** gives FD chromatograms.

Degradation condition	Pioglitazone		Rosiglitazone	
	% Assay	% deg	% Assay	% deg
Acid deg	85.1	14.9	85.1	14.9
Alkali deg	84.8	15.2	85.7	14.3
Peroxide deg	86.4	13.6	87.1	12.9
Reduction deg	87.5	12.5	88.8	11.2
Thermal deg	88.6	11.4	89.9	10.1
Hydrolysis deg	89	11	88.2	11.8

Table 5.11: As a consequence of forced deterioration

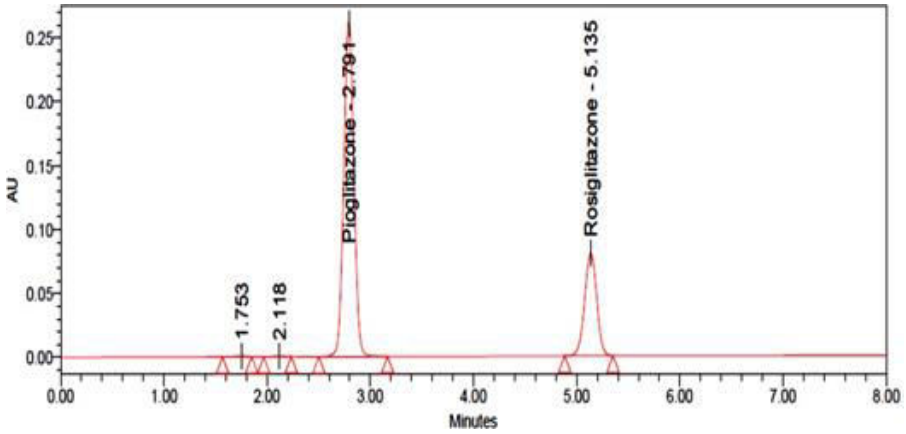

Figure 5.42: Analysis of the acid degradation chromatogram

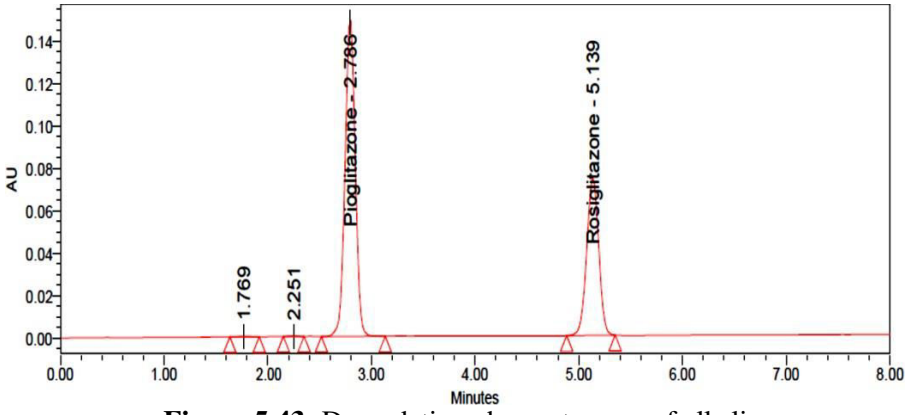

Figure 5.43: Degradation chromatogram of alkali

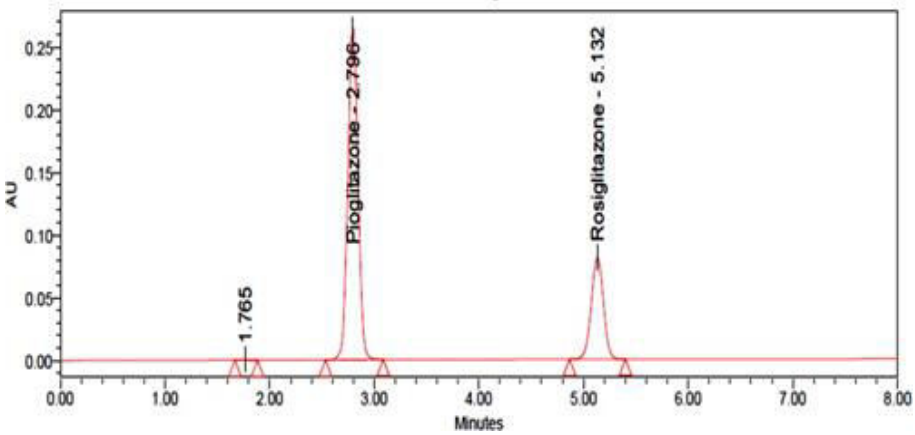

Figure 5.44: Chromatogram of peroxide degradation

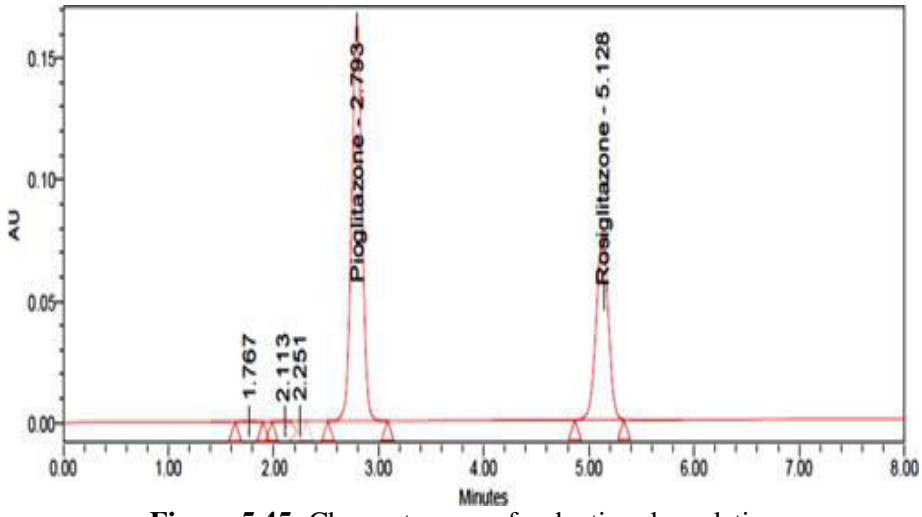

Figure 5.45: Chromatogram of reduction degradation

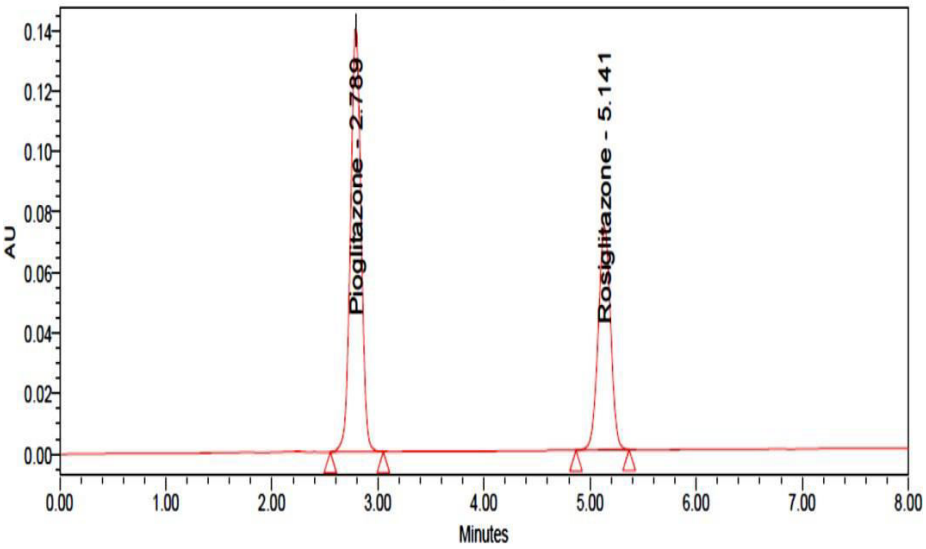

Figure 5.46: Chromatogram of Hydrolysis degradation

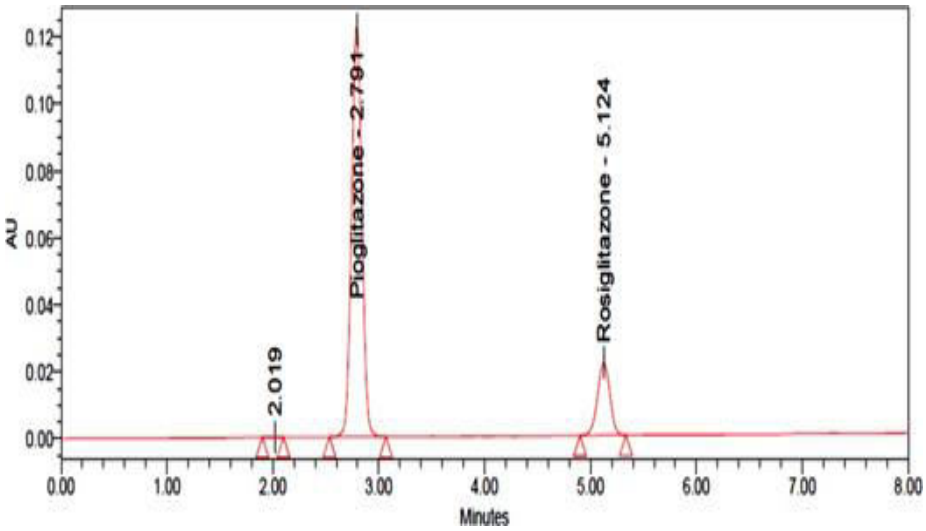

Figure 5.47: Chromatogram of Thermal degradation

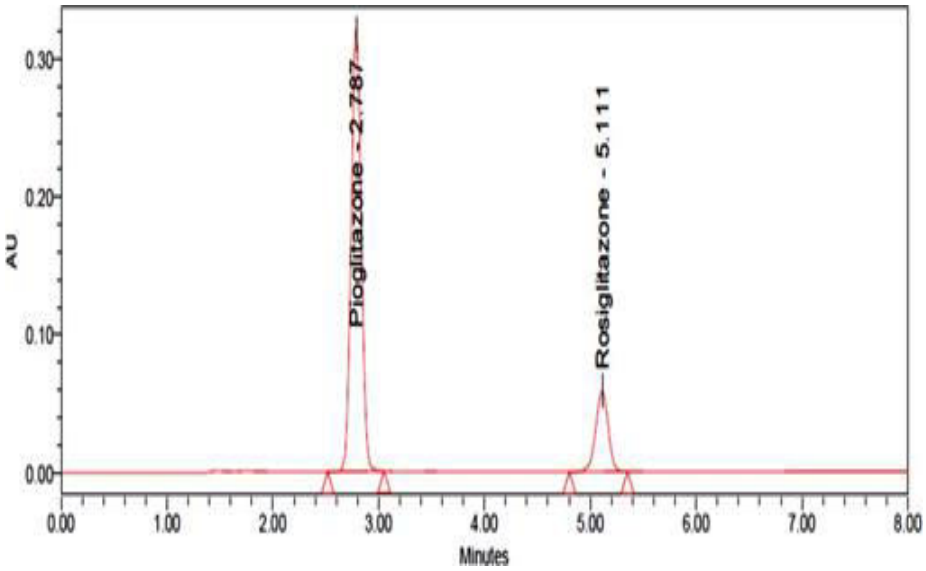

Figure 5.48: Chromatogram of control degradation

5.7.8 Stability [Table 5.12 and Fig 5.49-5.53]

Standard and experimental solutions were stored at 2-8°C and room temperature, respectively, for a total of 24 hours. In order to determine the percentage of divergence from initial to 24 hours, these solutions were injected into the apparatus. During the study, no significant deviations were found, confirming that solutions were stable for at least 24 hours.

	Pioglitazone		Rosiglitazone	
Stability	**Purity**	**% of deviation**	**Purity**	**% of deviation**
Initial	100	0.00	100	0.00
6 Hrs	99.8	-0.20	99.6	-0.40
12 Hrs	99.5	-0.50	99.3	-0.70
18 Hrs	99.3	-0.70	99	-1.00
24 Hrs	99	-1.00	98.8	-1.20

