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The method precision for the determination of assay was below 2.0%RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.

Tirthankar Choudhury completed: B. Pharm, M.Pharm, PhD, F.I.C.Teaching, has research experience of 12 years 8 Months. Present Designation: Professor, H.O.D. (Pharmaceutical Chemistry) Award & Achievement:-1) "Distinguished Professor" Award by IPES 2020 2) International Scientist Awards 2021:-
"Best Researcher Award" Award by VDGOOD Technology.

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RP-HPLC METHOD AND METHOD VALIDATION OF PIROXICAM BY USING SINGLE MOBILE PHASE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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DEDICATED TO MY
PARENTS, SON & FAMILY MEMBERS

TABLE OF CONTENTS

ABSTRACT

A rapid and precise Reverse Phase High Performance Liquid Chromatographic method has been developed for the validated of Piroxicam, in its pure form as well as in tablet dosage form. Chromatography was carried out on X-terra C18 (4.6 x 250mm, 5µm) column using a mixture of Acetonitrile and Water (40:60 v/v) as the mobile phase at a flow rate of 0.9ml/min, the detection was carried out at 227nm. The retention time of the Piroxicam was 5.430 ±0.02min respectively. The method produce linear responses in the concentration range of 10-50mg/ml of Piroxicam.

The method precision for the determination of assay was below 2.0%RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.

Keywords: Piroxicam, RP-HPLC, Validation, C18 Column

INTRODUCTION

1.1 Analytical chemistry[1]

Analytical chemistry is a scientific discipline used to study the chemical composition, structure and behaviour of matter. The purposes of chemical analysis are together and interpret chemical information that will be of value to society in a wide range of contexts. Quality control in manufacturing industries, the monitoring of clinical and environmental samples, the assaying of geological specimens, and the support of fundamental and applied research are the principal applications. Analytical chemistry involves the application of a range of techniques and methodologies to obtain and assess qualitative, quantitative and structural information on the nature of matter.

- **Qualitative Analysis** is the identification of elements, species and/or compounds present in sample.
- **Quantitative Analysis** is the determination of the absolute or relative amounts of elements, species or compounds present in sample.

Structural analysis is the determination of the spatial arrangement of atoms in an element or molecule or the identification of characteristic groups of atoms (functional groups). An element, species or compound that is the subject of analysis is known as analyte. The remainder of the material or sample of which the analyte(s) form(s) a part is known as the matrix.

The gathering and interpretation of qualitative, quantitative and structural information is essential to many aspects of human endeavour, both terrestrial and extra-terrestrials. The maintenance of an improvement in the quality of life throughout the world and the management of resources heavily on the information provided by chemical analysis. Manufacturing industries use analytical data to monitor the quality of raw materials, intermediates and finished products. Progress and research in many areas is dependent on establishing the chemical composition of man-made or natural materials, and the monitoring of toxic substances in the environment is of ever increasing importance. Studies of biological and other complex systems are supported by the collection of large amounts of analytical data. Analytical data are required in a wide range of disciplines and situations that include not just chemistry and most other sciences, from biology to zoology, butte arts, such as painting and sculpture, and archaeology. Space exploration and clinical diagnosis are two quite

desperate areas in which analytical data is vital. Important areas of application include the following.

Quality Control (**QC**) in many manufacturing industries, the chemical composition of raw materials, intermediates and finished products needs to be monitored to ensure satisfactory quality and consistency. Virtually all consumer products from automobiles to clothing, pharmaceuticals and foodstuffs, electrical goods, sports equipment and horticultural products rely, in part, on chemical analysis. The food, pharmaceutical and water industries in particular have stringent requirements backed by legislation for major components and permitted levels of impurities or contaminants. The electronic industry needs analyses at ultra-trace levels (parts per billion) in relation to the manufacture of semi-conductor materials. Automated, computer-controlled procedures for process-stream analysis are employed in some industries.

Monitoring and Control of Pollutants The presence of toxic heavy metals (e.g., lead, cadmium and mercury), organic chemicals (e.g., polychlorinated biphenyls and detergents) and vehicle exhaust gases (oxides of carbon, nitrogen and sulphur, and hydrocarbons) in the environment are health hazards that need to be monitored by sensitive and accurate methods of analysis, and remedial action taken. Major sources of pollution are gaseous, solid and liquid wastes that are discharged or dumped from industrial sites, and vehicle exhaust gases.

Clinical and Biological Studies The levels of important nutrients, including trace metals (e.g., sodium, potassium, calcium and zinc), naturally produced chemicals, such as cholesterol, sugars and urea, and administered drugs in the body fluids of patients undergoing hospital treatment require monitoring. Speed of analysis is often a crucial factor and automated procedures have been designed for such analyses.

Geological Assays The commercial value of ores and minerals are determined by the levels of particular metals, which must be accurately established. Highly accurate and reliable analytical procedures must be used for this purpose, and referee laboratories are sometimes employed where disputes arise.

Fundamental and Applied Research The chemical composition and structure of materials used in or developed during research programs in numerous disciplines can be of significance. Where new drugs or materials with potential commercial value are synthesized, a complete chemical characterization maybe required involving considerable analytical work. Combinatorial chemistry is an approach used in pharmaceutical research that generates very large numbers of new compounds requiring confirmation of identity and structure.

Analytical Techniques There are numerous chemical or physico-chemical processes that can be used to provide analytical information. The processes are related to a wide range of atomic and molecular properties and phenomena that enable elements and compounds to be detected and/or quantitatively measured under controlled conditions. The underlying processes define the various *Analytical Techniques*. The more important of these are listed in Table.No.1 together with their suitability for qualitative, quantitative or structural analysis and the levels of analyte(s) in a sample that can be measured. *Atomic, molecular spectrometry* and *chromatography*, which together comprise the largest and most widely used groups of techniques, can be further subdivided according to their physico-chemical basis. *Spectrometric techniques* may involve either the *emission or absorption* of *electromagnetic radiation* over a very wide range of energies, and can provide qualitative, quantitative and structural information for analytes from major components of a sample down to ultra-trace levels. The most important atomic and molecular spectrometric techniques and their principal applications are listed in Table.No.2.

Chromatographic techniques provide the means of separating the components of mixtures and simultaneous qualitative and quantitative analysis, as required. The linking of chromatographic and spectrometric techniques, called *hyphenation*, provides a powerful means of separating and identifying unknown compounds.

Electrophoresis's another separation technique with similarities to chromatography that is particularly useful for this parathion of charged species. The principal separation techniques and their applications are listed in **Table.No.3**.

Analytical Methods

An analytical method consists of a detailed, stepwise list of instructions to be followed in the qualitative, quantitative or structural analysis of a sample for one or more analytes and using a specified technique. It will include a summary and lists of chemicals and reagents to be used, laboratory apparatus and glassware, and appropriate instrumentation. The quality and sources of chemicals, including solvents, and the required performance characteristics of instruments will also be specified as will the procedure for obtaining a representative sample of the material to be analyzed. This is of crucial importance in obtaining meaningful results. The preparation or pre-treatment of the sample will be followed by any necessary standardization of reagents and/or calibration of instruments under specified conditions. Qualitative tests for the analyte(s) or quantitative measurements under the same conditions as those used for standards complete the practical part of the method. The remaining steps will be concerned with data processing, computational methods for quantitative analysis and the

formatting of the analytical report. The statistical assessment of quantitative data is vital in establishing the reliability and value of the data, and the use of various statistical parameters and tests is widespread. Many *Standard Analytical Methods* have been published as papers in analytical journals and other scientific literature, and in textbook form. Collections by trades associations representing, for example, the cosmetics, food, iron and steel, pharmaceutical, polymer plastics and paint, and water industries are available standards organizations and statutory authorities, instrument manufacturer's applications notes, the Royal Society of Chemistry and the US Environmental Protection Agency are also valuable sources of standard methods. Often, laboratories will develop their own *in-house methods* or adapt existing ones for specific purposes.

Method Development forms a significant part of the work of most analytical laboratories, and *method validation and* periodic revalidation is a necessity. Selection of the most appropriate analytical method should take into account the following factors:

- The purpose of the analysis, the required time scale and any cost constraints;
- The level of Analyte(s) expected and the detection limit required;
- The nature of the sample, the amount available and the necessary sample preparation procedure;
- The accuracy required for a quantitative analysis;
- The availability of reference materials, standards, chemicals and solvents, instrumentation and any special facilities;
- Possible interference with the detection or quantitative measurement of the analyte(s) and the possible need for sample clean-up to avoid matrix interference;
- The degree of selectivity available − methods may be selective for a small number of analytes or specific for only one.
- Quality control and safety factors.

Table.No.1. Analytical Techniques and Principal Applications

Technique	Property measured	Principal areas of application
Gravimetry	Weight of pure analyte or compound of known as stoichiometry	Quantitative for major or minor components
Titrimetry	Volume of standard reagent solution reacting with the analyte	Quantitative for major or minor Component
Atomic molecular spectrometry	Wavelength and intensity of electromagnetic radiation emitted/ absorbed by the analyte	Qualitative, quantitative or structural or for major down to trace level components
Mass spectrometry	Mass of analyte or fragments of it	Qualitative or structural for major down to trace level components isotope ratios
Chromatography and electrophoresis	Various physicochemical properties of separated analytes	Qualitative and quantitative separations of mixtures at major to trace levels
Thermal analysis	Chemical/physical changes in the analyte when heated or cooled	Characterization of single or mixed major/minor compounds
Electrochemical analysis	Electrical properties of the analyte in solution	Qualitative and quantitative for major to trace level components
Radiochemical analysis	Characteristic ionizing nuclear radiation emitted by the analyte	Qualitative and quantitative at major to trace levels

Table.No.2. Spectrometric Techniques and Principal Applications

Technique	Basis	Principal applications
Plasma emission spectrometry	Atomic emission after excitation in high temperature gas plasma	Determination of metals and some non-metals mainly at trace levels
Flame emission spectrometry	Atomic emission after flame excitation	Determination of alkali and alkaline earth metals
Atomic absorption spectrometry	Atomic absorption after atomization by flame or electro thermal means	Determination of trace metals and some non-metals
Atomic fluorescence spectrometry	Atomic fluorescence emission after flame excitation	Determination of mercury and hydrides of non-metals at trace levels
X-ray emission spectrometry	Atomic or atomic fluorescence emission after excitation by electrons or radiation	Determination of major and minor elemental components of metallurgical and geological samples
γ-spectrometry	γ-ray emission after nuclear excitation	Monitoring of radioactive elements in environmental samples
Ultraviolet/visible spectrometry	Electronic molecular absorption in solution	Quantitative determination of unsaturated organic
Infrared spectrometry	Vibrational molecular absorption	Identification of organic compounds
Nuclear magnetic resonance spectrometry	Nuclear absorption (change of spin states)	Identification and structural analysis of organic compounds
Mass spectrometry	Ionization and fragmentation of molecules	Identification and structural analysis of organic compounds

Table.No.3. Separation techniques and principal applications

Technique	Basis	Principal applications
Thin-layer chromatography	Differential rates of migration of analytes through a stationary phase by movement of a liquid or gaseous mobile phase	Qualitative analysis of mixtures
Gas chromatography	-Do-	Quantitative and qualitative determination of volatile compounds
High-performance liquid chromatography	-Do-	Quantitative and qualitative determination of non-volatile compounds
Electrophoresis	Differential rates of migration of analytes through a buffered medium	Quantitative and qualitative determination of ionic compounds

1.2 Chromatography [2]

1.2.1 Introduction

The chromatography was discovered by Russian Chemist and botanist *Micheal Tswett* (1872-1919) who first used the term chromatography (colour writing derived from Greek for colour – Chroma , and write – graphein) to describe his work on the separation of coloured plant pigments into bands on a column of chalk and other material such as polysaccharides, sucrose and insulin.

"*Chromatography is a method in which the components of a mixture are separated on an adsorbent column in a flowing system*".

The adsorbent material, or stationary phase, first described by Russian scientist named Tswett in 1906, has taken many forms over the years, including paper, thin layers of solids attached to glass plates, immobilized liquids, gels, and solid particles packed in columns. The flowing component of the system, or mobile phase, is either a liquid or a gas. Concurrent with development of the different adsorbent materials has been the development of methods

more specific to particular classes of analytes. In general, however, the trend in development of chromatography has been toward faster, more efficient.

"In his early papers of Tswett (1906) stated that chromatography is a method in which the component of a mixture are separated on an adsorbent column in a flowing system. Chromatography has progressed considerably from Tswett's time and now includes a number of variations on the basic separation process".

"Chromatography is a physical method of separation in which the component to be separated are distributed between two phases of which in stationary while other moves in a definite direction (IUPAC)"

1.2.2. Chromatographic Process[4]

Chromatographic separations are based on a forced transport of the liquid (mobile phase) carrying the analyte mixture through the porous media and the differences in the interactions at analytes with the surface of this porous media resulting in different migration times for a mixture components. In the above definition the presence of two different phases is stated and consequently there is an interface between them. One of these phases provides the analyte transport and is usually referred to as the mobile phase, and the other phase is immobile and is typically referred to as the stationary phase. A mixture of components, usually called analytes, are dispersed in the mobile phase at the molecular level allowing for their uniform transport and interactions with the mobile and stationary phases. High surface area of the interface between mobile and stationary phases is essential for space discrimination of different components in the mixture. Analyte molecules undergo multiple phase transitions between mobile phase and adsorbent surface. Average residence time of the molecule on the stationary phase surface is dependent on the interaction energy. For different molecules with very small interaction energy difference the presence of significant surface is critical since the higher the number of phase transitions that analyte molecules undergo while moving through the chromatographic column, the higher the difference in their retention. The nature of the stationary and the mobile phases, together with the mode of the transport through the column, is the basis for the classification of chromatographic methods.

1.2.3. Types of Chromatography

The mobile phase could be either a liquid or a gas, and accordingly we can subdivide chromatography into Liquid Chromatography (LC) or Gas Chromatography (GC). Apart from these methods, there are two other modes that use a liquid mobile phase, but the nature

of its transport through the porous stationary phase is in the form of either (a) capillary forces, as in planar chromatography (also called Thin-Layer Chromatography, TLC), or (b) electro osmotic flow, as in the case of Capillary Electro Chromatography (CEC).