Table no 5.12: Results of stability

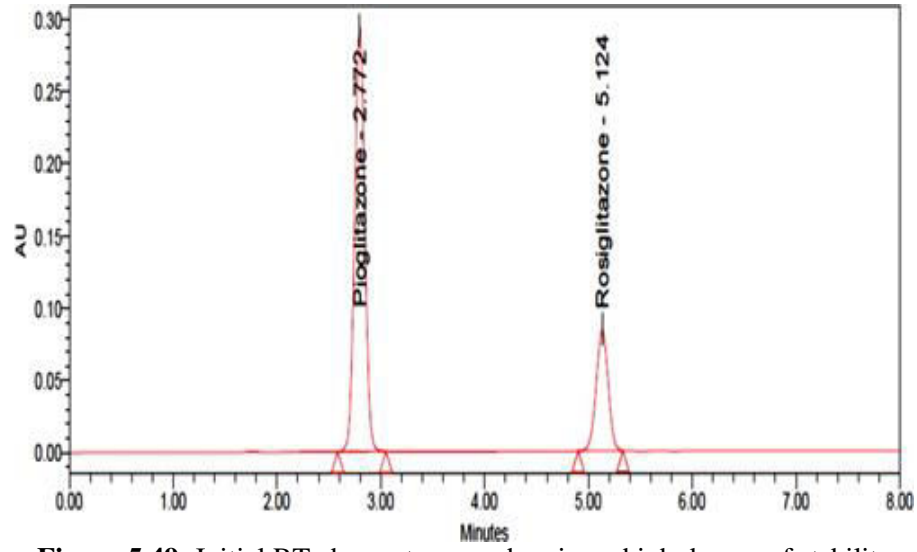

Figure 5.49: Initial RT chromatogram showing a high degree of stability

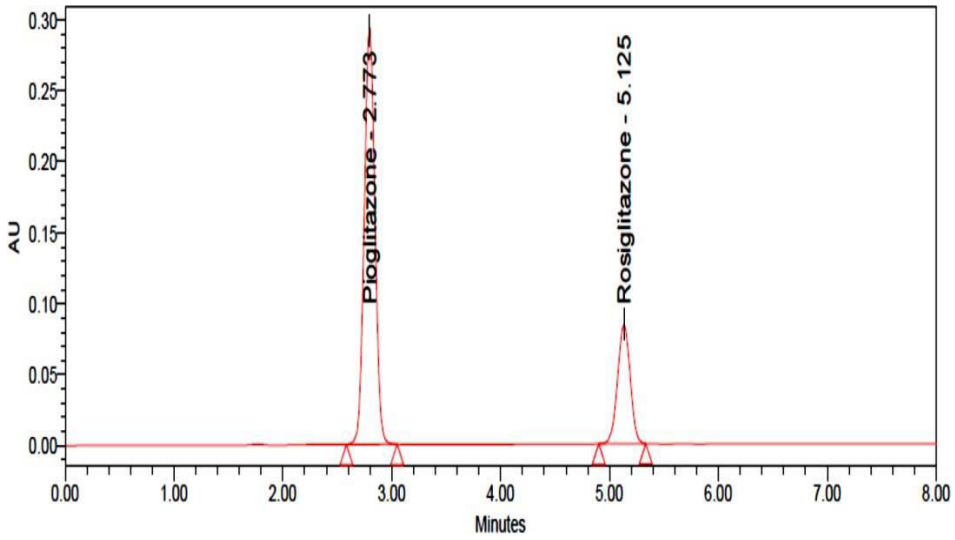

Figure 5.50: Six-hour-long RT stability chromatogram

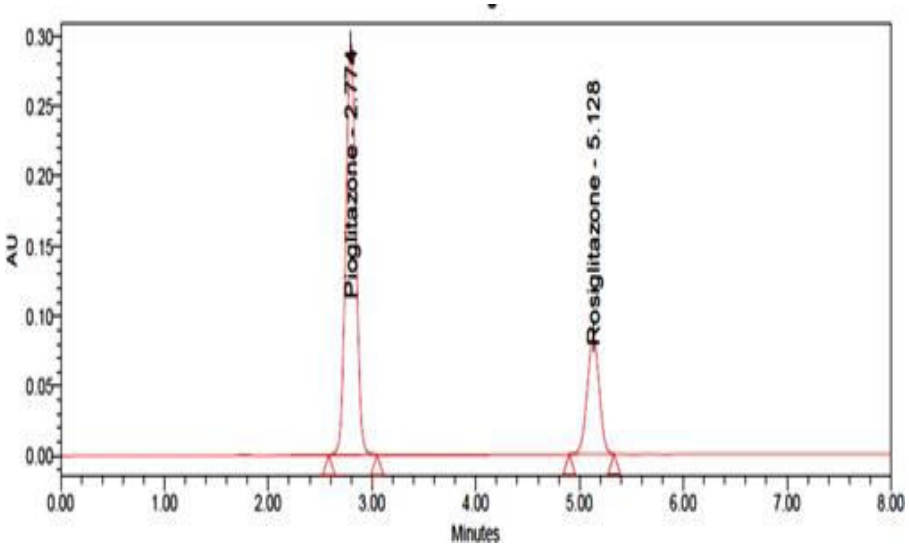

Figure 5.51: Stability chromatogram after 12 hours of RT

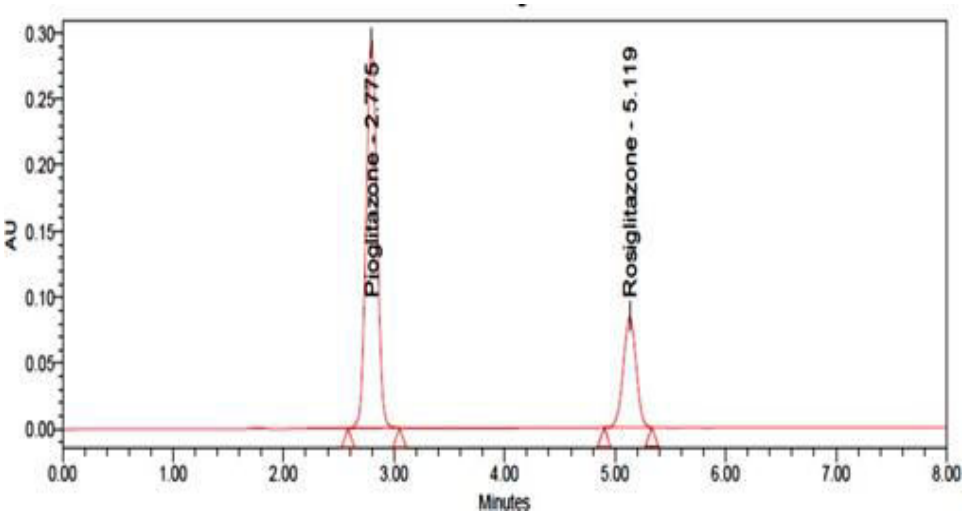

Figure 5.52: 18-Hour Stability Chromatogram

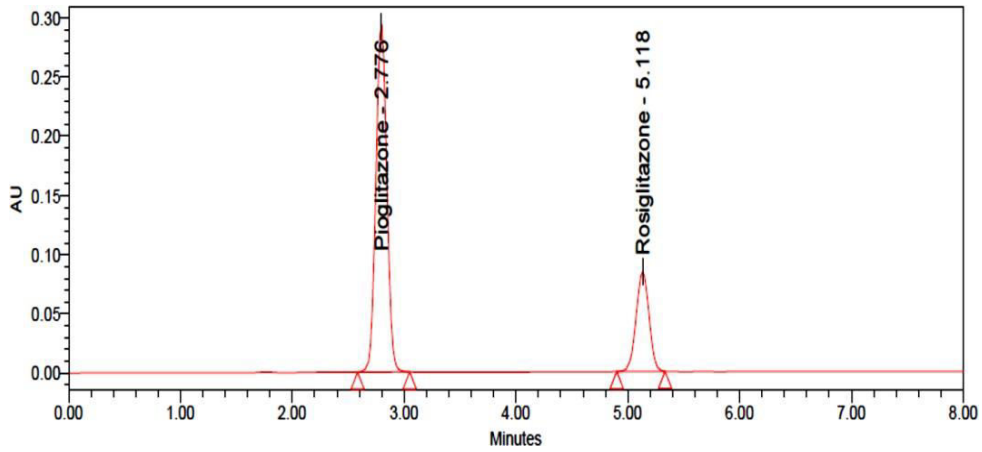

Figure 5.53: Stability chromatogram after 24 hours of RT

5.8CONCLUSION

In this procedure, Pioglitazone and Rosiglitazone were quantified according to ICH recommendations in bulk and pharmaceutical formulations. Accuracy, precision, linearity and reliability were discovered in the developed method. Simple sample preparation and good repeatability results make this method a good choice. It is possible to conduct regular drug research using the improved chromatographic approach.

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6.

NEW VALIDATED METHOD FOR THE ESTIMATION OF OLANZAPINE AND SAMIDORPHAN USING HPLC AND STUDY OF ITS DEGRADATION

6.1 OLANZAPINE

6.1.1Drug Profile of Olanzapine

Olanzapine, sold under the trade name **Zyprexa** among others, is an atypical antipsychotic (**1, 2**) primarily used to treat schizophrenia (**3, 4**) and bipolar disorder. For schizophrenia, it can be used for both new-onset disease and long-term maintenance. It is taken by mouth or by injection into a muscle. Common side effects include weight gain, movement disorders (**5**), dizziness, feeling tired, constipation, and dry mouth. Other side effects include low blood pressure (**6**) with standing, allergic reactions, neuroleptic malignant syndrome (**7, 8**), high blood sugar, seizures (**9**), gynecomastia (**10**), erectile dysfunction (**11**), and tardive dyskinesia (**12**). In older people with dementia, its use increases the risk of death. Use in the later part of pregnancy may result in a movement disorder in the baby for some time after birth. Although how it works is not entirely clear, it blocks dopamine and serotonin receptors (**13**).

Structure of Olanzapine [Fig 6.01]

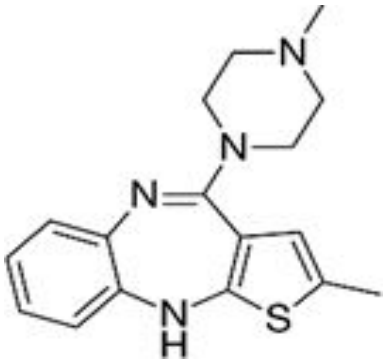

Figure 6.01: Olanzapine's chemical structure

6.1.2Name of the IUPAC:

2-Methyl-4-(4-methyl-1-piperazinyl)-10H-thieno[2,3-b][1,5]benzodiazepine

6.1.3Molecularformula: $C_{17}H_{20}N_4S$

6.1.4Molecularweight: 312.44 $g \cdot mol^{-1}$

6.1.5Category

Atypical antipsychotics, of which olanzapine is a member, are a class of medicine. It operates by altering the brain's normal chemical activity.

6.1.6Mechanism of Action

A number of different neuronal receptors are antagonised by the antagonism of olanzapine, including dopamine D1, D2 and the alpha-1 adrenergic and histamine H1 receptors. The olanzapine antagonism of these receptors results in the drug's action. The antagonistic impact of olanzapine on the dopamine D2 receptor in the mesolimbic pathway is critical because it prevents dopamine from acting at the post-synaptic receptor, as previously discussed. The D2 receptor binding of olanzapine is readily dissociable, allowing for some dopamine neurotransmission. But olanzapine has been shown to function in the frontal cortex's serotonin 5HT2A receptors in a way that is comparable to how dopamine D2 receptors have been affected by the drug. Allows for a reduction in the number of side effects.

6.1.7 Side effects of Olanzapine:

- Dizziness, feeling unsteady, or having trouble keeping your balance.
- Restlessness.
- Unusual behavior.
- Depression.
- Difficulty falling asleep or staying asleep.
- Weakness.
- Difficulty walking.
- Constipation.
- Sinus infection.
- Fracture of bone.
- Sore throat (pharyngitis)

6.1.8Contraindications:

- Breast cancer.
- Diabetes.
- A high prolactin level.
- High cholesterol.
- High amount of triglyceride in the blood.
- Excessive fat in the blood.
- Dehydration.
- Overweight.

6.1.9Absorption

In the 5 to 8 hours after oral dosing, peak plasma concentrations of Olanzapine may be expected. Food has no effect on the absorption. Unlike intravenous administration, oral bioavailability has not been established.

6.1.10 Uses

Certain mental/emotional disorders are addressed using the drug olanzapine (such as schizophrenia, bipolar disorder). It may also be used in conjunction with other antidepressant medications to treat depression as well. This medicine may help you feel less irritated, think more clearly and positively about yourself, and participate more actively in daily life by reducing your hallucinations. As an atypical antipsychotic, Olanzapine falls within the category. It works by assisting in the reestablishment of a healthy equilibrium in the brain between various natural components. Consult your physician about the potential downsides and upsides of any proposed therapy (especially when used by teenagers). Also check out the Precautions page.

6.1.11 Adult dose:

- 2.5mg

- 5mg
- 7.5mg
- 10mg
- 15mg
- 20mg

6.2SAMIDORPHAN

6.2.1Drug Profile of Samidorphan:

Samidorphan (INN, USAN) (developmental code names ALKS-33, RDC-0313), also known as 3-carboxamido-4-hydroxynaltrexone (**14**) is an opioid antagonist (**15**) that preferentially acts as an antagonist of the μ-opioid receptor (**16**) (MOR). It is under development by Alkermes for the treatment of major depressive disorder and possibly other psychiatric conditions. Samidorphan has been investigated for the treatment of alcoholism and cocaine addiction by its developer, Alkermes (**17**), showing similar efficacy to naltrexone (**18**) but possibly with reduced side effects. However, it has attracted much more attention as part of the combination product ALKS- 5461 (buprenorphine/samidorphan) (**19**), where samidorphan is combined with the mixed MOR weak partial agonist and κ-opioid receptor (KOR) (**20**) antagonist buprenorphine, as an antidepressant (**21**). Buprenorphine has shown antidepressant effects in some human studies, thought to be because of its antagonist effects at the KOR, but has not been further developed for this application because of its MOR agonist effects and consequent abuse potential. By combining buprenorphine with samidorphan to block the MOR agonist effects, the combination acts more like a selective KOR antagonist, and produces only antidepressant effects, without typical MOR effects such as euphoria or substance dependence being evident. Samidorphan is also being studied in combination with olanzapine, as ALKS-3831 (olanzapine /samidorphan), for use in schizophrenia (**22, 23**). A Phase 3 study found that the addition of samidorphan to olanzapine significantly reduced weight gain compared to olanzapine alone (**24**). The combination is now under review for approval by the US Food and Drug Administration.

Structure of Samidorphan [Fig 6.02]

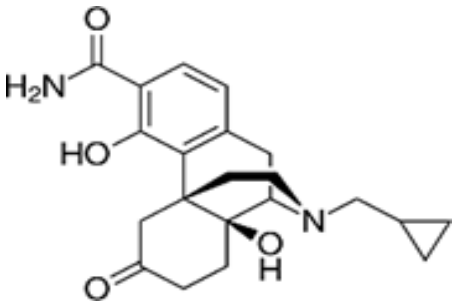

Figure 6.02: Samidorphan's chemical structure

6.2.2Name of the IUPAC: (1R,9R,10S)-17-(cyclopropylmethyl)-3,10-dihydroxy-13- oxo-17-azatetracyclo[7.5.3.$0^{1,10}$.$0^{2,7}$]heptadeca-2(7),3,5-triene-4-carboxamide

6.2.3Formula molecular: $C_{21}H_{26}N_2O_4$

6.2.4Molecular weight: 370.449 $g \cdot mol^{-1}$

6.2.5Category: Analgesics, Opioid Partial Agonists, buprenorphine and samidorphan (Pending FDA Approval)

6.2.6Mechanism of Action

As an opioid receptor modulator, samidorphan has been shown to be effective in both vitro and in vivo studies. 1,11 For example, samidorphan has been shown to have strong affinity for the receptors of all three classes of opioids (adrenergic, norepinephrine, and codeine) in vitro. 2,3,4,11 It works as an antagonist at both the - opioid receptor and the -opioide receptor in vitro when it signals via Gi proteins, as well as a partial agonist when it signals through GoA-GoB-Gz proteins. Samidorphan also operates as both an antagonist and agonist at the -opioid receptor. 2 Both the main N- dealkylated metabolites, as well as the major N-oxide metabolites, bind to the receptors for the three major opioids (Ki values of 0.26 for agonists, 23 for antagonists), with the former acting as an antagonist for the receptors and the latter acting as an agonist for them. 11 Samidorphan, in general, is predominantly an antagonist for -opioid receptors in vivo.

6.2.7Side effects of Samidorphan:

Samidorphan's in vitro profile suggests that it may cause somnolence, drowsiness, dizziness, and hallucinations in some people at the levels studied in human studies, in line with its in vitro profile.

6.2.8Contraindications:

Hypersensitivity, Type 1 diabetes, diabetic ketoacidosis, and a hyperosmolar hyperglycemic condition are all contraindications to the use of samidorphan.

6.2.9Absorption

The -opioid receptor antagonist samidorphan (SAM) is a brand-new compound on the market. In healthy individuals, we examined the effects of food and age on the oral pharmacokinetics (PK) of SAM after various delivery techniques.

6.2.10 Uses:

Adults with bipolar I disorder may benefit from the combination of samidorphan and olanzapine for the acute treatment of manic and mixed episodes, or as maintenance therapy, as well as for schizophrenia.

6.2.11 Adult dose:

Olanzapine 10 mg/samidorphan 10 mg PO qDay

6.3LITERATURE RESURVEY

Shanmuga Pandiyan P, Saibaba SV, Method validation and development of RP-HPLC method for the determination of Olanzapine in bulk and tablet dosage form: Objective: To design and test a reverse phase-high performance liquid chromatographic technique for the determination of olanzapine in bulk and tablet dosage form.

Methods: XTerra C18 (150—3.5 mm inner diameter, 5 Î14m) was used for chromatographic analysis with a flow rate of 0.6 ml/minutes and a buffer (potassium dihydrogen phosphate) and methanol (45:55 percent v/v) as the mobile phase. The measurement was made at a wavelength of 247 nm.

Results: The olanzapine calibration curve was linear between 30 and 70 Î14g/ml. The commercial formulation was found to have a mean percent assay of 100.2 percent, and the percent recovery ranged from 98 to 102 percent. The precision research revealed a 2% relative standard deviation.

Conclusion: The devised approach is simple, exact, and quick, therefore it may be used to estimate olanzapine dose in bulk and tablet form.

Noely Camila Tavares Cavalcanti Bedor [1], Carlos Eduardo Miranda de Sousa, Danilo César Galindo Bedor, Daniel da Mota Castelo Branco, Felipe Nunes Bonifácio, Davi Pereira de Santana, Leila Bastos Leal, The development and validation of a method for quantifying olanzapine in human plasma by liquid chromatography tandem mass spectrometry and its application in a pharmacokinetic study: 1. Liquid chromatography tandem mass spectrometry tandem mass spectrometry was used to develop and test a fast technique for quantifying olanzapine (OLZ) in human plasma. There was an internal standard (IS) of Venlafaxine utilised in this study, which was prepared by extracting liquid-liquid samples from 400 millilitres of human plasma. 2. 5-micron-pore acetonitrile C18 (ACE C18) was used for the chromatography. In isocratic mode, water and acetonitrile (50:50 v/v) were used as solvents A and B, respectively, with 0.1 percent formic acid. One millilitre per minute was the flow rate. The OLZ and IS had retention times of 0.78 and 1.04 minutes, respectively. Both the OLZ and the IS were detected using tandem mass spectrometry in positive electrospray ionisation mode with multiple reaction monitoring. 3. OLZ and IS retention periods were not affected by the matrix, and the mean recovery of OLZ was 90%. In the concentration range of 1-20 ng/mL, the assay was linear (R(2) = 0.9976). This study found that the accuracy was 1.66 percent for both intra-day and inter-day precision. 4. A pharmacokinetic research in which healthy participants were given 10- mg OLZ tablets and their plasma OLZ levels were tracked over time was effectively applied to this validated approach. Ninety percent of the confidence intervals were within the regulatory authorities' suggested range of 80-125% for the OLZ test and reference medicine (Zyprexa(®)).

Fahad Pervaiz*, Muhammad Usman Minhas, Mahmood Ahmad and Muhammad Sohail, Development and Validation of Reverse Phase High Performance Chromatography Method for Determination of Olanzapine in Microsample Rat Plasma: Application to Preclinical Pharmacokinetic Study: Purpose: To develop a sensitive and reliable RP-HPLC technique for the measurement of olanzapine in micro-samples of rat plasma utilising UV detection. Methods: Overnight fasting rats (n = 6) were given a single oral dose of olanzapine (7 mg/kg). Dichloromethane and n-hexane were used in a liquid-liquid extraction to remove the drug from rat plasma samples (80:20). For chromatographic separation, C18 hypersil- BDS reverse phase chromatographic column C18 hypersil-BDS was employed, and the mobile phase consisted of a 50:30:20 acetonitrile to methanol solution (50:30:20, methanol:acetonitrile) that was flown at 1.2 ml/min. Using a 214 nm UV detector, olanzapine's concentration was determined. The method's precision and accuracy have been verified. Endogenous interference had no effect on the separation of the chemicals of interest. A coefficient of regression (r2) of 0.9986 was reported for the concentration range of 1 - 500 ng/ml in rat plasma. Solid phase extraction had a similar recovery rate to liquid-liquid extraction. It took 5.0 and 13.4 minutes for olanzapine to be retained in the system and 13.4 minutes for the internal standard (fluoxetine). While inter-day and intra-day precision were both 12.5%, the lowest limit of quantification (LLOQ) was set at 1 ng/ml. The method's accuracy ranged from 94% to 105%, and there was no significant difference in findings between two analysts (p = 0.626). Olanzapine's mean Cmax was 412.7 ng/ml, tmax was 1 h, and t1/2 was 2.54 h, all while the drug's half-life was 2.54 h. For precision and accuracy that are within the FDA's guidelines for bioanalytical assay validation, the proposed technique has been tested and found to be accurate and reliable. Olanzapine preclinical pharmacokinetics in rats were effectively assessed using this approach.

Nidhi Jain, Ankur Joshi, Sapna Malviya ,Narendra Vyas, Anil Kharia, Development and validation of RP-HPLC method for the estimation of Olanzapine in marketed formulation: For the analysis of Olanzapine in commercialised formulation, a simple, accurate, precise, and quick RP-HPLC technique was designed and validated. For this experiment, we used an Acetonitrile/Potassium Dihydrogen Phosphate Buffer (pH 6): Acetonitrile (60:40) (v/v) flow rate, and UV detection at 258 nm with a constant room temperature as the stationary phase. 20 l

injection volume and 5 min chromatographic duration were both used for this experiment. The correlation coefficient for the proposed approach was found to be 0.998 and linear for concentrations ranging from 5 to 25 Î14g/ml. Recovery research evaluated the validity and reliability of the suggested technique. There was a range of 99.58 percent - 100.50 percent recovery of the additional requirements (80%, 100%, and 120%). Temperature, flow rate, and the mobile phase ratio were all varied to assess the method's resilience.