Fig.No.1. Showing flow chart for classification of chromatography[4]

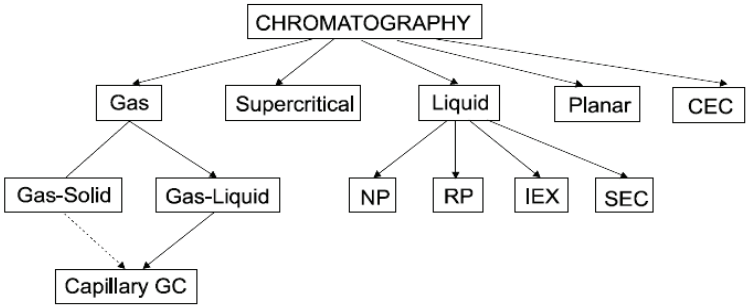


Table.No.4. Principles and classification of chromatography

Technique	Stationary Phase	Mobile Phase	Format	Principal sorption mechanism
Paper chromatography (PC)	Paper (cellulose)	Liquid	Planar	Partition (adsorption, ion-exchange, exclusion)
Thin-layer chromatography (TLC)	Silica, cellulose, ion-exchange, resin, controlled porosity solid	Liquid	Planar	Adsorption (partition, ion-exchange, exclusion)
Gas chromatography (GC)				
Gas-liquid chromatography (GLC)	Liquid	Gas	Column	Partition
Gas-solid chromatography (GSC)	Solid	Gas	Column	Adsorption
Liquid Chromatography (LC)				
High Performance Liquid Chromatography (HPLC)	Solid or bonded-phase	Liquid	Column	Modified partition (adsorption)
Size-Exclusion Chromatography (SEC)	Controlled porosity solid	Liquid	Column	Exclusion
Ion-Exchange Chromatography (IEC), Ion Chromatography (IC)	Ion-exchange resin or bonded-phase	Liquid	Column	Ion-exchange

Chiral Chromatography (CC)	Solid chiral Selector	Liquid	Column	Selective adsorption

1.3 METHODS IN CHROMATOGRAPHY[5]

1. According to nature of stationary and mobile phase

- Solid- Liquid chromatography
- Liquid-Liquid chromatography
- Gas- Solid chromatography
- Gas -Liquid chromatography

2. According to principle of separation

A. Adsorption chromatography

- Gas Solid chromatography
- Thin layer chromatography
- Column chromatography
- High performance liquid chromatography
- Affinity phase chromatography
- Hydrophobic Interaction chromatography (HIC)

B. Partition chromatography

- Gas liquid chromatography
- Paper partition chromatography
- Column partition chromatography

3. Based on modes of chromatography

- Normal phase chromatography
- Reversed phase chromatography

4. Other types of chromatography

- Size exclusion chromatography (SEC)
- Gel permeation chromatography
- Gel chromatography
- Gel Filtration
- Gel permeation chromatography
- Ion exchange chromatography
- Chiral chromatography

1. Adsorption chromatography

Chromatography in which separation is based mainly on difference between the adsorption affinities of the sample components for the surface of an active solid. The analyte interact with solid stationary surface and are displaced with eluent for active sites on surface.

2. Partition chromatography

This method results from a thermodynamic distribution of analytes between two liquid phases. On the basis of relative polarities of stationary and mobile phase, partition chromatography can be divided in to normal phase and reverse phase chromatography. In normal phase chromatography, the stationary phase bed is strongly polar in nature (e.g. Silica gel) and the mobile phase is non-polar (such as n-hexane or tetrahydrofuran). Polar sample are thus retained on polar surface of the column packing longer than polar material while in reverse phase chromatography, the stationary bed is non-polar (hydrophobic in nature, while the mobile phase is polar liquid, such as mixture of water and methanol or acetonitrile. Here the more non polar the material is, the longer it will retain.

3. Size-exclusion chromatography

This involves a solid stationary phase with controlled pore size. Solids are separated according to molecular size, with the large molecule unable to enter the pores eluted first.

4. Ion- exchange chromatography

Involves a solid stationary phase with anionic or cationic groups on the surface to separation, HPLC and HPTLC methods have widely been exploited in pharmaceutical analysis because of its simplicity, precision, accuracy and reproducibility of result.

5. Solid-Phase Extraction [SPE]

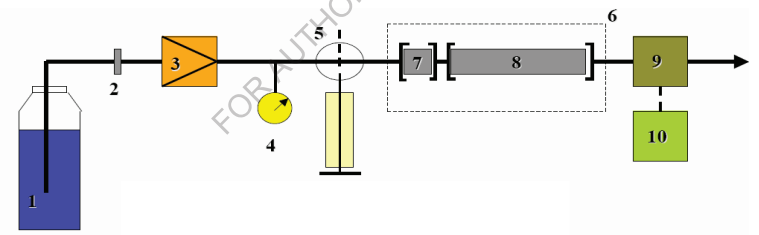
A sample preparation technique that uses LC principles to isolate, enriches, and/or purifies analytes from a complex matrix applied to a miniature chromatographic bed. *Offline* SPE is done with larger particles in individual plastic cartridges or in micro-elution plate wells, using low positive pressure or vacuum to assist flow. *Online* SPE is done with smaller particles in miniature HPLC columns using higher pressures and a valve to switch the SPE column online with the primary HPLC column, or offline to waste, as appropriate. SPE methods use step gradients to accomplish bed conditioning, sample loading, washing, and elution steps. The goal is to remove matrix interferences and to isolate the analyte in a solution, and at a concentration, suitable for subsequent analysis.

1.3.1. High Performance Liquid Chromatography (HPLC) [6]

The acronym *HPLC*, coined by the Late Prof. Csaba Horvath for his 1970 Pittconpaper, originally indicated the fact that high pressure was used to generate the flow required for liquid chromatography in packed columns. In the beginning, pumps only had a pressure capability of 500 psi [35 bars]. This was called *high pressure liquid chromatography*, or HPLC. The early 1970s saw a tremendous leap in technology. These new HPLC instruments could develop up to 6,000 psi [400 bars] of pressure, and incorporated improved injectors, detectors, and columns. With continued advances in performance during this time [smaller particles, even higher pressure], the acronym HPLC remained the same, but the name was changed to high performance liquid chromatography.

High Performance Liquid Chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantitative the compounds that are present in any sample that can be dissolved in a liquid. Today, compounds in trace concentrations as low as *parts per trillion* (ppt) may easily be identified. HPLC can be, and has been, applied to just about any sample, such as pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples, and industrial chemicals.

Fig.No.2. High-Performance Liquid Chromatography [HPLC] System



1 = eluent reservoir
2 = filter
3 = high pressure pump with pulse dampener
4 = pressure gauge
5 = sample injection valve with syringe
6 = column oven
7 = guard column
8 = column
9 = detector
10 = recorder (integrator, PC etc.)

1.3.2. Types of HPLC techniques[7]

1. Based on modes of separation

- Normal phase chromatography
- Reversed phase chromatography

2. Based on principal of separation

- Adsorption chromatography
- Partition chromatography
- Ion exchange chromatography
- Ion pair chromatography
- Size exclusion or Gel permeation chromatography
- Affinity chromatography
- Chiral phase chromatography

3. Based on elution technique

- Isocratic elution
- Gradient elution

4. Based on scale of operation

- Analytical HPLC
- Preparative HPLC

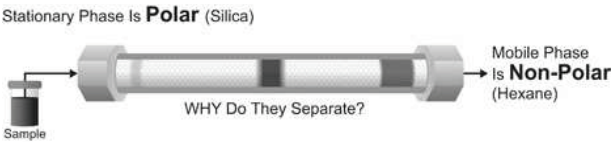
5. Based on types of analysis

- Qualitative analysis
- Quantitative analysis

1.3.3. Normal Phase Chromatography

Normal phase HPLC (NP-HPLC) was the first kind of HPLC chemistry used, and separate analytes, based on polarity. This method uses a polar stationary phase and a non-polar mobile phase, and is used when the analyte is fairly polar in nature. The polar analyte associates with and is retained by the polar stationary phase. Absorption strengths increase with increase in analyte polarity, and the interaction between the polar analyte and the polar stationary phase increases the elution time. The interaction strength not only depends on the functional groups in the analyte molecule, but also on stearic factors and structural isomers is often resolved from one another. Use of more polar solvents in the mobile phase will decrease the retention time of the analyte while more hydrophobic solvents tend to increase retention times. Particularly polar solvents in a mixture tend to deactivate the column by occupying the stationary phase surface.

Fig.No.3. Normal-Phase Chromatography

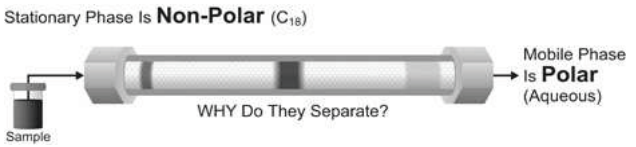


1.3.4. Reversed Phase Chromatography (RPC)

Reversed phase HPLC (RP-HPLC) consists of a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. The retention time is therefore longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily. Retention Time (R_t) is increased by the addition of polar solvent to the mobile phase and decreased by the addition of more hydrophobic solvent. The pharmaceutical industry regularly employs RPC to qualify drugs before their release.

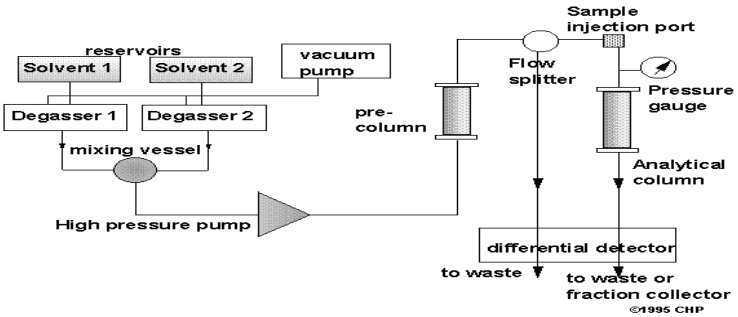
RPC operates on the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar analyte, and the non-polar stationary phase. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand in the aqueous eluent. The energy released in this process is proportional to the surface tension of the eluent (water: 73 erg/cm², methanol: 22 erg/cm²) and to the hydrophobic surface of the analyte and the ligand respectively. The retention can be decreased by adding less-polar solvent (MeOH, ACN) into the mobile phase to reduce the surface tension of water. Gradient elution uses this effect by automatically changing the polarity of the mobile phase during the course of the analysis.

Fig.No.4. Reversed-Phase Chromatography



1.3.5. Isocratic flow and gradient elution

Fig.No.5. High-Pressure-Isocratic System



A separation in which the mobile phase composition remains constant throughout the procedure is termed isocratic (meaning constant composition). The word was coined by Csaba Horvath, who was one of the pioneers of HPLC. The mobile phase composition does not have to remain constant. A separation in which the mobile phase composition is changed during the separation process is described as a gradient elution. One example is a gradient starting at 10% methanol and ending at 90% methanol after 20 minutes. The two components of the mobile phase are typically termed "A" and "B"; A is the "weak" solvent which allows the solute to elute only slowly, while B is the "strong" solvent which rapidly elutes the solutes from the column. In reverse-phase chromatography, solvent A is often water or an aqueous buffer, while B is an organic solvent miscible with water, such as acetonitrile, methanol, THF, or isopropanol.

1.3.6. Working Principle of HPLC [8]

The components of a basic High-Performance Liquid Chromatography [HPLC] system are shown in the simple diagram in figure 5. A reservoir holds the solvent [called the mobile phase, because it moves]. A high-pressure pump [solvent delivery system or solvent manager] is used to generate and meter a specified flow rate of mobile phase, typically millilitres per minute. An injector is able to introduce [inject] the sample into the continuously flowing mobile phase stream that carries the sample into the HPLC column.

The column contains the chromatographic packing material needed to effect the separation. This packing material is called the stationary phase because it is held in place by the column hardware. A detector is needed to see the separated compound bands as they elute from the HPLC column. The mobile phase exits the detector and can be sent to waste, or collected, as desired. When the mobile phase contains a separated compound band, HPLC provides the ability to collect this fraction of the elute containing that purified compound for further study. This is called preparative chromatography. The detector is wired to the computer data station,

the HPLC system component that records the electrical signal needed to generate the chromatogram on its display and to identify and quantitative the concentration of the sample constituents. Since sample compound characteristics can be very different, several types of detectors have been developed. For example, if a compound can absorb Ultra Violet light, a UV-absorbance detector is used. If the compound does not have either of these characteristics, a more universal type of detector is used, such as an Evaporative-Light-Scattering Detector [ELSD]. The most powerful approach is the use multiple detectors in series. For example, a UV and/or ELSD detector may be used in combination with a Mass Spectrometer [MS] to analyze the results of the chromatographic separation. This provides, from a single injection, more comprehensive information about an analyte. The practice of coupling a mass spectrometer to an HPLC system is called LC/MS.

1.4 Components of HPLC[9]

- Solvent
- Solvent Delivery System (Pumps)
- Injector
- Column
- Detectors
- Recorder (Data Collection)

Fig.No.6. Schematic of a modular HPLC instrument

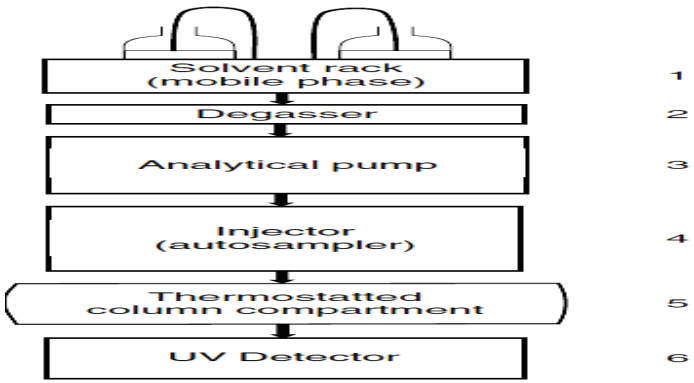
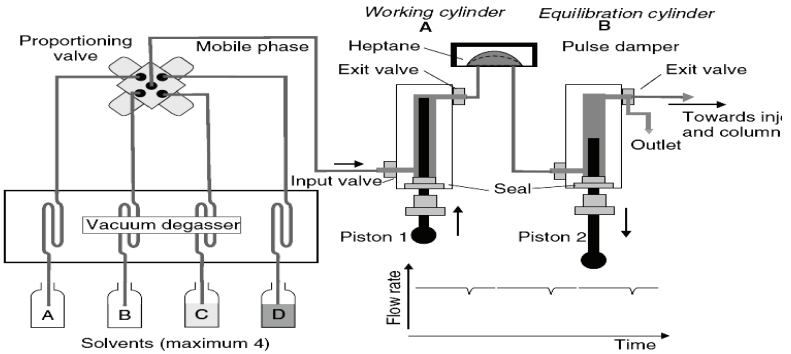


Fig.No.7. Schematic of the dual-headed reciprocating pump



The most simplified way of explaining the cycle of operation, without taking into account the compressibility of the solvents, is as follows. From the moment when the outlet valve of cylinder a closes and its entrance valve open, the piston in A, moving backwards, sucks the eluent through the inlet check valve and the chamber fills. Meanwhile cylinder B is open and its piston moves forward to force the mobile phase towards the injector and the column. The volume displaced by piston B is half of that available in the chamber of piston A. With chamber A full, the entrance valve of a closes and the corresponding outlet valve opens. Piston a now advances and pushes out the contents of the chamber. Half of this volume is expelled directly towards the column, the other half serves to fill cylinder B as piston B retracts. A pulse absorber is located between the two cylinders (diagram courtesy of Agilent Technologies).

1.4.1. Solvent

- Compatible with the instrument (pumps, seals, fittings, detector etc).
- Compatible with the stationary phase.
- Readily available (often use liters/day) and of adequate purity.
- Spectroscopic and trace-composition usually.
- Not too compressible (causes pump/flow problems).
- Free of gases (which cause compressibility problems).
- The polarity index is a measure of the relative polarity of a solvent. It is used for identifying suitable mobile phase solvents.
- Increasing eluent strength or polarity index values mean increasing solvent polarity, and
- The analyte(s) and samples must be mobile phase and stationary phase compatible.