Duygu Yeniceli Uğur , Sakine Atila Karaca *, Development of a validated HPLC method for simultaneous determination of olanzapine and aripiprazole in human plasma: Antipsychotic medications, olanzapine and aripiprazole, may be detected in spiked human plasma using an HPLC approach that is simple and quick. Experimentation was used to conduct optimization research. Fractional factorial design was used to pick the most significant analytical parameters, and Box-Behnken design was used to establish the optimal amounts of these parameters. A monolithic column (Rp-18, 100-4.6 mm) set at 35 with a flow rate of 0.8 ml/min was used to accomplish separation. Mobile phase consisted of phosphate buffer (pH 3.14, 20 mM) and an acetonitrile-based solution. olanzapine and aripiprazole had retention durations of 2.34 and 6.90 minutes, respectively, according to the study. For olanzapine, the approach had a linear range of 0.125-50.0 g/ml, whereas for aripiprazole, the range was 0.500- 50.0 g/ml. The olanzapine and aripiprazole quantitation limits were 0.069 and 0.498 g/ml, respectively. Based on recovery values of 99-102 percent and relative standard deviations of less than 2 percent, the approach was confirmed to be accurate and precise. Both the technique and analytes were tested for their robustness.

6.4 EXPERIMENTAL

6.4.1Chemical and Reagents:

An Indian company, Merck (India) Ltd. (Worli, Mumbai), supplied the acetonitrile and ortho phosphoric acid (HPLC grade). APIs for Olanzapine and Samidorphan were obtained from Spectrum Pharma Research Solutions Pvt. Ltd., Hyderabad, for use as standard reference materials in this study

6.4.2Instrumentation:

Waters alliance e2695 HPLC system (quaternary pump, PDA detector-2998) was used. Empower 2.0 software was used to process the data.

6.4.3Standard Solution Preparation:

Transfer 20 milligrammes of Olanzapine and 10 mg of Samidorphan into a 100 ml volumetric flask and add app. 70 ml of diluent, dissolved using a sonicator for 30 minutes and then diluted to the desired strength. Dilutions are needed to further dilute the aforementioned solution to 50 ml, therefore add 5 ml of diluents.

Preparation of Sample Solution

Add 70 mL of diluent to a volumetric flask filled to a weight of 20 mg Olanzapine and 10 mg Samidorphan. Weigh and transfer the sample accurately. Sonicate to dissolve and dilute to the desired concentration with diluent. Filter the solution through a 0.45 nylon syringe filter after diluting to 50 ml.

6.5 Method Development Analytical method development:

A successful attempt has been made in the proposed project to establish a simple accuracy for Olanzapine and Samidorphan analysis using RP-HPLC.

6.5.1Method development parameters:

Selection of following parameters in method development is very important.

- Mode of chromatography

- Wavelength
- Column
- Mobile phase composition
- Solvent delivery system
- Flow rate
- Injection volume

6.5.1.1 Selection of Mode of Chromatography:

Selected mode of chromatography: Reversed phase chromatography Basis of selection : Polarity of the molecule

Reason for selection : as Olanzapine and Samidorphan is polar

molecule it elutes at faster along with mobile phase

6.5.1.2 Detector Wavelength Selection:

The choice of a detector wavelength is critical to completing the analytical procedure. PDA detector and wavelength are used to determine precise wavelength of the standard API, which is manufactured and injected into the chromatographic system using PDA detector and wavelength.

Selected wave length: 261 nm

Basis for selection: Maximum absorbance of analytes and impurities

Reason for selection: Olanzapine & Samidorphan having maximum absorbance 261 nm.

6.5.1.3 Selection of column:

Column Selected: Symmetry C_{18} column (150x4.6 mm, 3.5 µ)

Basis for selection: Based on the polarity, and chemical differences among analytics **Reason for selection:** It has good physiochemical surface qualities and is compatible with many different organic solvents, as well as an extensive variety of bonding chemistries.

6.5.1.4 Selection of the mobile phase composition and of the buffer:

It is vital to note that the buffer and its intensity have an impact on peak symmetry and separation. Peak tailings during chromatography may modify the ionic form if the right buffer strength is not used to cover the column injection load.

Mobile phase preparation:

Solution A: Acetonitrile

Solution B: 0.1% OPA

6.5.1.5 Selection of the rate of flow

Even in reverse phase separation for the resolution of tiny molecules, flow rate is indicated as a significant element. However, in large-scale inverted phase chromatography, adding the sample solution at a high flow rate has a significant impact on the analytical results. Dynamic binding capacity might vary depending on the flow rate employed during sample loading. When scaling up the purification process, the dynamic binding capacity must be estimated to determine the optimal flow rate for loading. Based on flow factor, retention duration, column composition, separation impurity, and peak symmetry, a flow rate of 1 ml/min has been chosen in this system.

6.5.1.6 Selection of injection volume:

For API estimate, a volume of 10 to 20 µl is often advised. Extraction has been a problem thus the test concentration may be kept low and injection volume can be increased to 50 µl. Although it is crucial to make sure there is not too much pressure on the column volume that you set. For Olanzapine and Samidorphan, the injection volume is 10 µl.

6.6 RESULTS AND DISCUSSION

6.6.1Trials in optimization of chromatographic condition: Trial-1 [Fig 6.03]

Mobile phase	:	Acetonitrile and Water pH-4 adjusted with OPA (80:20)
Column	:	X-Bridge phenyl (150x4.6mm, 3.5µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	10 min
Wavelength	:	200-400 nm Observation: Peak resolution is very less

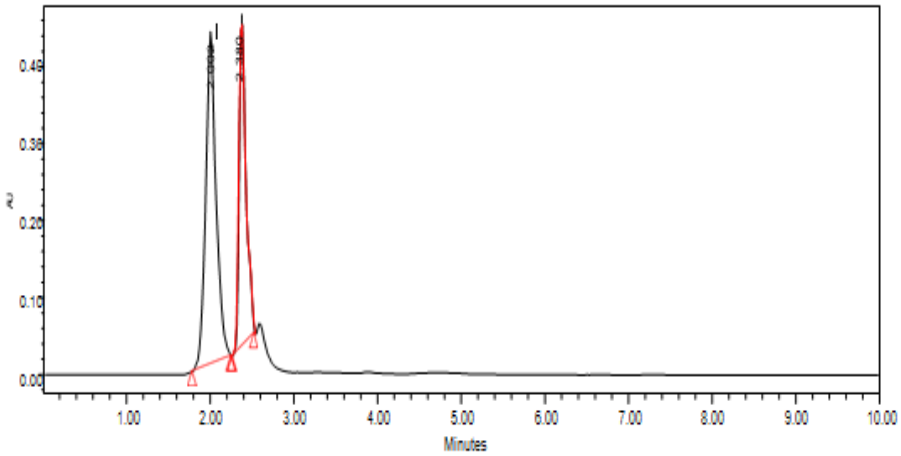

Figure 6.03: chromatogram of trial-1

Trial-2 [Fig 6.04]

Mobile phase	:	Acetonitrile and Water pH-4 adjusted with OPA (70:30)
Column	:	X-Bridge phenyl (150x4.6mm, 3.5µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	10 min
Wavelength	:	261 nm
Observation	:	Two peaks plate count not within the limit

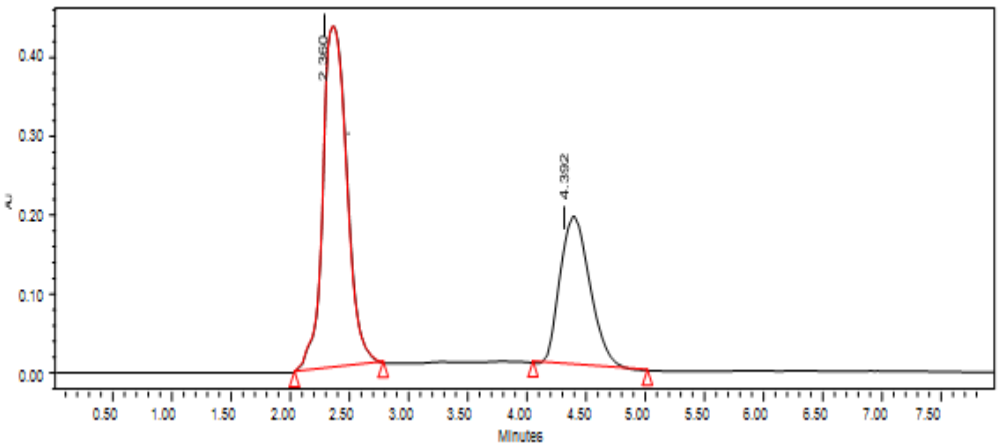

Figure 6.04: Chromatogram of trial-2

Trial-3 [Fig 6.05]

Mobile phase	:	Acetonitrile and 0.1% TFA (70:30)
Column	:	X-Bridge phenyl (150x4.6mm, 3.5µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	6 min
Wavelength	:	261 nm
Observation	:	Peak shape is not good

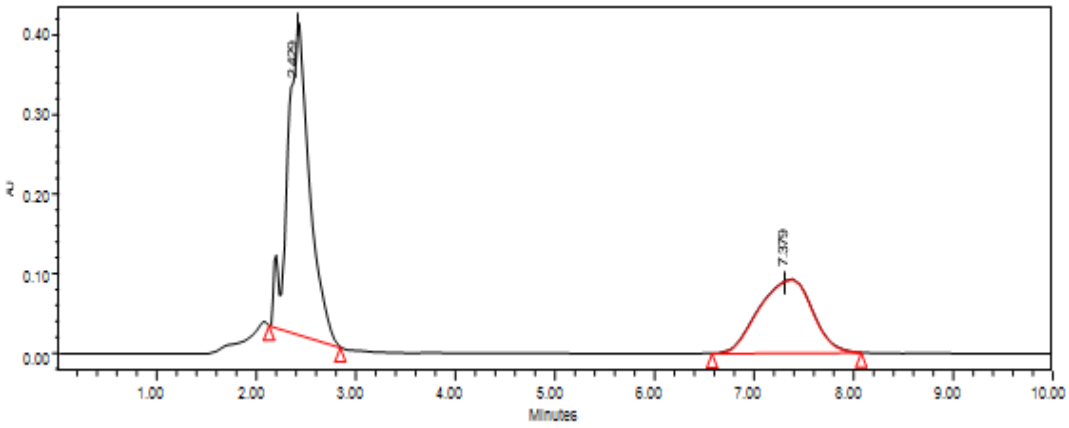

Figure 6.05: Chromatogram of trial-3

Trial-4 [Fig 6.06]

Mobile phase	:	Acetonitrile and 0.1% TFA (65:35)
Column	:	Symmetry shield RP-18 150x4.6mm, 3.5µ
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	10 min

Wavelength	:	261 nm
Observation	:	Height is not within the limit

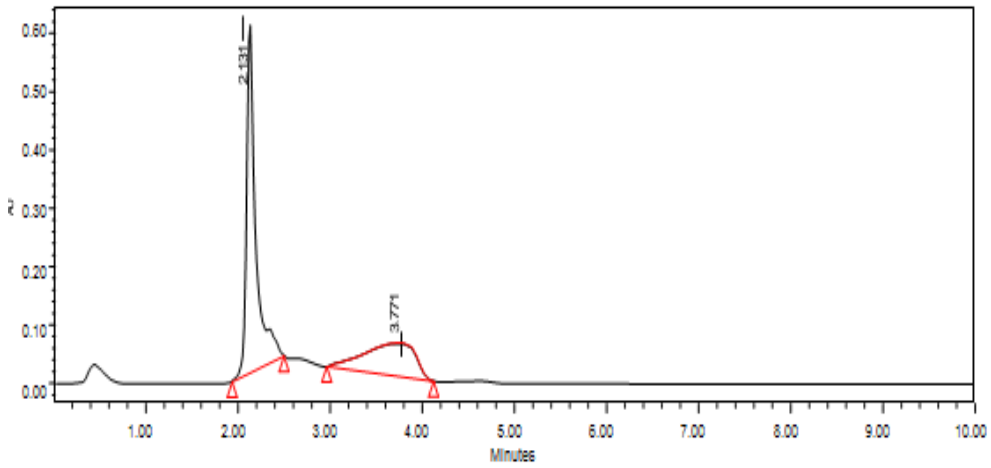

Figure 6.06: Chromatogram of trial-4

Trial-5 [Fig 6.07]

Mobile phase	:	Acetonitrile and 0.1% TFA (60:40)
Column	:	Symmetry shield RP-18 150x4.6mm, 3.5µ
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	9 min
Wavelength	:	261 nm
Observation	:	First peak is separated into two peaks

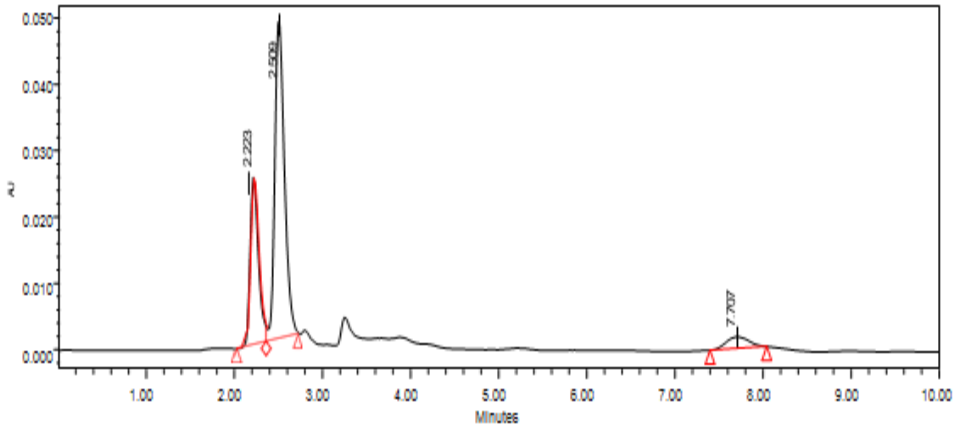

Figure 6.07: Chromatogram of trial-5

Trial-6 [Fig 6.08]

Mobile phase	:	Acetonitrile and 0.1% TFA (50:50)
Column	:	Symmetry C_{18} column (150x4.6mm, 3.5 µ)
Rate of flow	:	1ml/min

Volume of injection	:	10µl
Period of run	:	8 min
Wavelength	:	261 nm
Observation	:	Broad peaks are observed

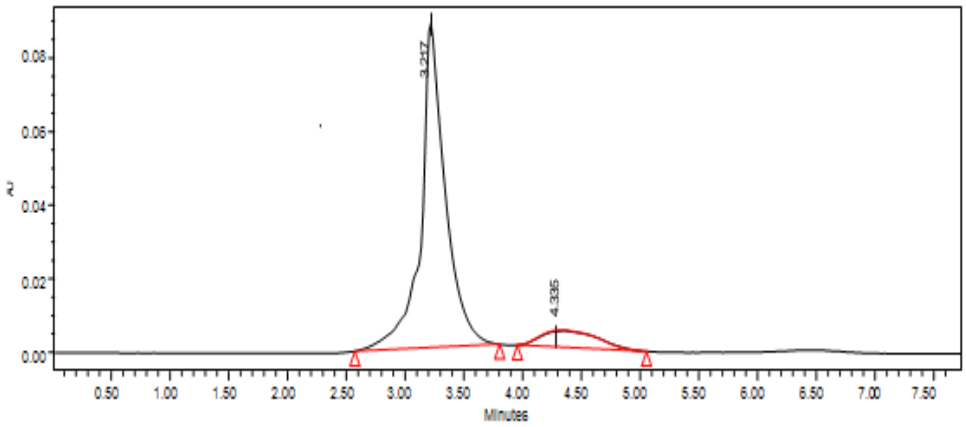

Figure 6.08: Chromatogram of trial-6

Trial-7 [Fig 6.09]

Mobile phase	:	Acetonitrile and 0.1% OPA (60:40)
Column	:	Symmetry C_{18} column (150x4.6mm, 3.5 µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	8 min
Wavelength	:	261 nm
Observation	:	Second peak response is very low

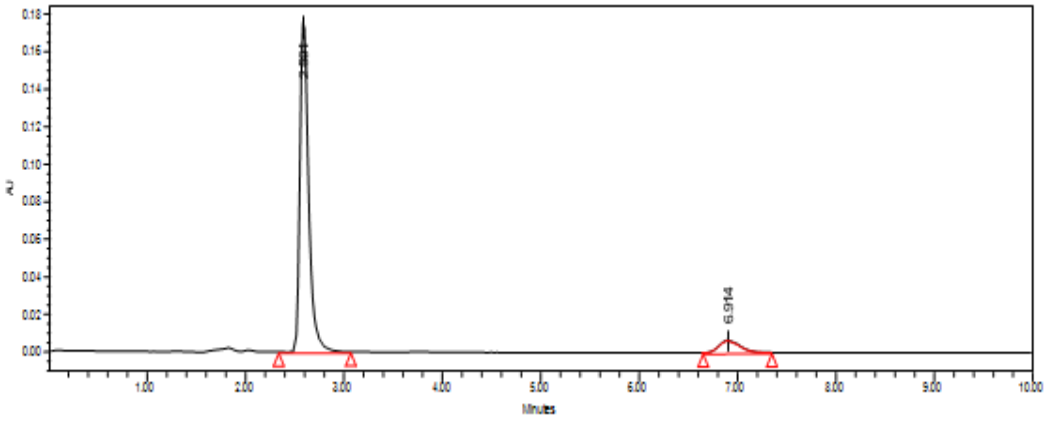

Figure 6.09: Chromatogram of trial-7

Trial-8 [Fig 6.10]

Mobile phase	:	Acetonitrile and 0.1% OPA (50:50)

Column	:	Symmetry C_{18} column (150x4.6mm, 3.5 µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	10min
Wavelength	:	261 nm
Observation	:	More than two peaks are obtained

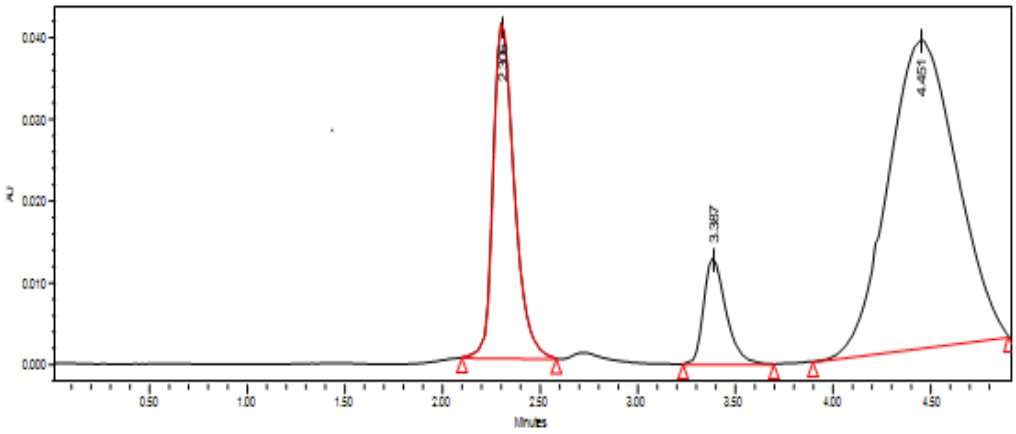

Figure 6.10: Chromatogram of trial-8

Trial-9 [Fig 6.11]

Mobile phase	:	Acetonitrile and 0.1% OPA (45:55)
Column	:	Symmetry C_{18} column (150x4.6mm, 3.5 µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	4 min
Wavelength	:	261nm
Observation	:	Height is not within the limit

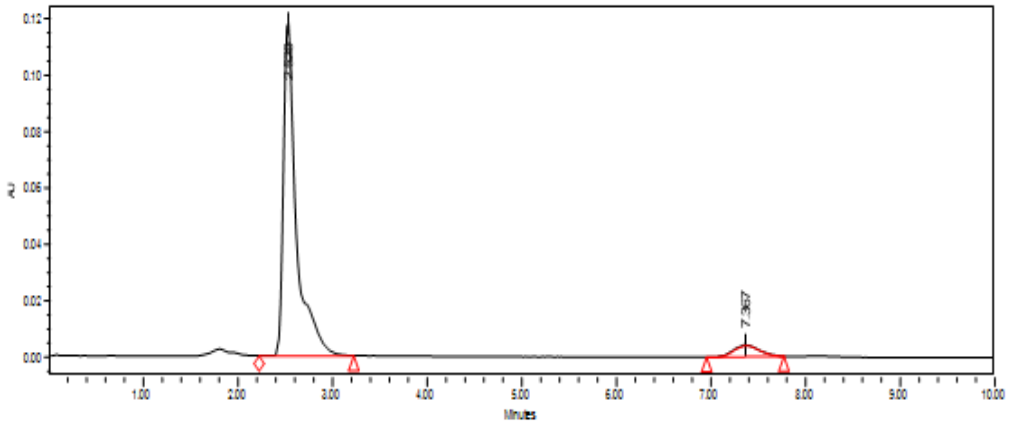

Figure 6.11: Chromatogram of trial-9

Trial-10 [Fig 6.12]

Mobile phase	:	Acetonitrile and 0.1% OPA (40:60)
Column	:	Symmetry C_{18} column (150x4.6mm, 3.5 µ))
Rate of flow	:	1 ml/min
Volume of injection	:	10µl
Period of run	:	10 min
Wavelength	:	261 nm
Observation	:	suitable for validation

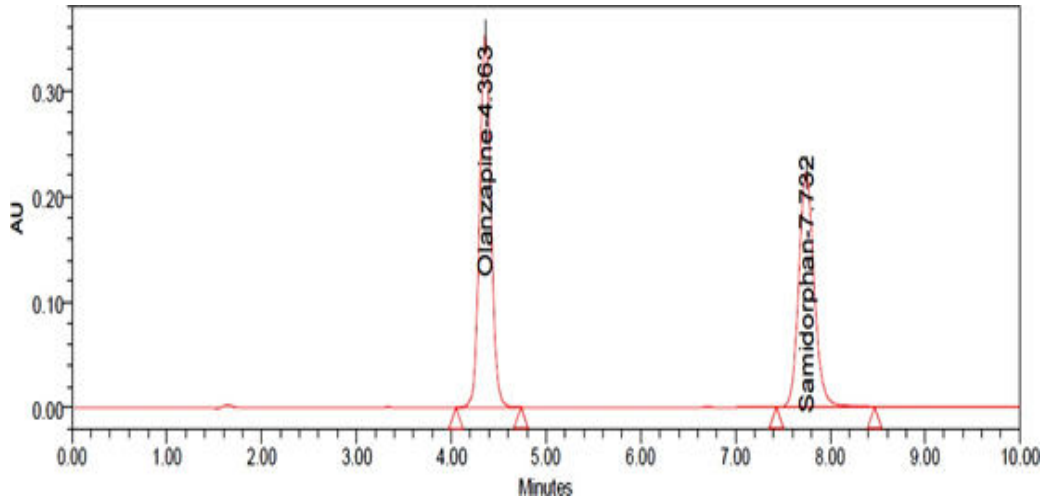

Figure 6.12: Chromatogram of trial-10

The chromatographic results of all trials were shown in **table 6.01**.

Trial No.	RT	Area	% Area	USP Resolution	USP Tailing	USP Plate count
	2.002	3702369	60.37		1.11	1195
1	2.380	2430354	39.63	1.89	1.40	4982
	2.360	6206948	64.92		1.07	805
2	4.392	3353907	35.08	4.96	1.29	1322
	2.429	5966161	63.30		1.10	645
3	7.379	3458818	36.70	7.16	0.91	858
	2.131	4133104	65.80		1.94	3422
4	3.771	2148671	34.20	2.79	0.70	230
	2.223	165387	29.30			2597.86
5	2.509	366996	65.02	1.51	1.27	2560.10
	7.707	32087	5.68	14.66	1.02	4493.36
	3.217	1271098	89.23		0.98	1505
6	4.335	153454	10.77	1.84	1.43	369
	2.591	1088876	91.90		1.48	4434
7	6.914	95923	8.10	15.48	1.21	5141
	2.306	309374	23.29		1.37	2320
8	3.387	101744	7.66	5.25	1.37	4377
	4.451	917010	69.05	2.39	1.02	719

	2.529	1121570	93.46		2.29	2300
9	7.367	78494	6.54	13.06	1.17	3432
	4.363	3600412	55.45		1.09	5362
10	7.732	2354785	44.55	6.58	1.01	8956

Table 6.01: Chromatographic results of all trials

6.6.2Optimized method [Table 6.02 and Fig 6.13]

S.NO	Parameter	Chromatographic condition
1	Mobile phase	Acetonitrile: 0.1% OPA (40:60)
2	Column	Symmetry C_{18} (150x4.6 mm, 3.5 µ)
3	Rate of flow	1ml/min
4	Column temperature	25ºC
6	Wavelength	261 nm
7	Volume of injection	10µl
8	Period of run	10 min
9	Retention time	Olanzapine Retention time-4.363 Samidorphan retention time-7.732

Table 6.02: Optimized method chromatographic conditions

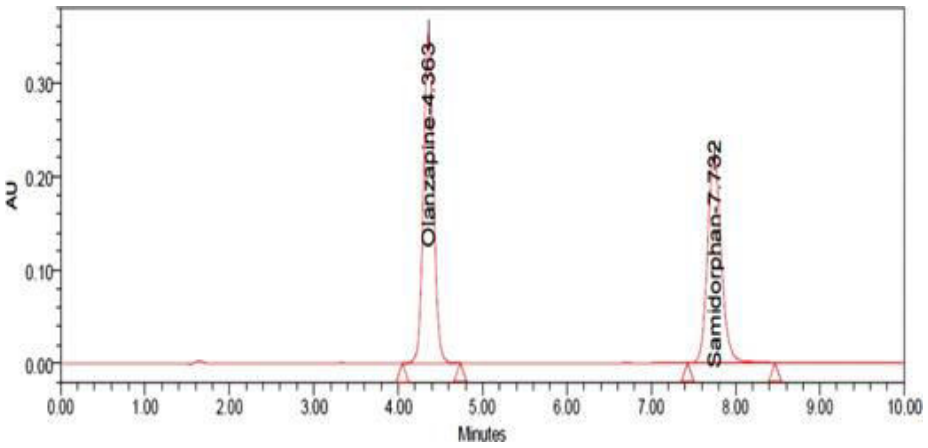

Figure 6.13: Chromatogram of standard

6.6 Validation of Method

It has been shown to be accurate, specific and precise in compliance with the ICH Q2 (R1) requirements for device appropriateness, as well as robustness, LOD, LOQ and stability.

6.6.3Specificity:

Specificity is the ability to test the analyte in the presence of other components, such as contaminants or excitements, that may be presumed to be present in the sample solution and the norm solution, without any interference. Olanzapine and Samidorphan were spiked into the blank samples for testing. **Figure 6.14** gives blank chromatogram.

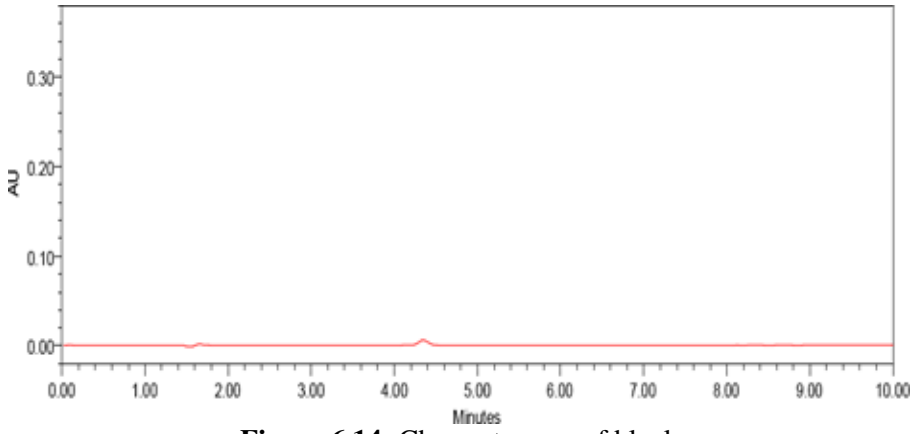

Figure 6.14: Chromatogram of blank

6.6.4Linearity:

This method's capacity to provide outcomes inside a predetermined framework is known as its linearity. It was determined that the peak area correlated with the concentration of analytes in the sample: six standard solutions were used. The peak area in relation to the normal solution concentration was used to construct the regression equations and to produce the calibration curve. The least squares technique of least squares was used to get the slope and correlation coefficient.

Linearity Stock Solution Preparation:

To prepare the volumetric flask, measure out 20 mg of Olanzapine and 10 mg of Samidorphan and add 70ml of diluents. For 15 minutes, sonicate to dissolve the diluents.

10 percent solution preparation: (2ppm of Olanzapine and 1ppm of Samidorphan)

Dilution of the stock solution in a new 50 ml volumetric flask was carried out using the diluents stated above.

25 percent solution preparation: (5ppm of Olanzapine and 2.5ppm of Samidorphan)

One of the stock solutions was diluted in a 50 ml volumetric flask with diluents to the mark in another 50 ml volumetric flask.

50 percent solution preparation: (10ppm of Olanzapine and 5ppm of Samidorphan)

One of the stock solutions was diluted in a 50 ml volumetric flask with diluents to the mark in another 50 ml volumetric flask.

100 percent solution preparation: (20ppm of Olanzapine and 10ppm of Samidorphan)

5 ml of the above-mentioned stock solution was diluted with the diluents to the appropriate concentration in a separate 50 ml volumetric flask.

125 percent solution preparation: (25ppm of Olanzapine and 12.5ppm of Samidorphan)

6.25 ml of the above-mentioned stock solution was diluted with the diluents to the appropriate concentration in a separate 50 ml volumetric flask.

150 percent solution preparation: (30ppm of Olanzapine and 15.0ppm of Samidorphan)

Dilution of the stock solution in a new 50 ml volumetric flask was carried out in the same manner.

Procedure:
Use the chromatographic technique to inject and measure the peak area at each step. Coefficients of correlation may be calculated by drawing a line graph of peak area (Y-axis peak area) and concentration (X-axis concentration).

Range:
This is the interval between the upper and lower phases of analysis, which has been shown to be dependable, accurate, and linear.

Acceptance criteria:
The correlation coefficient should not be less than 0.999.

Table 6.03 gives linearity results and **figures** from **6.15-6.23** gives linearity chromatograms.

S.No	Olanzapine		Samidorphan	
	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area
1	2.00	355342	1.00	212053
2	5.00	942105	2.50	635013
3	10.00	1802635	5.00	1252468
4	20.00	3600412	10.00	2354785
5	25.00	4365329	12.50	2925436
6	30.00	5254521	15.00	3405684
Slope	174856		229090	
Intercept	33364		35323	
CC	0.9998		0.9992	