1.4.2. Pumps

- It drives the mobile phase from the reservoir to the column.
- Because of the small particles used in modern HPLC, modern pumps need to operate reliably and precisely at pressures of 10,000 p.s.i. or at least 6,000 p.s.i.
- To operate at these pressures and remain sensibly inert to the wide variety of solvents used, HPLC pumps usually have sapphire pistons, stainless steel cylinders and return valves fitted with sapphire balls and stainless steel seats.
- For analytical purposes HPLC pumps should have flow rates that range from 0 to 10 ml/min., but for preparative HPLC, flow rates in excess of 100 ml/min may be required.
- The level of constancy is required because most HPLC detectors are flow sensitive and errors in quantization will result from changes in flow rate.

TYPES

- Syringe pumps
- Reciprocating pumps
- Pneumatic pumps

Other errors are added to this due to capillary action and the small dimensions/cavities inside the injector.

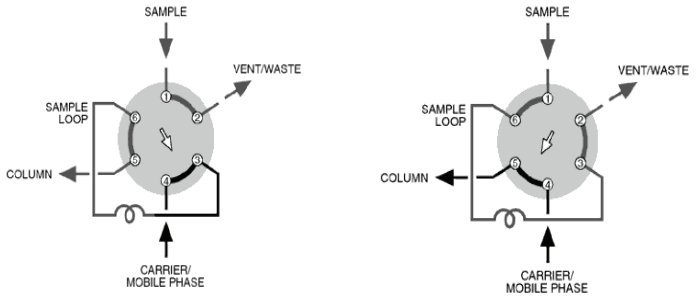
1.4.3. Injectors

- There are three types of injectors, they are
    1. Septum injectors
    2. Stop flow injectors
    3. Rheodyne injectors

There are two modes of sample injection in LC, they are

1. Load position
2. Inject position

Fig.No.8. Injectors port used in HPLC.



LOAD
(The sample loop)

INJECT
(Move the sample loop in the mobile phase)

1.4.4. Columns

- The column is one of the most important components of the LC chromatograph because the separation of the sample components is achieved when those components pass through the column.
- The Liquid Chromatography apparatus is made out of stainless steel tubes with a diameter of 3 to 5mm and a length ranging from 10 to 30cm.
- Normally, columns are filled with silica gel because its particle shape, surface properties and pore structure help to get a good separation.

Types of columns

1. Guard column
2. Analytical column

Guard Column

- Placed between injector and analytical column
- Same material as that of analytical column
- Does not contributes to any separation
- Eliminate particulate and impurities
- Extend life time of analytical column
- Protect the system component

Analytical Column

Most important part of HPLC technique which decides efficiency of separation.

Column Material

- ❖ Stainless steel
- ❖ Glass
- ❖ Polyethylene
- ❖ Polyether ether ketone (PEEK)

Column Dimensions

Column length10-30 cm long

Internal diameter 5-10 mm

Packing Techniques

- ➢ Dry packing
- ➢ Wet / slurry packing

Column packing material

- ➢ **Micro Porous Supports**

  3-10 µm in diameter

  Composed of silica, alumina, ion exchange resin.

- ➢ **Pellicular Supports**

  40 µm in diameter

  Porous particles are coated onto an inert solid core such as glass bed.

- ➢ **Bonded Phases**
  - ❖ Stationary phase is chemically bonded onto inert support.
  - ❖ Liquid- liquid partition system.
  - ❖ Stationary phase coated on to the inert support.
  - ❖ Both micro porous and pellicular supports are used for supporting liquid Phase.

Liquid- liquid partition system

- ❖ Stationary phase coated on to the inert support.
- ❖ Both micro porous and pellicular supports are used for supporting liquid Phase.

Disadvantages

- Developing solvent may gradually wash off the liquid Phase with repeated use.
- To overcome this problem bonded phases have been developed.
- Ex.- silica, silicone polymer.

- **For adsorption chromatography**
  - Adsorbents such as silica or alumina are available as micro porous forms.
  - Pellicular systems generally have a high efficiency but low sample capacity and so micro porous supports are preferred.
  - Spherical shape gives good efficiency and flow properties.

Disadvantages

- Developing solvent may gradually wash off the liquid Phase with repeated use.
- To overcome this problem bonded phases have been developed.
- Ex.- silica, silicone polymer etc.,

Stationary phase

Polar (normal phase): Silica, alumina, Cyano, amino or diol terminations on the bonded phase.

Non-polar (reversed phase): C18 to about C8 terminations on the bonded phase, Phenyl and Cyano terminations on the bonded phase.

- Mixtures of functional groups can be used.

Packed particles in a column require:

- Frits at the ends of the column to keep the particles in.
- Filtering of samples to prevent clogging with debris
- High pressure pumps and check-valves
- Often a Guard Column to protect the analytical column

1.4.5. General HPLC column care[9]

The correct use of an HPLC column is extremely important for the life time of a column and therefore for the benefit of your HPLC analysis. The following lines will give us some guidelines for the use, cleaning and storage of HPLC columns. These guidelines depend on

the one hand on the nature of the chromatographic support (silica, polymers or others) and on the other hand on the surface chemistry of the corresponding stationary phase.

Silica based columns

General Guidelines

Silica is the ideal support for HPLC columns. It offers a large mechanical stability, excellent physicochemical surface properties, a wide range of bonding chemistries and is compatible with a broad range of organic solvents. However, the following points are extremely important to know when working with silica based HPLC columns.

pH Stability

In general, HPLC columns are stable within a pH range of 2 to 8. If you are measuring a pH value, the measurement must be done in the aqueous media before mixing the eluent with organic solvents. Modern HPLC columns can be used outside that pH range. The new bonding chemistries allow use down to pH-1 for some stationary phases. However, please check vendor's product information before using silica based column outside the pH range of 2 to 8. However, best lifetimes are obtained between pH 2.0 and pH 6.8. Stationary phases based on ultra-pure silica gel can also be used at higher pH ranges, up to pH 11, depending on the chemical nature of the modifier used in the mobile phase. Large bases (like Pyrolidine) are not able to attack the surface of the silica and therefore can be used at higher pH values. If you are working at pH values above 8 with small bases as modifier (like Ammonia), we highly recommend using stationary phases based on Polymers or Zirconium dioxide.

Mechanical Stability

Stationary phases based on silica are mechanically very stable. The packed columns show no pressure limit and can be used at more than 40 Mpa (6000 psi) without any problem. However, please avoid pressure shocks on the column. Pressure shocks lead to channelling in the column, which results in peak splitting in the corresponding chromatogram.

Mobile Phases (Eluents)

Silica based stationary phases are compatible with all organic solvents in the above mentioned pH range. Please use the highest quality solvents available (HPLC grade). Also, please filter all prepared buffer through a 0.5µm filter before using them in your HPLC

system. The use of non pure solvents in HPLC causes irreversible adsorption of impurities on the column head. These impurities block adsorption sites, change the selectivity of the column and lead to peak splitting in the chromatogram. In gradient elution, impurities cause so called "Ghost Peaks". Ghost peaks are peaks that always appear in the same position on the chromatogram. Their origin is not the sample, but the impurities from the solvents or solvent additives. Therefore, it is highly recommended to run a gradient without injection in the beginning of each method to determine the ghost peaks. To avoid irreversible adsorption at the column head, you should always use a pre-column. The use of a pre-column increases the life time of a column dramatically. In addition, a pre-column can filter solid parts stemming from pump seals or injection rotors. An alternative to a pre-column is an in-line filter. These filters are attached directly to the column. These filters get rid of solid parts in the eluent but will not avoid irreversible adsorption of organic impurities.

Proper storage of HPLC columns

For short term storage, i.e. overnight, columns can be stored in the eluent used in last analysis.

For middle term storage, i.e. 2 days or over the weekend, columns should be flushed with pure water to prevent microbial growth.

For long term storage

Silica based columns should be stored in an aprotic solvent.

- The water content should not be higher than 50%. The best storing solvent is acetonitrile.
- *Caution:* Please make sure that all buffers are washed out of the column before flushing with Acetonitrile. Buffer salts are mainly not soluble in Acetonitrile and can block the capillaries and the column.

FACTORS AFFECTING COLUMN EFFICIENCY

- Dimensions of column
- Particle size of adsorbent
- Nature of solvent
- Temperature of column
- Pressure
- Packing of column

Equilibration time

- The equilibration time of a column depends on the column dimensions. In general, a column is equilibrated after flushing with 20 column volumes. The equilibration time for the most important column dimensions is summarized in the following Table. No.5.
- Shorter equilibration times are possible if you simply increase the flow rate. It is no problem to do that if no chromatography is done. However, 20 column volumes are necessary to ensure a 100% equilibration.

Table.No.5. Equilibration times

Column dimension	Column volume [ml]*	Flow rate [ml/min]	Equilibration time [min]
250 x 4.6 mm	2.91	1.00	58
150 x 4.6 mm	1.74	1.00	35
100 x 4.6 mm	1.16	1.00	23
50 x 4.6 mm	0.58	1.00	12
250 x 4.0 mm	2.20	1.00	44
125 x 4.0 mm	1.10	1.00	22
250 x 2.0 mm	0.55	0.25	44
150 x 2.0 mm	0.33	0.25	26
50 x 2.0 mm	0.11	0.25	9

Regeneration of a column

- Irreversible adsorption of impurities stemming from the matrix on the column head can cause changes in selectivity or peak splitting. Often those “dirty columns” can be regenerated by applying the following protocols.

Regeneration of RP packing’s

- RP- packing’s are C18, C8, and C4, C1, C30, CN or Phenyl stationary phases.
- Flush the column with 20 column volumes water
- Flush the column with 20 column volumes acetonitrile
- Flush the column with 5 column volumes isopropanol

- ❖ Flush the column with 20 column volumes heptane
- ❖ Flush the column with 5 column volumes isopropanol

Regeneration of NP (Normal Phase) packing's

- ➢ NP-packing's are Silica, Diol, Nitro and Amino stationary phases.
- ➢ Flush the column with 20 column volumes Heptane
- ➢ Flush the column with 5 column volumes Isopropanol
- ➢ Flush the column with 20 column volumes Acetonitrile
- ➢ Flush the column with 20 column volumes Water
- ➢ Flush the column with 20 column volumes Acetonitrile
- ➢ Flush the column with 5 column volumes Isopropanol
- ➢ Flush the column with 20 column volumes Heptane

Regeneration of Ion Exchange Pickings

Ion exchange packing's are Anion or Cation exchangers (WCX, SCX, WAX and SAX) Flush the column with 20 column volumes of the same eluent, but double the buffer concentration. Follow the regeneration protocol for RP packing's (see above) Flush with 20 column volumes of water equilibrate the column now to the original conditions.

Polymer Based Columns

Polymer based stationary phases show higher pH stability but lower mechanical stability, compared to silica based columns. Also, polymer based packing's are not compatible with all organic solvents. They swell or shrink in some organic solvents. Unfortunately, the pressure stability and solvent compatibility are different for the different nature of polymers and from manufacturer to manufacturer. Therefore, no general rules for the column care of polymer based materials can be given. Always read the instructions for the use of those columns. In case of doubt please contact the corresponding manufacturer.

1.4.6 Detectors of HPLC [10-14]

A chromatography detector is a device used liquid chromatography (LC) to visualize components of the mixture being eluted off the chromatography column.

There are two general types of detectors: destructive and non-destructive.

- ❖ The destructive detectors perform continuous transformation of the column effluent (burning, evaporation or mixing with reagents) with subsequent measurement of

some physical property of the resulting material (plasma, aerosol or reaction mixture).

- ❖ The non-destructive detectors are directly measuring some property of the column effluent (for example UV absorption) and thus affords for the further analyte recovery.

The detector for an HPLC is the component that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram. It is positioned immediately posterior to the stationary phase in order to detect the compounds as they elute from the column.

Characteristics of Ideal LC Detector

- ❖ Low drift and noise level (particularly crucial in trace analysis).
- ❖ High sensitivity.
- ❖ Fast response.
- ❖ Wide linear dynamic range (this simplifies quantitation).
- ❖ Low dead volume (minimal peak broadening).

There are many types of detectors that can be used with HPLC. Some of the more common detectors include:

- ❖ Refractive Index (RI)
- ❖ Ultra-Violet (UV)
- ❖ Fluorescent
- ❖ Radiochemical
- ❖ Electrochemical
- ❖ Near-Infra Red (Near-IR)
- ❖ Mass Spectrometry (MS)
- ❖ Nuclear Magnetic Resonance (NMR)
- ❖ Light Scattering (LS)

1. Refractive Index (RI) Detectors

- ➢ Measure the ability of sample molecules to bend or refraced light. This property for each molecule or compound is called its refractive index.
- ➢ For most RI detectors, light proceeds through a bi-modular flow-cell to a photo detector.

- Detection occurs when the light is bent due to samples eluting from the column and this is read as a disparity between the two channels.

Types of RI detector

Two types of RI are available, they are:

- Deflection type (most popular)
- Reflection type (measure changes in % reflected light at glass-liquid interface)

Deflection type RI Detector

The reason it's called deflection type is because deflection is created in a rectangular sample cell by separating the compartment into two parts with a diagonal glass divider.

Operating Principles

- ✓ Light from the source is focused onto the sample cell, which consist of sample and the reference chamber**.**
- ✓ After deflection from the mirror, light is diverted through an optical zero adjustment (beam splitter) into the detector, which actually consist of two photo-cells, P1 and P2.
- ✓ When a solute elutes off the column the RI of the sample compartment
- ✓ This causes a change in the amount of deflected light, which in turn changes the relative amount falling on P1 and P2.

Disadvantages of RI Detector

a) Not-suitable for gradient elution, changes in solvent composition change RI. Therefore, baseline shifts and *S/N* are affected.

b) Require careful control of the column and detector temperature.

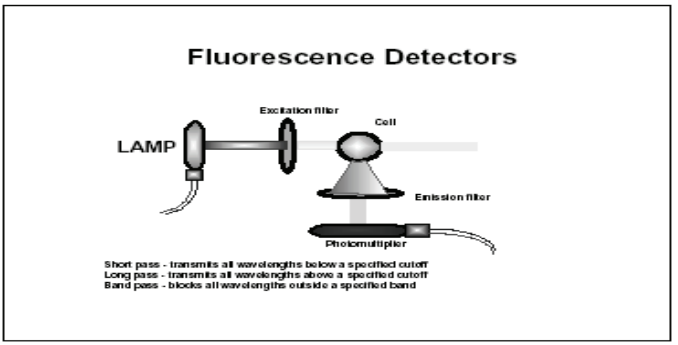
c) Moderate sensitivity10^{-9} to 10^{-10}g, not useful for trace analysis.

2. Fluorescence Detector

Measure the ability of a compound to absorb then re-emit light at given wavelengths.

- ❖ Each compound has a characteristic fluorescence.

Fig.No.9. Fluorescence detector



- The excitation source passes through the flow-cell to a photo detector while a monochromator measures the emission wavelengths.
- It has sensitivity limit of 10^{-9} to 10^{-11}gm/ml.