Table 6.03: Results of linearity

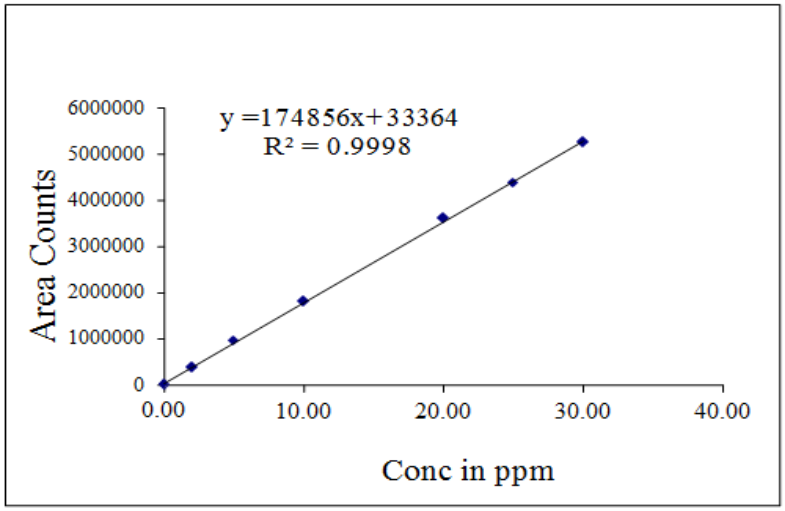

Figure 6.15: Calibration plot of Olanzapine

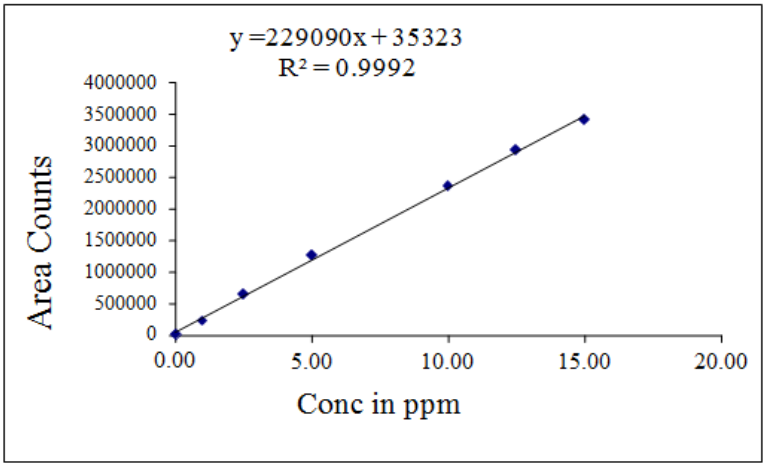

Figure 6.16: Calibration plot of Samidorphan

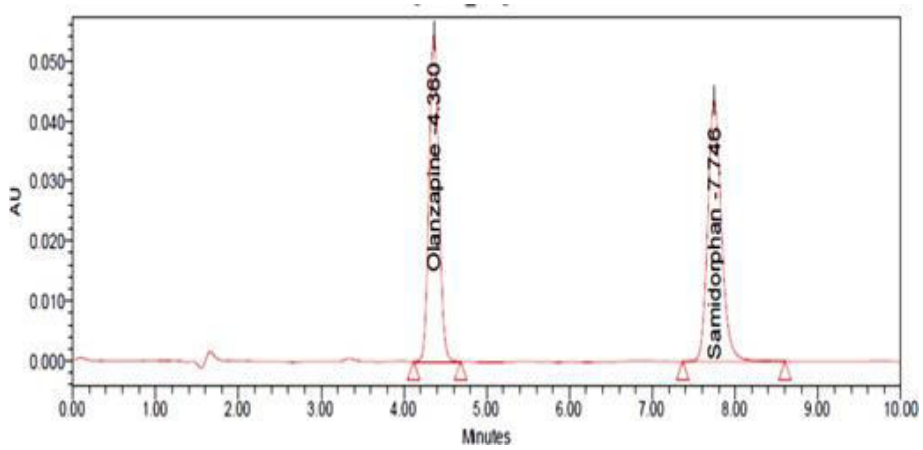

Figure 6.17: 10 percent linearity chromatogram

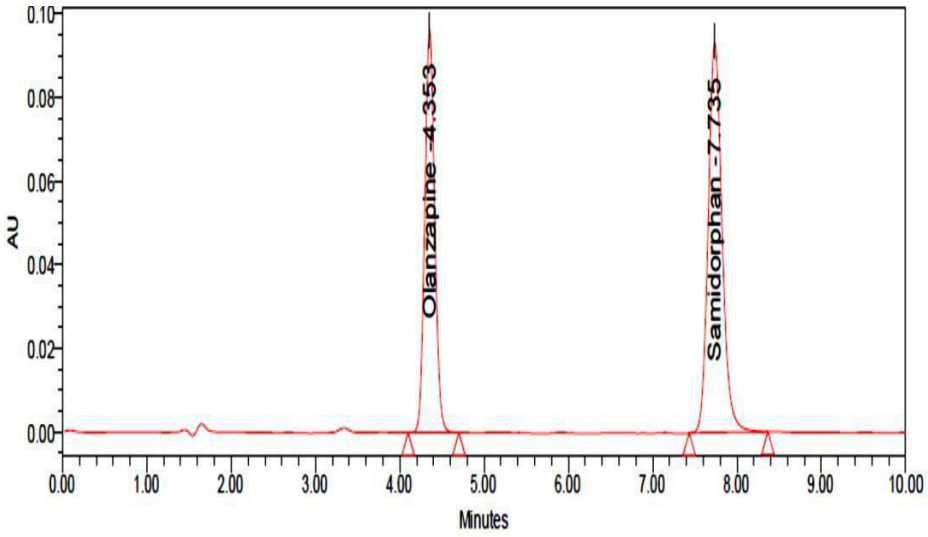

Figure 6.18: 25 percent linearity chromatogram

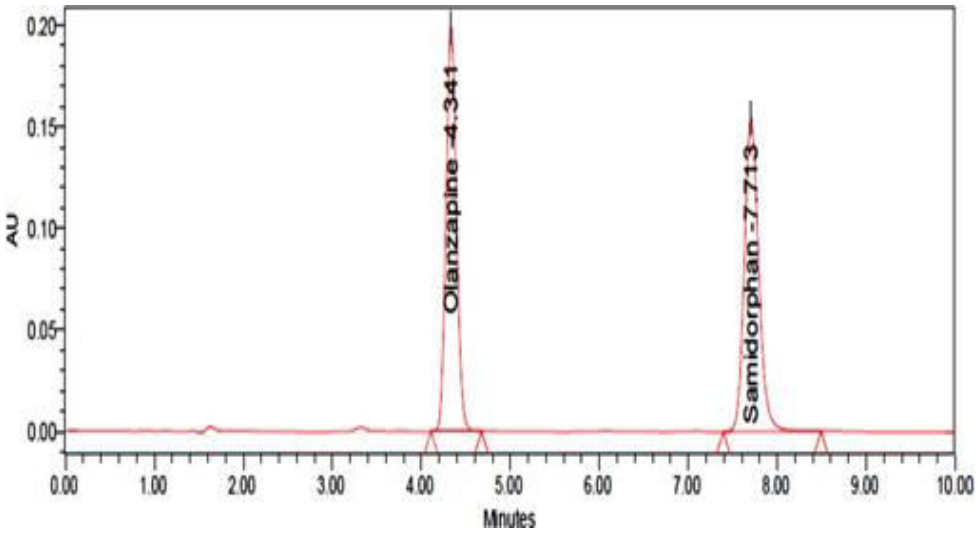

Figure 6.19: 50 percent linearity chromatogram

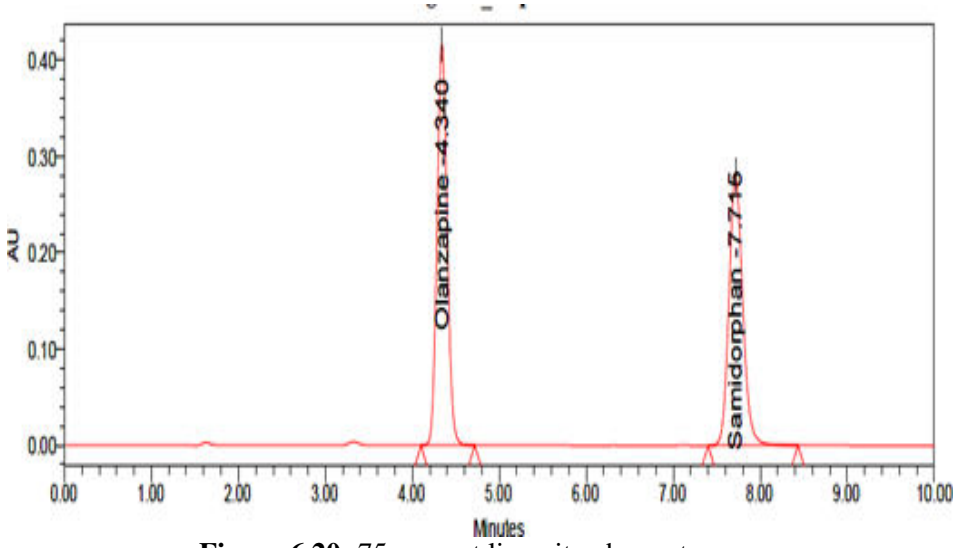

Figure 6.20: 75 percent linearity chromatogram

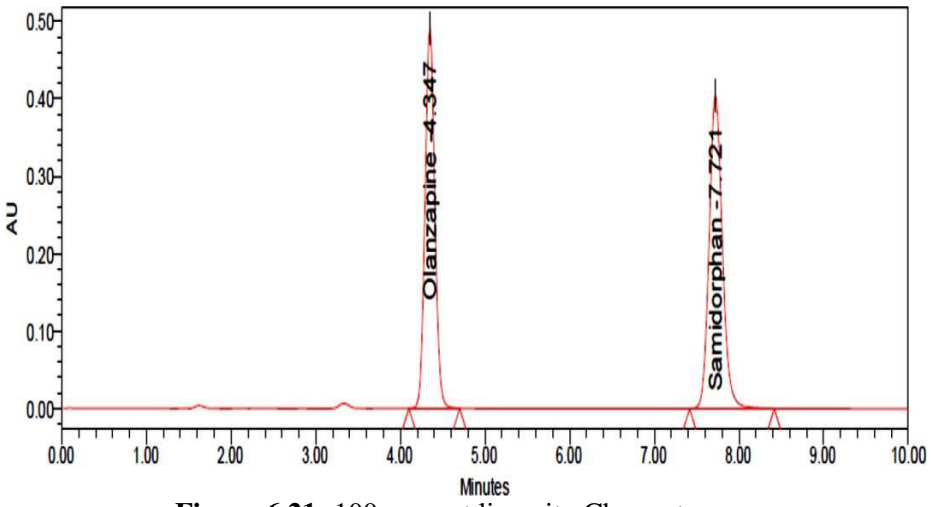

Figure 6.21: 100 percent linearity Chromatogram

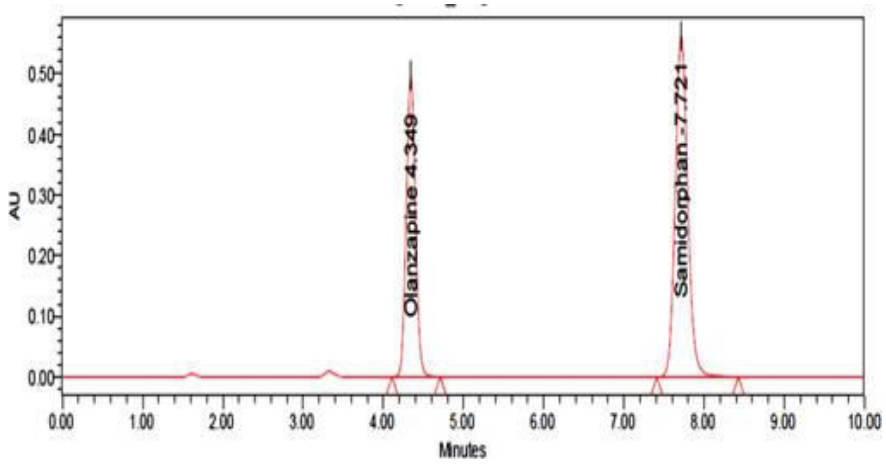

Figure 6.22: 125 percent linearity chromatogram

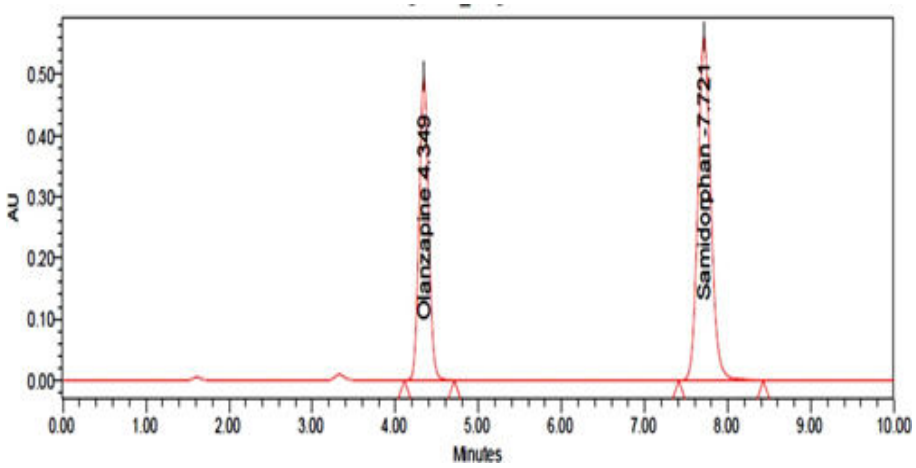

Figure 6.23: 150 percent linearity chromatogram

6.6.5Accuracy:

50 percent solution preparation (with respect to the concentration of the target assay) Sonicate 10 mg of Olanzapine and 5 mg of Samidorphan in a clean and dry volumetric flask to dissolve thoroughly and get the diluents level up to the diluents level, then transfer the mixture to a new flask.

When the volumetric flask has been filled to the desired level, pipette 5 ml of the aforesaid solution into it. In this case, 10ppm of Olanzapine, and 5ppm Samidorphan

100 percent solution preparation (with respect to target assay concentration) Apply diluents and sonicate to thoroughly dissolve Olanzapine and Samidorphan in a clean and dry volumetric flask before adding diluents and bringing the diluent level up to the diluents' levels.

Add diluents to a volumetric flask to get the desired concentration, then add 5 ml of the aforesaid solution. Olanzapine (20 ppm) and Samidorphan (10 ppm) are used.

150 percent solution preparation (with respect to target assay concentration) Diluents should be added to the volumetric flask to dissolve the Olanzepine and Samidorphan. Sonicate the mixture to get it up to diluent level.

In a 50ml volumetric flask, pipette 5ml of the aforementioned stock solution and dilute it with diluents to the desired volume. Olanzapine (30ppm) and Samidorphan (15ppm) were used.

Procedure:
Inject the standard solution with a 50 percent accuracy, 100 percent accuracy, or 150 percent accuracy level of precision.

Acceptance criteria:
The rate of recovery for each stage should be between 98-102 percent.

Table 6.04 gives accuracy results and **figures** from **6.24-6.26** shows accuracy chromatograms.

Accuracy	Amount of Olanzapine	% Recovery	Amount of Samidorphan	% Recovery
50	10	99.8	5	98.9
100	20	99.5	10	99.6
150	30	99.9	15	100.2

Table 6.04: Accuracy results of Olanzapine and Samidorphan

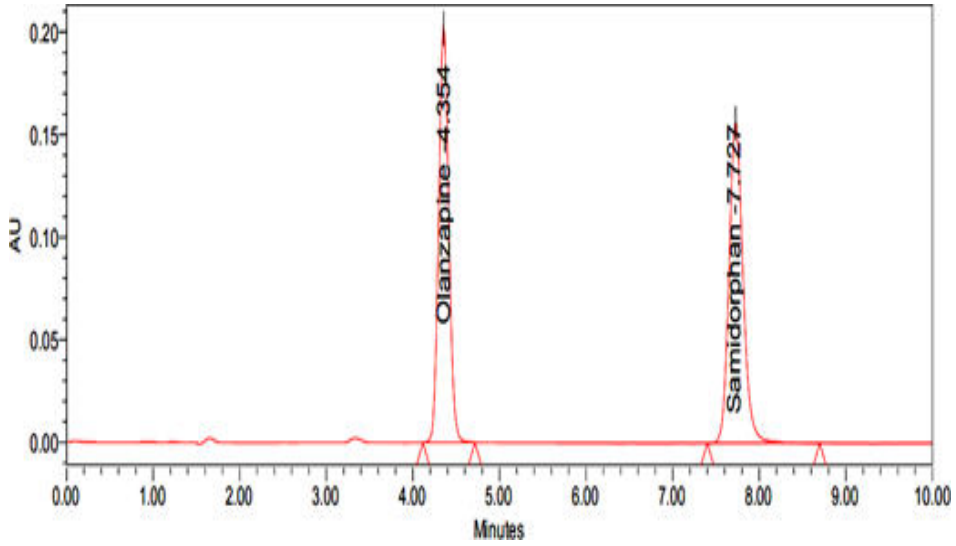

Figure 6.24: 50 percent accuracy chromatogram

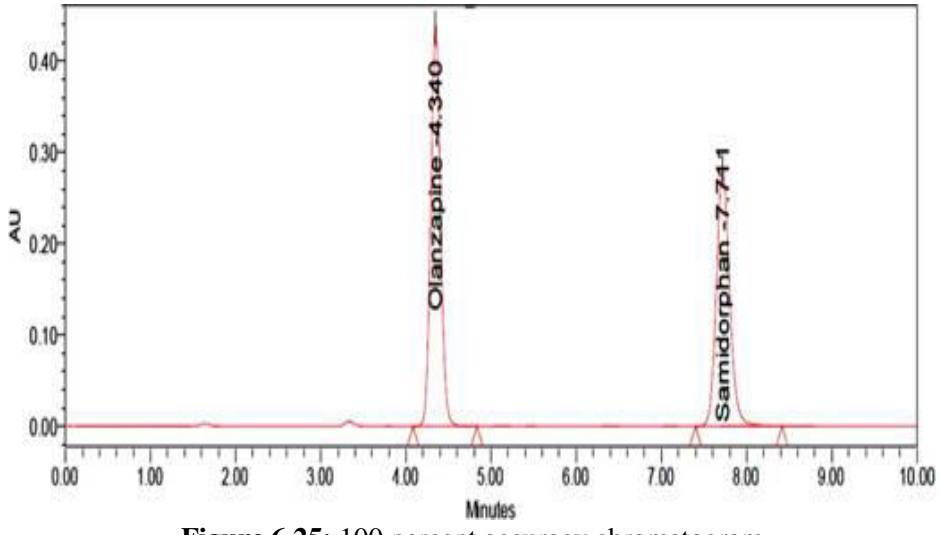

Figure 6.25: 100 percent accuracy chromatogram

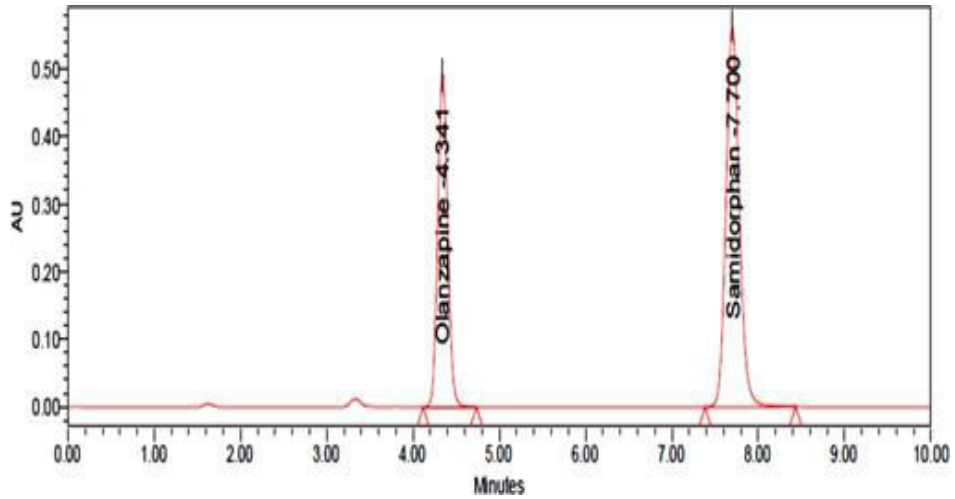

Figure 6.26: 150 percent accuracy chromatogram

6.6.6Precision:

Generally, the closer a set of measurements from various homogeneous samplings are, the more accurate an analytical technique is considered to be. Six injections of Olanzapine (20ppm) and Samidorphan (10ppm) were spiked to ensure the accuracy of the injection method. **Table 6.05** gives precision results and **figures** from **6.27-6.32** shows precision chromatograms.

S.No	Conc. of Olanzapine (µg/ml)	Area of Olanzapine	Conc. of Samidorphan (µg/ml)	Area of Samidorphan
1	20	3629082	10	2311078
2	20	3627503	10	2323358
3	20	3680656	10	2332502
4	20	3621863	10	2313639
5	20	3650588	10	2329357
6	20	3629914	10	2327574
Mean	3639934		2322918	
Std dev	22230.82		8734.66	
% RSD	0.611		0.376	

Table 6.05: Results of system precision

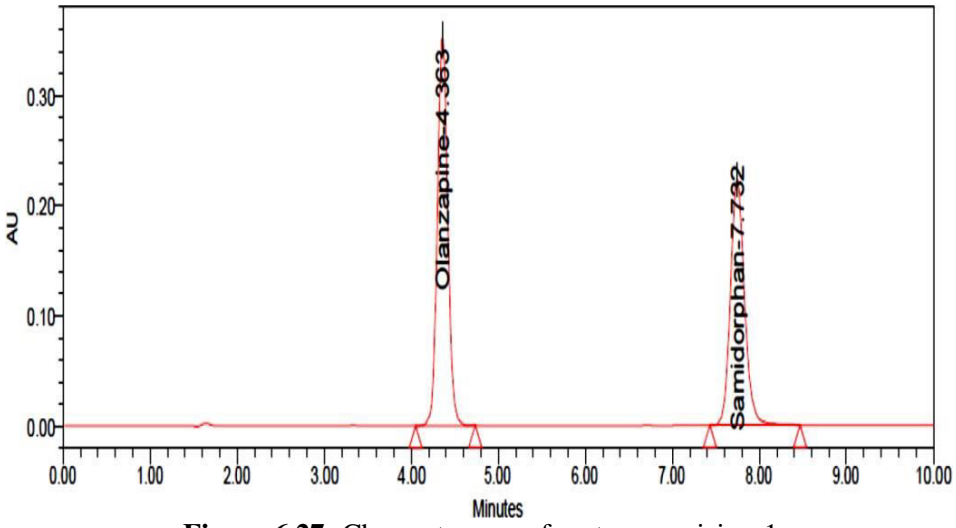

Figure 6.27: Chromatogram of system precision-1

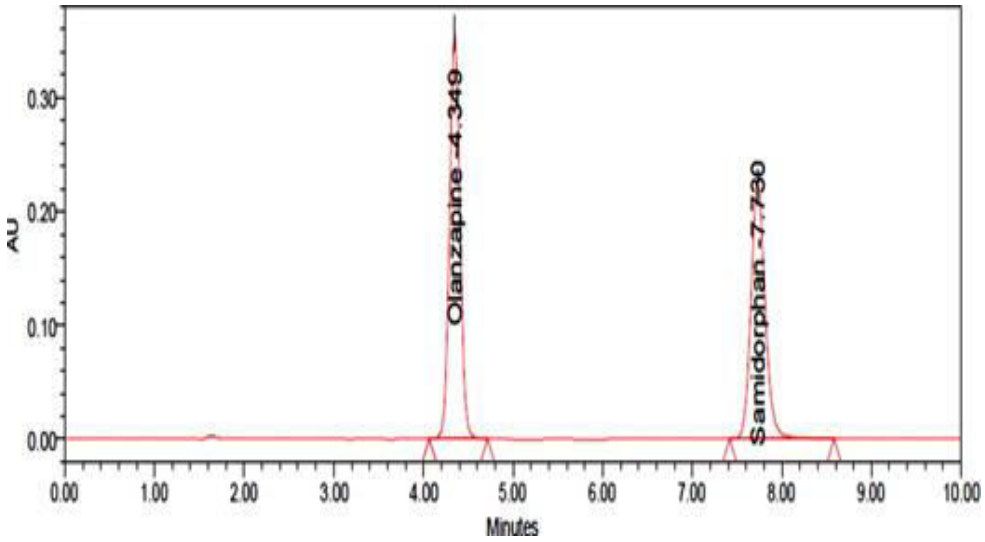

Figure 6.28: Chromatogram of system precision-2

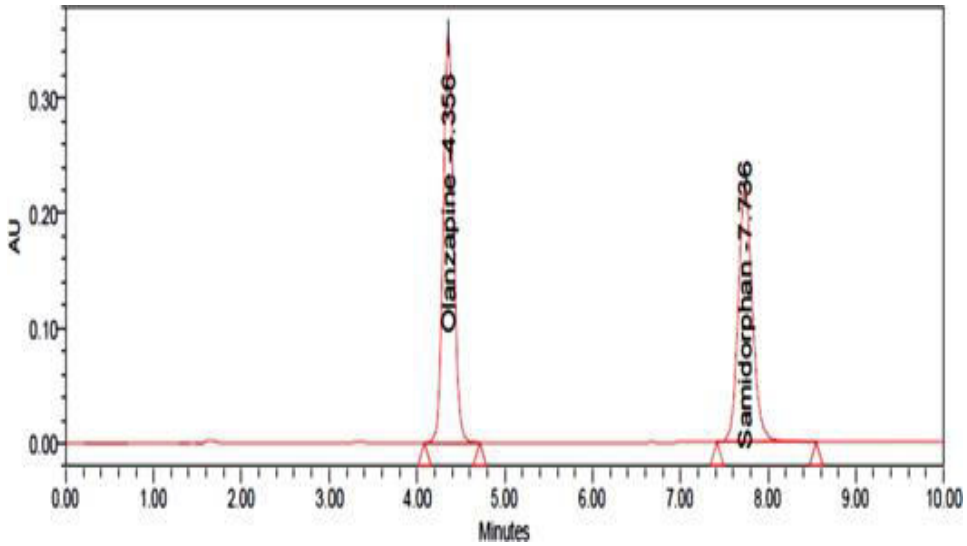

Figure 6.29: Chromatogram of system precision-3

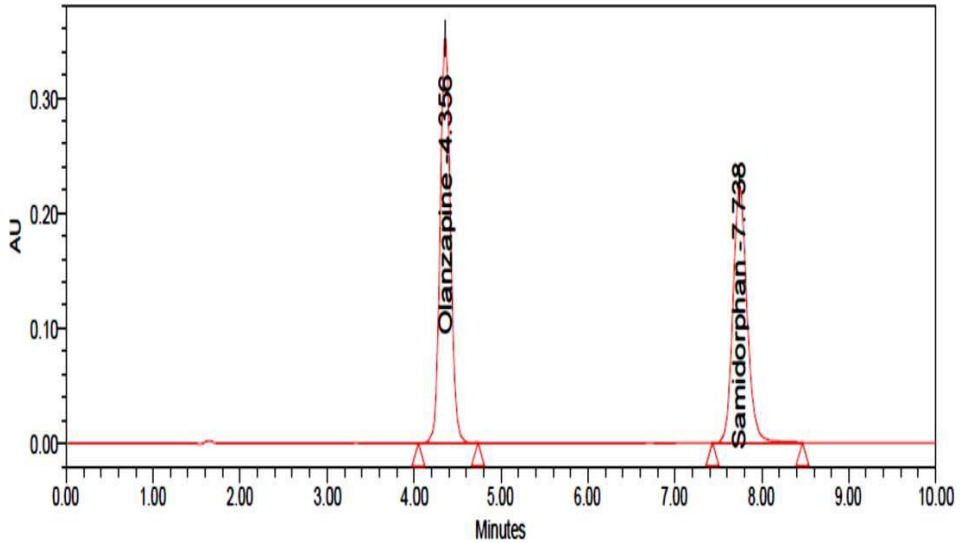

Figure 6.30: Chromatogram of system precision-4

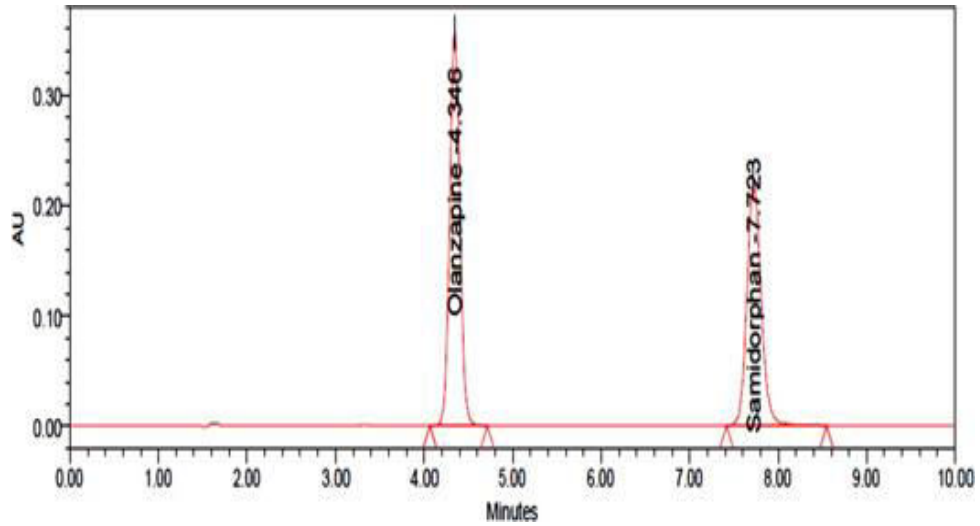

Figure 6.31: Chromatogram of system precision-5

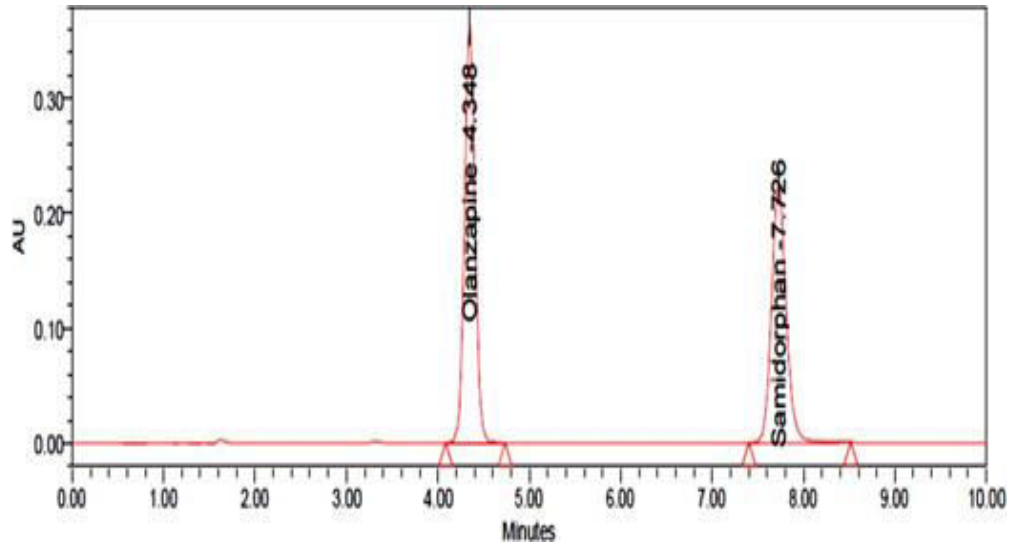

Figure 6.32: Chromatogram of system precision-6 Method precision

[Table 6.06 and Fig 6.33]

S.No	Olanzapine area	Samidorphan area
1	3635245	2365021
2	3501269	2345017
3	3542815	2315026
4	3546251	2385697
5	3536215	2345726
6	3524864	2311004
Mean	3656043	2344582
Std dev	26195.038	28673.099
% RSD	0.72	1.22

Table 6.06: Results of method precision

Acceptance criteria: Area six standard injection results should have an RSD percent of at least 2%.

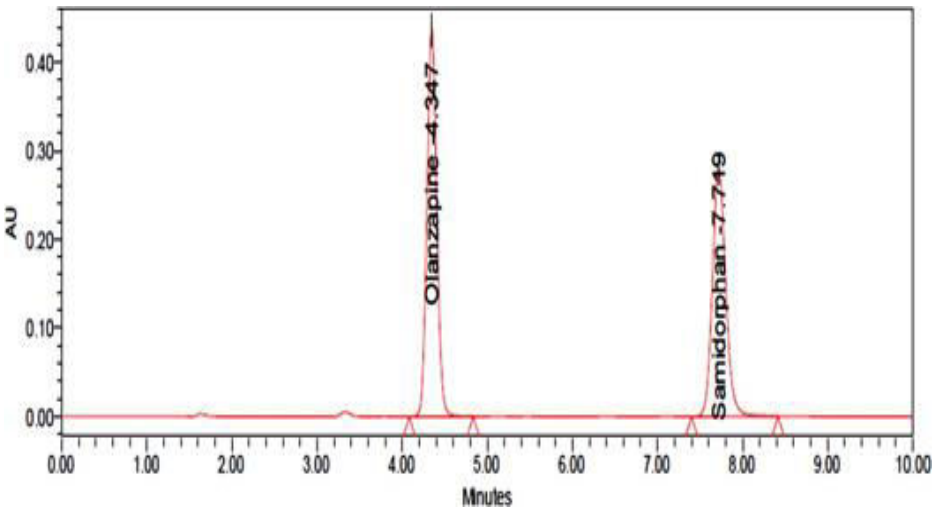

Figure 6.33: Chromatogram of method precision Intermediate precision (Day-Day precision)

[Table 6.07 and Fig 6.34]

Area of Olanzapine	Relative standard deviation	Area of Samidorphan	Relative standard deviation
3652130	0.68	2365201	1.16
3647518		2345187	
3652894		2365294	
3621457		2301546	
3695821		2359612	
3636259		2316325	

Table 6.07: Results of intermediate precision

Acceptance criteria: The RSD percentage for the six normal injection results should not be more than 2%.

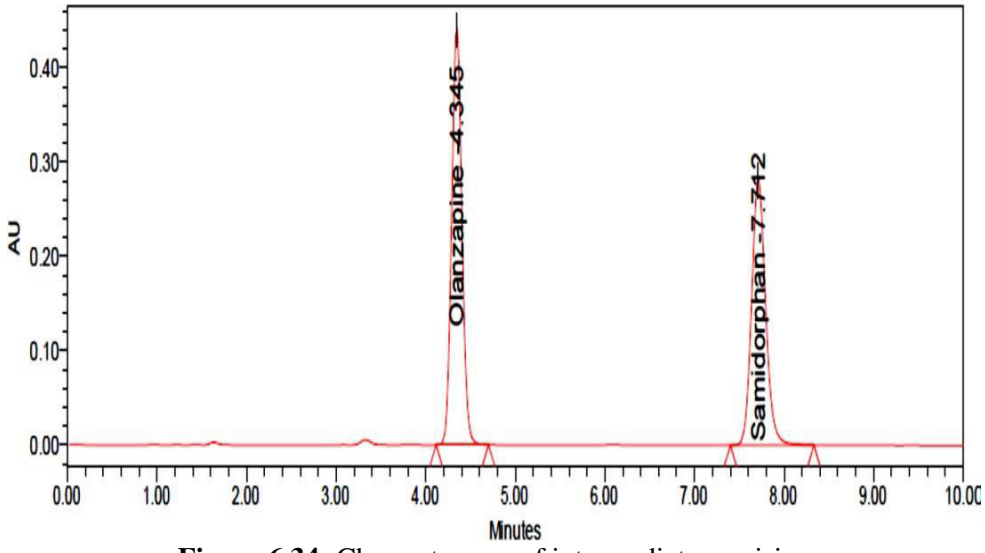

Figure 6.34: Chromatogram of intermediate precision

6.6.7Limit of detection (LOD) and limit of quantification (LOQ):

Calibrating with a calibration curve yielded the LOD and LOQ values. Low- concentration standard solution injections were used to estimate LOD and LQ for the chemical using RP-HPLC. In terms of LOD, Olanzapine's is 0.02g/ml, whereas Samidorphan's is 0.01g/ml, and their s/n values are 4 and 6. There is a LOQ concentration of 0.066g/ml for Olanzapine, while their s/n values are 26 and 23 for Samidorphan. **Table 6.08** gives sensitivity results and **Figures 6.35 and 6.36** gives LOD and LOQ chromatograms.