Working

- This excitation λ of light then passes through the column effluent in the flow cell.
- Radiation from a xenon or deuterium lamp passes through an excitation filter, which provides essentially monochromatic light of desired wavelength to excite the sample.
- When the sample molecule passes through the column effluent they are excited and emit light (fluorescence) at a longer λ.
- A second (emission) filter is positioned at 90^0 to the first filter to collect the emitted light.
- In this way only the light emitted from the sample fluorescence will pass on PMT for quantitation of the emission signal.

3. Radiochemical detection

It involves the use of radio labeled material, usually tritium (3H) or carbon-14 (14C). It operates by detection of fluorescence associated with *beta*-particle ionization and it is most popular in metabolite research.

Types of Detector:

a) Homogeneous: Where addition of scintillation fluid to column effluent causes fluorescence.

B) Heterogeneous: Where lithium silicate and fluorescence caused by beta-particle emission

interact with the detector cell.

It has sensitivity limit up to 10^{-9} to 10^{-10} gm/ml.

4. Mass spectrometry (ms) detectors

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

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

B) Chemical Ionization A less aggressive method which utilizes ionized gas to remove electrons from the compounds eluting from the column.

C) Fast Atom Bombardment (FAB) Xenon atoms are propelled at high speed in order to ionize the eluents from the column.

It has detection limit of 10^{-8} to 10^{-10} gm/ml.

5. Light-scattering (LS) detectors

When a source emits a parallel beam of light which strikes particles in solution, some light is reflected, absorbed, transmitted or scattered.

Two forms of LS detection may be used to measure the later occurrences. **A) Nephelometry** This is defined as the measurement of light scattered by a particulate solution. This method enables the detection of the portion of light scattered at a multitude of angles. The sensitivity depends on the absence of background light or scatters since the Detection occurs at the black or null background.

B) Turbidimetry This is defined as the measure of the reduction of light transmitted due to particles in solution. It measures the light scatter as a decrease in the light that is transmitted through the particulate solution. Therefore, it quantifies the residual light transmitted. Sensitivity of this method depends on the sensitivity of the machine employed, which can range from a simple spectrophotometer to a sophisticated discrete.

6. Nuclear magnetic resonance (NMR) detectors

- Certain nuclei with odd- numbered masses, including H and 13C, spin about an axis in a random fashion.
- However, when placed between poles of a strong magnet, the spins are aligned either parallel or anti-parallel to the magnetic field, with the parallel orientation favored since it is slightly lower in energy.

- ❖ The nuclei are then irradiated with electromagnetic radiation which is absorbed and places the parallel nuclei into a higher energy state; consequently they are now in "resonance" with the radiation.
- ❖ Each H or C will produce different spectra depending on their location and adjacent molecules or elements in the compound, because all nuclei in molecules are surrounded by electron clouds which change the encompassing magnetic field and thereby alter the absorption frequency.

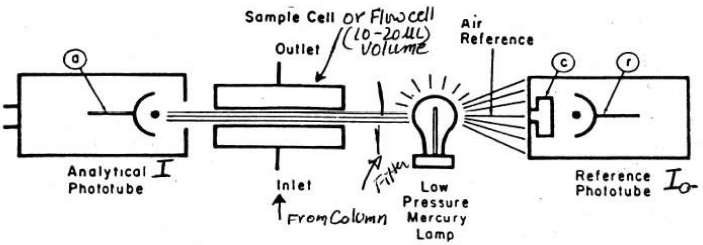
7. Near-Infrared detectors

- ➢ Operates by scanning compounds in a spectrum from 700 to 1100 nm.
- ➢ Stretching and bending vibrations of particular chemical bonds in each molecule are detected at certain wavelengths.
- ➢ This is a method which offers several advantages
    - ❖ Speed (sometimes less than 1 second)
    - ❖ Simplicity of preparation of sample
    - ❖ Multiple analyses from single spectrum
    - ❖ Non-consumption of the sample

8. Ultra-Violet (UV) detector

It is divided in to three types they are fixed wavelength, variable wavelength, and diode array detectors.

Fig.No.10. Ultra-Violet (UV) Detector



It measures the ability of a sample to absorb light. This can be accomplished at one or several wavelengths

- Fixed wavelength measures at one wavelength, usually 254 nm
- Variable wavelength measures at one wavelength at a time, but can detect over a wide range of wavelengths
- Diode Array measures a spectrum of wavelengths simultaneously
- UV detectors have a sensitivity to approximately 10^{-8} or 10^{-9} gm/ml.

A. Fixed Wavelength Detectors

a) It is the most common and inexpensive detector. The use of suitable λ is determined by the nature of the light source used.

b) Deuterium lamp can be used over a range of wavelength (covers a continuum of wavelengths), hence covering most of the UV spectral region.

B. Variable Wavelength Detectors

Provides detection of eluted peak at any selected intensity.

a) It is less sensitive than fixed wavelength but the detection wavelength can be varied.

b) Deuterium source is mostly used because it provides continuum source. This can be combined with a suitable monochromator in dual beam mode.

C. Diode Array detectors (DAD)

- A diode array consists of a number of photosensitive diodes placed side by side and insulated from one another in the form of a multi-layer sandwich.
- The common use of a diode array is to monitor light that has passed through a liquid sensor cell as in a multi-wavelength liquid chromatography detector.
- There are two major advantages of diode array detection:

  1. In the first, it allows for the best wavelength to be selected for actual analysis. This is particularly important when no information is available on molar absorptivities at different wavelengths.

  2. The second major advantage is related to the problem of peak purity. Often, the peak shape in itself does not reveal that it actually corresponds to two (or even more) components.

1.5. Chromatographic parameters [4]

1. Retention time (R_t)
2. Efficiency (N)
3. Retention volume (V_r)
4. Resolution factor (R_s)
5. Column Efficiency (N)
6. HETP (High Equivalent Theoretical Plates)
7. Capacity factor (mass distribution ratio, D_m)
8. Symmetry factor (A_s)
9. Tailing Factor (T)

Retention time

Retention time is the difference in time between the points of injection and eluted from a column. Retention time is measured in minutes or seconds. Retention time is also proportional to the distance moved on a chart paper, which can be measured in cm or mm.

Retention volume

The distance of the peak maxima from the injection point expressed in time units is called retention time (t_R), and it serves as an identifier for the given analyte on that particular system. Retention time is probably the most widely used descriptor of the analyte behaviour, and it is the most easily measurable parameter. However, even though it is easily measurable,

it is the least universal parameter. Analyte retention time is dependent on the mobile phase flow rate; the faster the flow rate, the smaller the analyte retention time. It is also dependent on the flow rate stability. The product of the analyte retention time and the mobile-phase flow rate is the retention volume (V_R).

Retention volume (V_R) = Retention time (t_R) x flow rate

HETP (High Equivalent Theoretical Plates)

A theoretical plate can be of any height, which decides the efficiency of separation. If HETP is less the column is more efficient. If HETP is more, the column is less efficient. The height equivalent to a theoretical plate (HETP) is given by

HETP=Length of column (L)/N

Where

N = plates per meter

RT = retention time of the components.

W = width of the base of the component peak using tangent method.

L = column length in meters

Capacity factor (mass distribution ratio, D_m)

This factor determines the retention of a solute and can be calculated from the chromatogram using the following formula:

$$D_m = \frac{(t_R - t_M)}{t_M}$$

Where,

t_R = retention time of the solute

t_M = retention time of an unretained component

A low D_m value indicates that the peak elutes close to the solvent front, which may compromise selectivity. A minimum D_m value of 1 is recommended for the peak of interest.