Drug name	LOD (µg/ml)	LOQ (µg/ml)
Olanzapine	0.02	0.066
Samidorphan	0.01	0.033

Table 6.08: Sensitivity parameter values

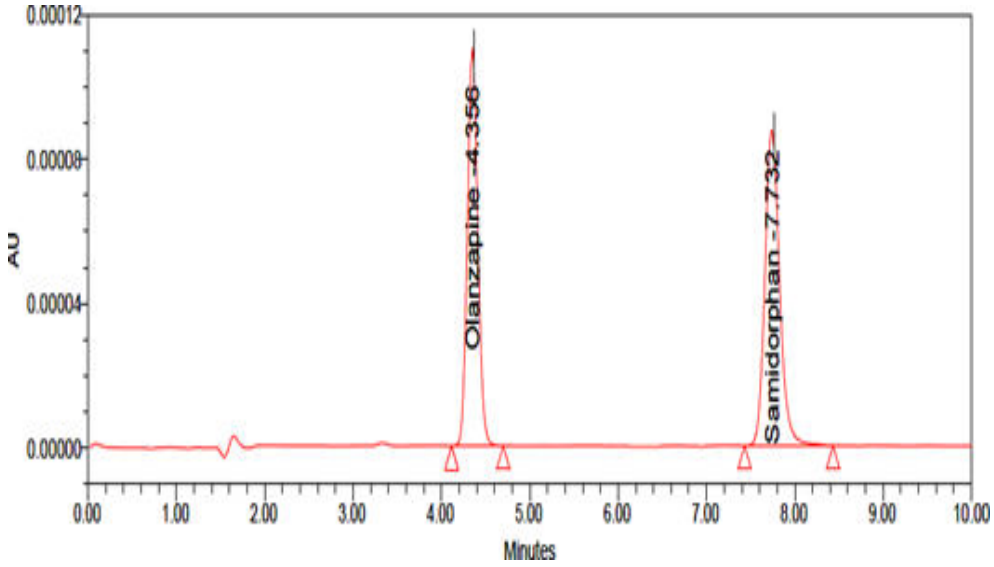

Figure 6.35: LOD chromatogram

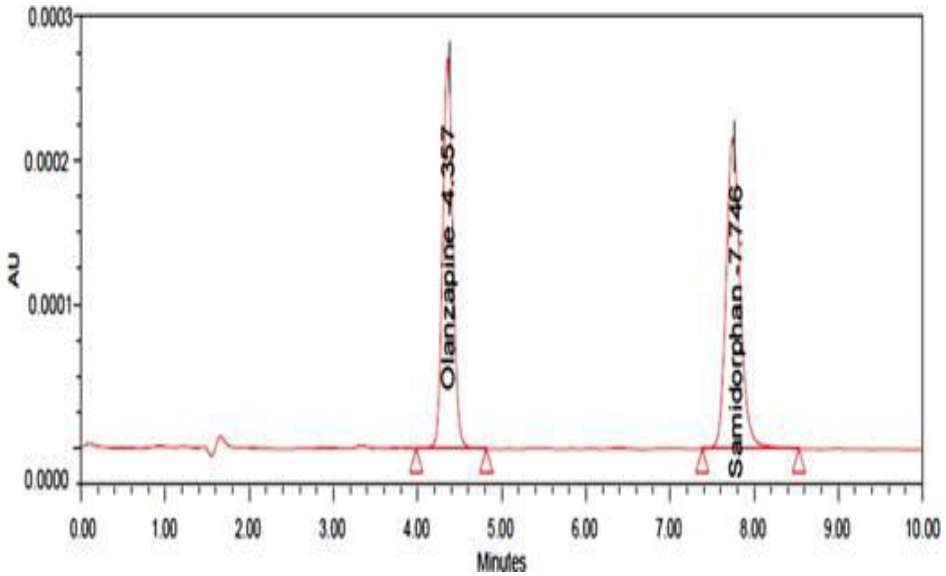

Figure 6.36: LOQ chromatogram

6.6.8Robustness:

Tests were carried out on known systems, such as flow rate or mobile phase in organic percentage, to see whether they could withstand these different circumstances. Active pharmaceutical components and contaminants were not considerably changed, and the period of retention, plate count, and tailing factor were not significantly altered. Thus, this strategy proved to be reliable. **Table 6.09, 6.10** gives robustness results and **Figures** from **6.37-6.40** gives robustness chromatograms.

Parameter	Olanzapine					
	Condition	**RT (min)**	**Peak area**	**Resolution**	**Tailing**	**Plate count**
Flow rate	Less flow (0.8 ml/min)	5.389	3926148		1.09	8583
	More flow (1.2 ml/min)	3.623	3371083		1.06	5257
Organic phase	Less organic (36:64)	4.939	3744312		1.08	7860
	More organic (44:56)	5.389	3418591		1.06	5875

Table 6.09: Robustness results of Olanzapine

Parameter	Samidorphan					
	Condition	**RT (min)**	**Peak area**	**Resolution**	**Tailing**	**Plate count**
Flow rate	Less flow (0.8ml /min)	9.544	2574532	14.93	1.13	13908
	More flow (1.2 ml/min)	6.410	2091546	12.23	1.11	10042
Organic phase	Less organic (36:64)	9.414	2411085	16.32	1.12	13390
	More organic (44:56)	9.544	2248765	11.06	1.12	10376

Table 6.10: Robustness results of Samidorphan

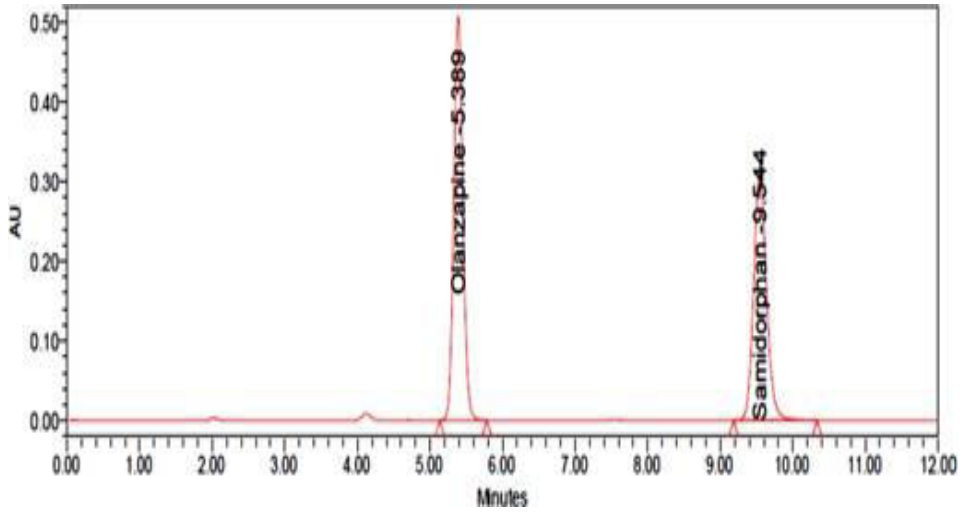

Figure 6.37: Less flow rate chromatogram (0.8ml/min)

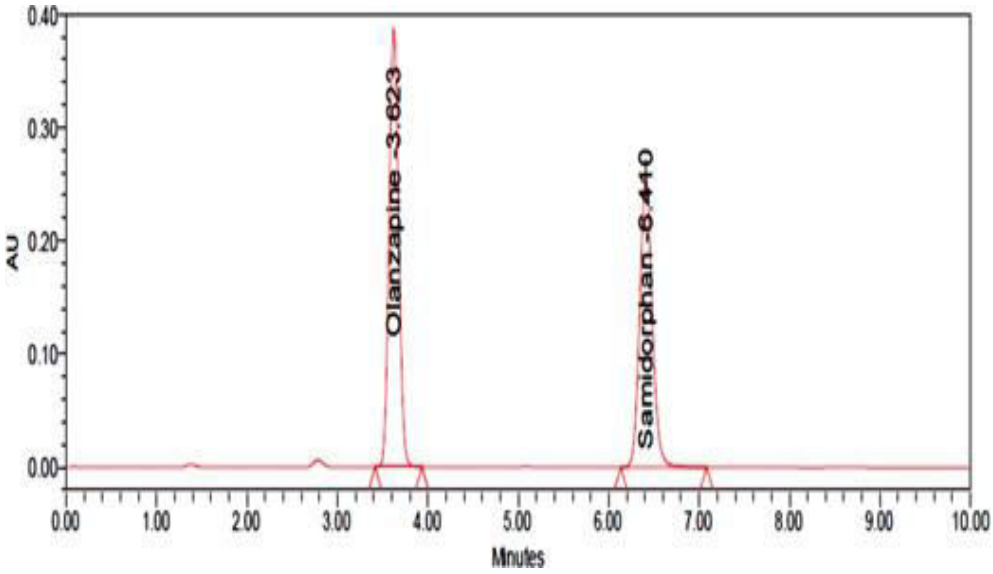

Figure 6.38: More flow rate chromatogram (1.2ml/min)

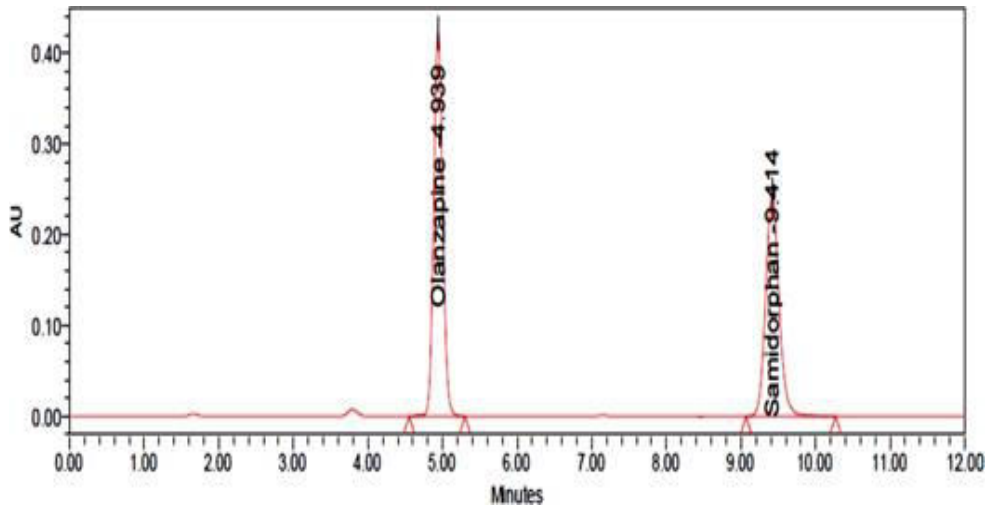

Figure 6.39: Less organic chromatogram (36:64)

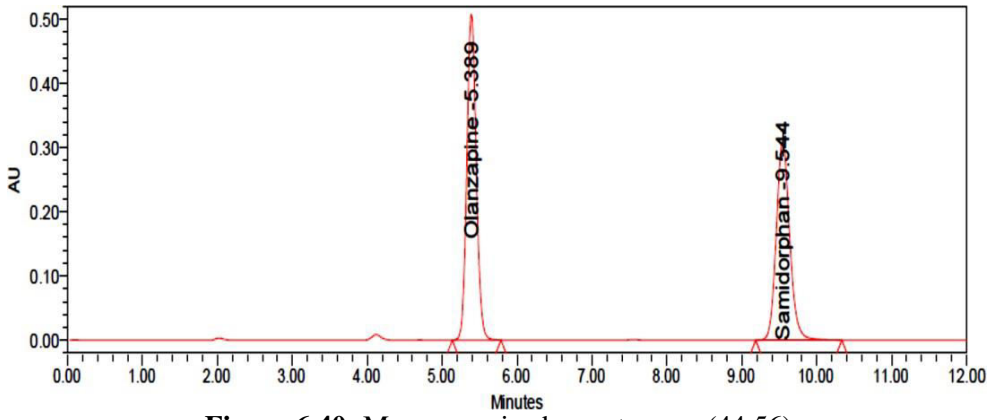

Figure 6.40: More organic chromatogram (44:56)

6.6.9 Forced Degradation Studies:

To partly degrade Olanzapine and Samidorphan, the samples were treated to various conditions of forced degradation. Forced degradation studies have been conducted to determine that the

procedure may be used to degrade materials. For this reason, the research includes information on what conditions could lead to instability in order to guarantee the formulation process is always safe.

Stock Solution Preparation:
Sonicate Olanzapine and Samidorphan to dissolve for 30 minutes and make up the diluents mark in a 100 ml volumetric flask with 70 ml diluents applied.

Acid Degradation:
1 ml of 1N Hcl was added to a volumetric flask of 50 ml containing 5 ml of sample stock solution and left for 15 minutes. To make up the diluents, add 1ml of 1N NaOH and mix well.

Alkali Degradation:
A volumetric flask holds 50 ml of the sample stock solution, and 1 ml of 1N NaOH is added. The flask is then let to sit for 15 minutes. After 15 minutes, add 1 ml of 1N Hcl and dilute with diluents until desired consistency is achieved.

Peroxide Degradation:
5 ml of the sample stock solution was transferred to a 50 ml volumetric flask, and 0.3 ml of 30% hydrogen peroxide was added before the flask was diluted to the desired level.

Reduction Degradation:
Sample stock solution was placed into a 50 ml volumetric flask and diluted with 30 percent sodium bi-sulphate solution to the diluents mark.

Photolytic Degradation:
For six hours, the sample solution was exposed to the sun's rays. HPLC was used to purify the final product. FD results were shown in **table 6.11** and the chromatograms were shown in **figure 6.41-6.45**.

Stress parameter	% of degradation	
	Olanzapine	Samidorphan
Acid degradation (1N HCl+ reflux+ 24 hrs)	13.3	14.5
Alkali degradation (1N NaOH+ reflux+ 24 hrs)	12.8	14.0
Peroxide degradation (30% Peroxide+ reflux+ 24 hrs)	13.7	13.2
Photo degradation (UV light (200 W h/m^2) and fluorescent light (1.2 million lux-hrs))	11.2	11.8

Table 6.11: Forced degradation results

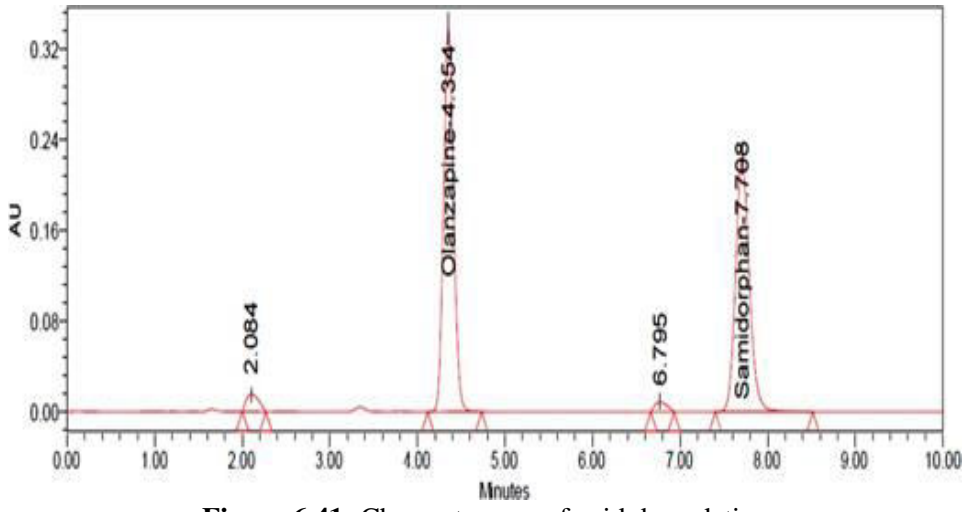

Figure 6.41: Chromatogram of acid degradation

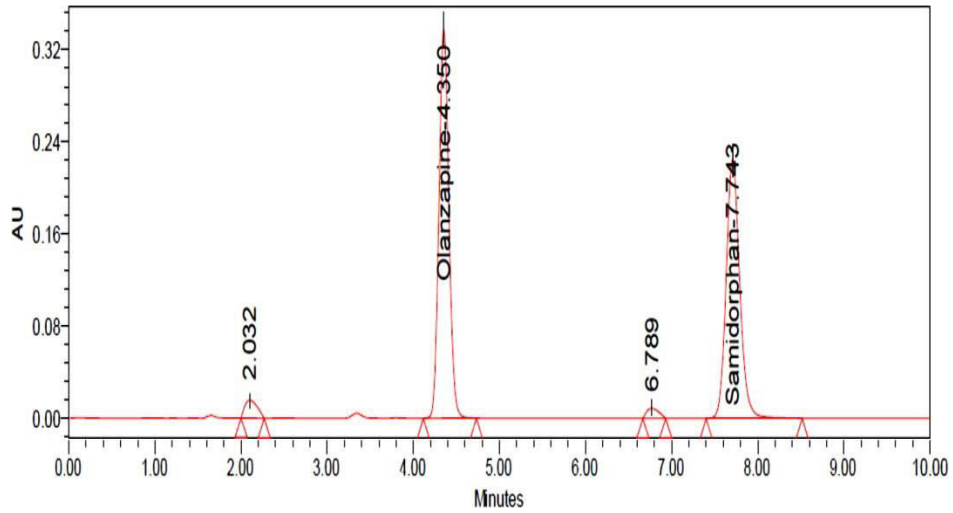

Figure 6.42: Chromatogram of alkali degradation

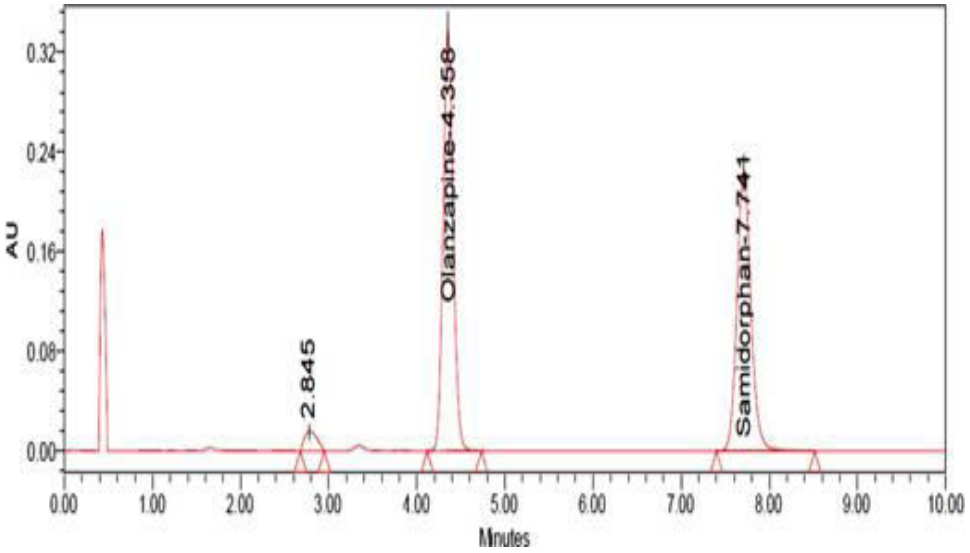

Figure 6.43: Chromatogram of peroxide degradation

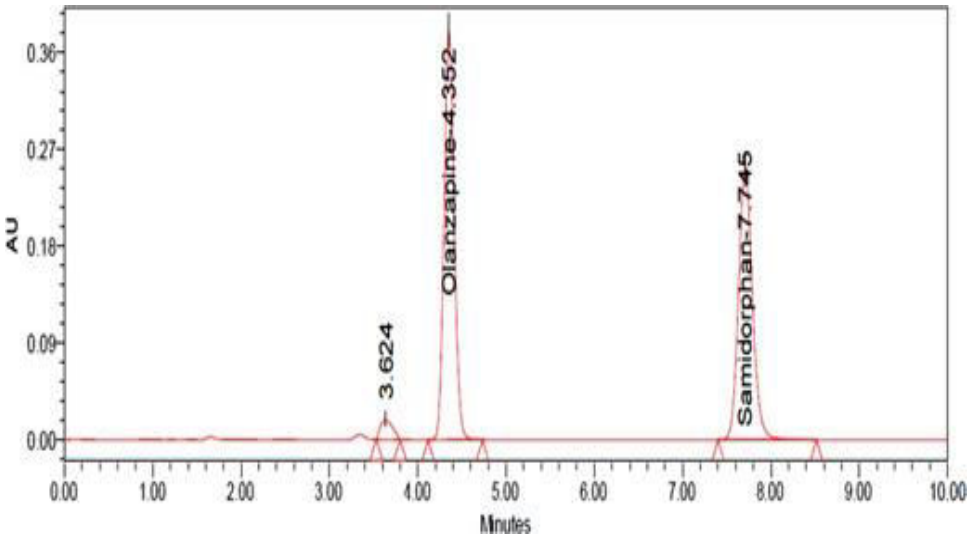

Figure 6.44: Chromatogram of Photolytic degradation

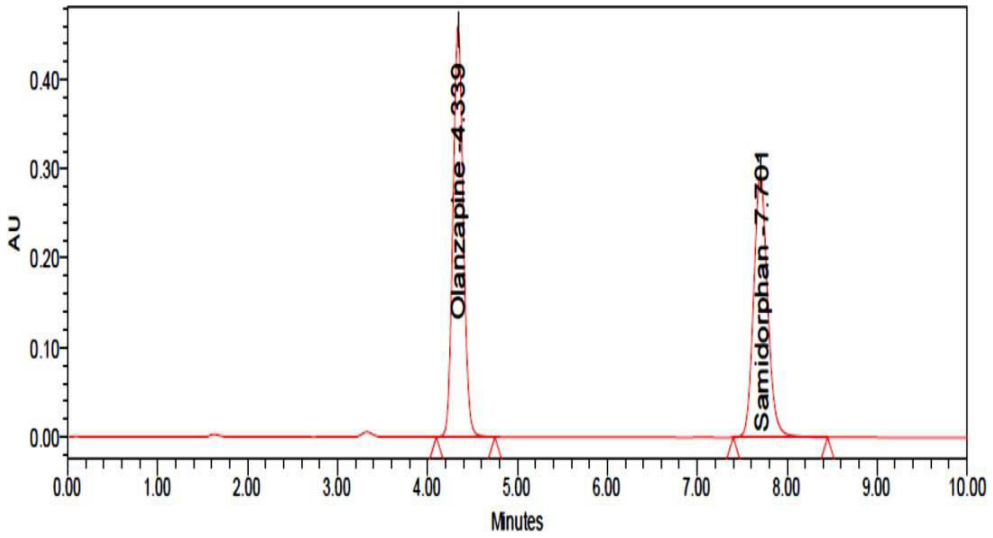

Figure 6.45: Chromatogram of control degradation

6.6.10 Stability:

At room temperature and between 2-8°C for up to 24 hours, the normal and sample solutions were stored. In order to determine the percentage of divergence from initial to 24 hours, these solutions were injected into the apparatus. Analysis showed that the solutions were stable for at least 24 hours, and no major deviations were found. Stability results were shown in **table 6.12** and the chromatograms were shown in **figure 6.46-6.50**.

Stability	Olanzapine		Samidorphan	
	Purity	% of deviation	Purity	% of deviation
Initial	100	0.00	100	0.00
6 Hrs	99.6	-0.40	99.7	-0.30
12 Hrs	99.5	-0.50	99.4	-0.60
18 Hrs	99.2	-0.80	98.9	-1.10
24 Hrs	98.8	-1.20	98.7	-1.30

Table 6.12: Results of stability

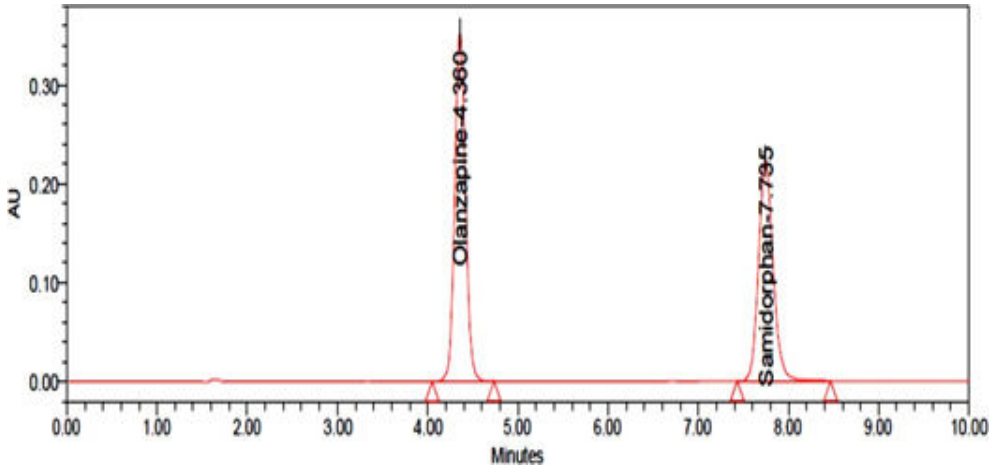

Figure 6.46: Chromatogram of stability initial RT

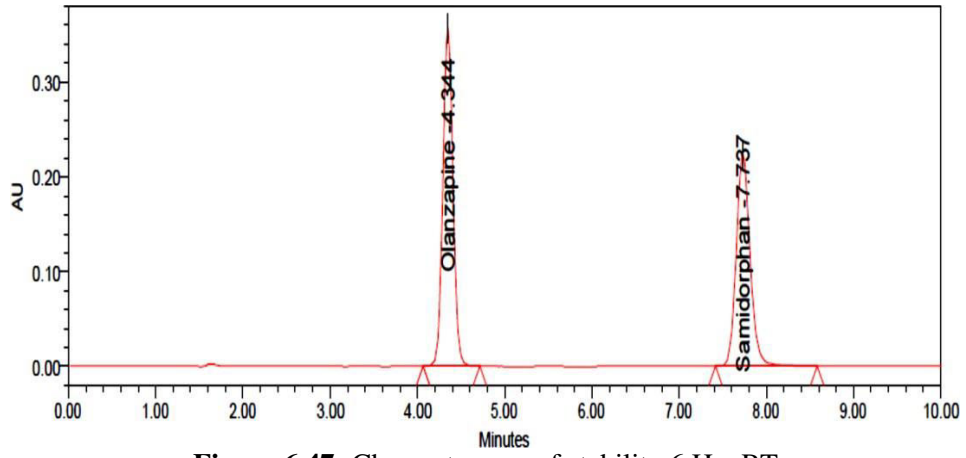

Figure 6.47: Chromatogram of stability 6 Hrs RT

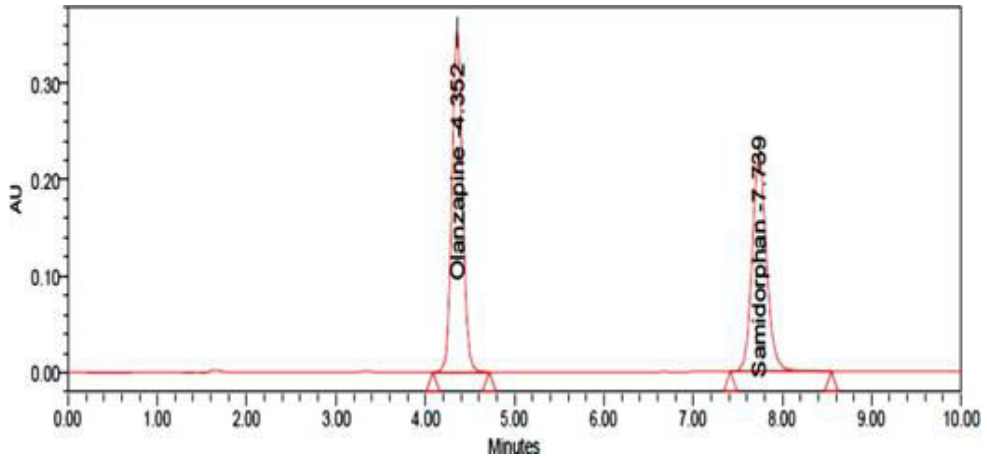

Figure 6.48: Chromatogram of stability 12 Hrs RT

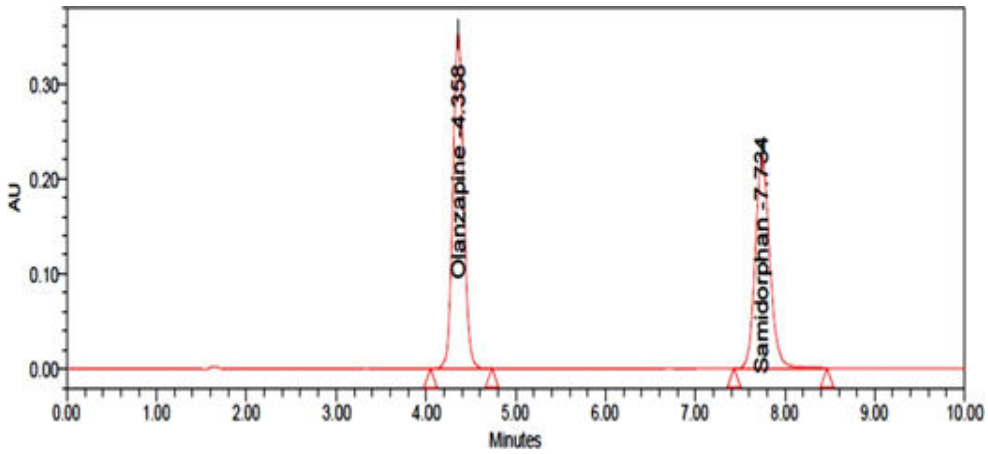

Figure 6.49: Chromatogram of stability 18 Hrs RT

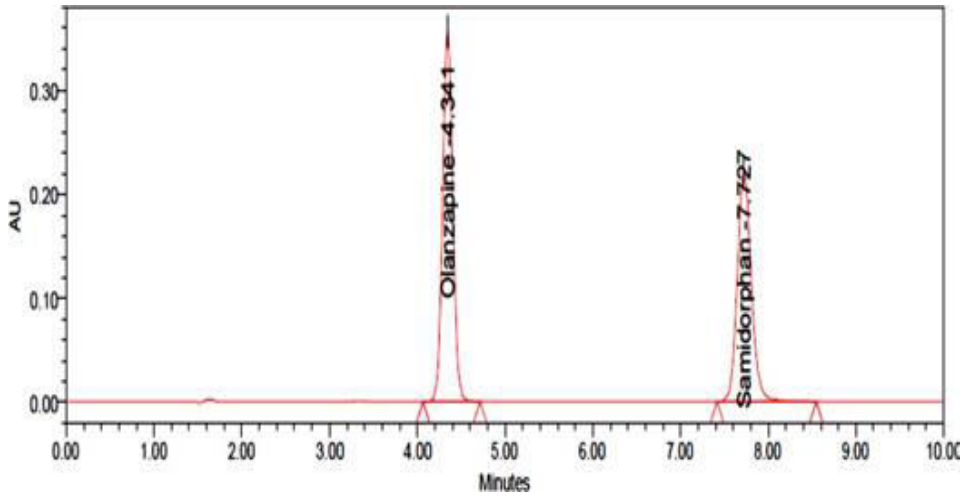

Figure 6.50: Chromatogram of stability 24 Hrs RT

6.7 CONCLUSION

In this research, a new HPLC technique for the simultaneous measurement of Olanzapine and Samidorphan in bulk and pharmaceutical dosage form was devised. It was fast, inexpensive, sensitive, and readily accessible. HPLC techniques haven't been published for this procedure, which is one of its primary benefits Shorter run times, lower costs, ease of use, sensitivity, dependability, and repeatability are all advantages of this approach. When a high number of samples must be examined, these characteristics are critical. This method's robustness and robustness were both determined to be within acceptable limits throughout the validation of all of the method's many parameters. Because the RSD values for each parameter are less than 2%, this implies that the approach is legitimate and the results achieved by this method are consistent. Since Olanzapine and Samidorphan formulations are routinely tested in quality control labs, the suggested approach may be employed without any prior separation.

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SUMMARY AND CONCLUSION

The analytical methods assume a great importance in quality assurance of many pharmaceutical industries due to i) development of new drugs ii) continuous changes in manufacturing processes for existing drugs and iii) setting up of threshold limits for individual and total impurities of drugs by regulatory authorities. This is accomplished by modern pharmaceutical analytical techniques which include the physico- chemical methods of analysis.

Our objective in the present research is to develop new liquid chromatographic methods for the assay of some selected drugs. This research deals with the chemistry of chromogenic reagents, the reactions used in the present investigation. The general methodology for developing new chromatographic methods optimization of experimental conditions (effect of pH, reagent concentration and order of addition, keeping time and temperature during each addition, effect of solvent, color development and stability) optical characteristics. Selectivity, precision, standard deviation, percent range of error, testing of significance by F-test, accuracy (comparison of the proposed and reference methods of pharmaceutical formulation, testing of significance by t-test and recovery experiments.

This research also includes the study of HPLC system components (solvent delivery systems, solvent degassing systems, gradient elution devices, sample introduction systems liquid chromatography detectors, column packing materials inclusive of bonded phase, derivatization, gradient elution), performance calculations (relative retention, theoretical plates, plates per meter, height equivalent to theoretical plate, capacity factor, resolution, peak asymmetry), linear fit properties of solvents used in chromatography and validation of analytical methods(recovery, response function, sensitivity, precision and accuracy).

Keeping this in view, an attempt was made by the author in the present investigation to develop new analytical methods for some of the important drugs in pure and pharmaceuticals dosage forms. All the methods described in the thesis are simple, rapid, reliable and valid. The methods could be used not only for quality control but also for process development of bulk drugs.

LIST OF ABBREVIATIONS

RP-HPLC	:	Reverse Phase High Performance Liquid Chromatography
ICH	:	International Conference on Harmonization
µL	:	Micro litre
MeOH	:	Methanol
H2O	:	Water
IR	:	Infra Red
ACN	:	Acetonitrile
Min	:	Minute
Hr	:	Hour
Conc	:	Concentration
µg	:	Micro gram
UV	:	Ultra violet
NLT	:	Not less than
^{O}C	:	Degrees Celsius
S.No	:	Serial Number
mL	:	Milli litre
Psi	:	Pounds per square inch
IP	:	Indian Pharmacopeia
LC	:	liquid chromatography
MS	:	Mass spectroscopy
GC	:	Gas Chromatography
TLC	:	Thin Layer Chromatography
UV	:	Ultra Violet
APCI	:	Atmospheric Pressure Chemical Ionization
APPI	:	Atmospheric Pressure Photo Ionization
DC	:	Duennschicht chromatographie
R_F	:	Retardation Factor
DDA	:	Data Dependent Acquisition
Da	:	Dalton
MS^n	:	Mass Spectrometric Technique
MCM	:	Million Cubic Meters
FDA	:	Food and Drug Administration
RSD	:	Relative Standard Deviation

S/N	:	Signal to noise ratio
S/B	:	Signal to Background Ratio
BCS	:	Biopharmaceutics Classification System
API	:	Active Pharmaceutical Ingredient
TNF	:	Tumor Necrosis Factor
PDA	:	Photo Diode Array Detector
ODS	:	Octadecyl-Silica
OPA	:	Ortho Phosphoric Acid
RT	:	Retention Time
USP	:	United States pharmacopeia
PPM	:	Parts Per Million
LOD	:	Limit of Detection
LOQ	:	Limit of Quantification
Org	:	Organic
ICH	:	International Conference on Hormonization
PDGFR	:	Platelet Derived Growth Factor Receptor
VEGF	:	Vascular Endothelial Growth Factor
DMSO	:	Dimethylsulfoxide
DMF	:	Dimethylformamide
DNA	:	Deoxyribonucleic Acid
RNA	:	Ribonucleic Acid
QTRAP	:	Quadrupole Linear Ion Trap
RPM	:	Revolutions per Minute
TFA	:	Tri Fluoro Acetic Acid
ISTD	:	Internal Standard
LLOQ	:	Lower Limit of Quantification

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ABOUT THE BOOK

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