Void volume

The volume of the liquid phase in the column is called "*void volume*" (ω). Several other names are also used in the chromatographic literature: "dead volume," "hold-up volume," and sometimes "retention volume of non retained component." In this book we will be using term "void volume."

$$t_R = \frac{V_R}{F}, \qquad t_0 = \frac{V_0}{F}$$

Void time can be interpreted as part of the total analyte retention time that the analyte actually spends in the mobile phase moving through the column, and for the rest of the retention time the analyte sits on the stationary phase surface.

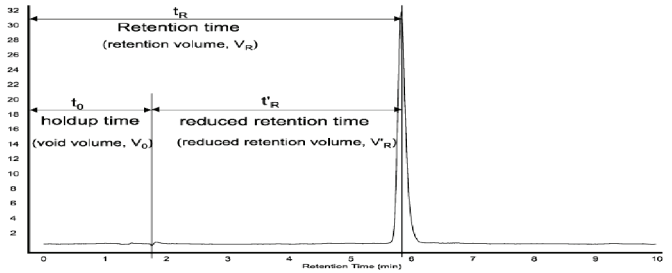
Retention Factor

The ratio of the reduced retention volume to the void volume is a widely used dimensionless parameter called *retention factor*, *k*.

$$k = \frac{V_R - V_0}{V_0} = \frac{V_R'}{V_0} = \frac{t_R - t_0}{t_0}$$

Retention factor (sometimes called capacity factor) is a very convenient chromatographic descriptor since it is dimensionless and independent on the mobile phase flow rate and column dimensions. Ideally if the retention of the same analyte was measured on two instruments equipped with columns of different dimensions with the same type of a stationary phase and the same mobile phase, theoretically the retention factors of that analyte on both systems should be identical.

Fig.No.11. Showing retention factor



Resolution

The distance between the peak maxima reflects the selectivity of the system. The greater the distance, the higher the selectivity. The width of the chromatographic peak reflects the system band broadening and thus efficiency. Resolution, *R*, is defined as the ratio of the distance between two peaks to the average width of these peaks (at baseline), and this descriptor encompasses both the efficiency and selectivity.

For the resolution of a so-called "critical pair" of analytes (two analytes in the mixture that have minimal distance between them compared to all other analytes in the

$$R = 2\frac{t_{R,2} - t_{R,1}}{w_2 + w_1}$$

Mixture), if they have relatively high retention factors ($k\_ > 5$) that their peak widths can be assumed as equal, reduces to

$$R = \frac{t_{R,2} - t_{R,1}}{w}$$

Peak width could be expressed from equation

$$w = \frac{4t_R}{\sqrt{N}}$$

If we select the retention of the second analyte for the calculation of the peak width, then applying equation in to below equation

$$R = \frac{t_{R,2} - t_{R,1}}{t_{R,2}} \cdot \frac{\sqrt{N}}{4}$$

Relatively simple algebraic conversion will bring us to so-called Master Resolution Equation

$$R = \frac{\alpha - 1}{\alpha} \cdot \frac{k_2}{1 + k_2} \cdot \frac{\sqrt{N}}{4}$$

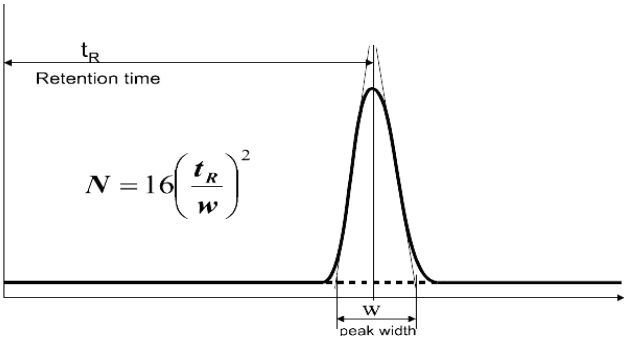
Efficiency (N)

The efficiency is the measure of the chromatographic band broadening and the number of the theoretical plates (*N*) in the column and is usually calculated using the following equation

$$N = 16\left(\frac{t_r}{t_w}\right)^2$$

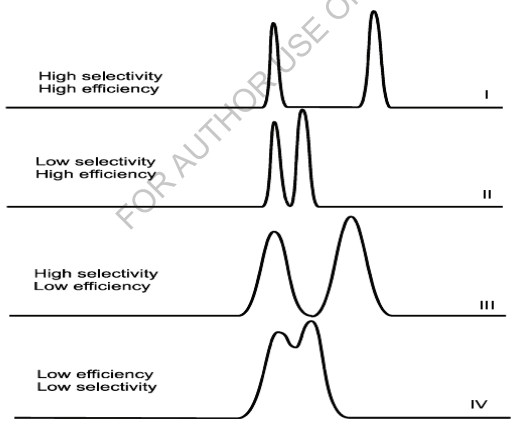
Where t_R is the analyte retention time and *w* is the peak width measured in time units as the distance between the intersections of the tangents to the peak inflection points with the baseline, as shown in Figure.no.12. Column efficiency is mainly dependent on the kinetic factors of the chromatographic system such as molecular diffusion, mass-flow dynamics, properties of the column packing bed, flow rate, and so on. The smaller the particles and the more uniform their packing in the column, the higher the efficiency. The faster the flow rate, the less time analyte molecules have for diffusive band-broadening. At the same time, the faster the flow rate, the further analyte molecules are from the thermodynamic equilibrium with the stationary phase. This shows that there should be an optimum flow rate that allows achievement of an optimum efficiency for a given column.

Fig.No.12. Efficiency



Efficiency and selectivity are complementary chromatographic descriptors. A column with high efficiency can generate narrow chromatographic zones and allows the separation of analytes with low selectivity.

Fig.No.13. Efficiency



Selectivity The ability of the chromatographic system to discriminate different analytes is called selectivity. Selectivity is determined as the ratio of the retention factors of two analytes, or the ratio of the reduced retention times.

$$\alpha = \frac{k_2}{k_1} = \frac{t_{R_2} - t_0}{t_{R_1} - t_0}$$

Tailing factor (T) the Tailing factor T, a measure of peak symmetry is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced. In some cases, values less than 1 maybe observed. As peak asymmetry increases integration and hence precision becomes less reliable.

Where,

$$T = W_{0.05} / 2f$$

$W_{0.05}$ = width of peak at 5% height

f = Distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline Limit: ≤2

1.5. Analytical Method Development[16-17]

A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible and it should allow the use of sophisticated tools such as computer modeling. The important factors, which are to be taken into account to obtain reliable quantitative analysis, are

- Careful sampling and sample preparation.
- Appropriate choice of the column.
- Choice of the operating conditions to obtain the adequate resolution of the mixture.
- Reliable performance of the recording and data handling systems.
- Suitable integration/peak height measurement technique.
- The mode of calculation best suited for the purpose.
- Validation of the developed method.

Careful sampling and sample preparation[17]

Before beginning method development it is need to review what is known about the sample in order to define the goals of separation. The sample related information that is important is summarized in following.

The sample related summarized relation

- Number of compounds present, chemical structures
- Molecular weights of compounds

- pKa values of compounds, UV spectra of compounds
- Concentration range of compounds in samples of interest
- Sample solubility

The chemical composition of the sample can provide valuable clues for the best choice of initial conditions for an HPLC separation.

Table No. 6 Table showing special samples and customized conditions

Sample	Requirements
Inorganic ions	Detection is primary problem; use ion chromatography.
Isomers	Some isomers can be separated by reversed-phase HPLC and are then classified as regular samples better separations of isomers are obtained using either (1) normal-phase HPLC or (2) reversed-phase separations with cyclodextrin-silica columns.
Enantiomers	These compounds require "chiral" conditions for their separation.
Biologicals	Several factors make samples of this kind "special": molecular conformation, polar functionality, and a wide range of hydrophobicity.

Choice of the column

The selection of the column in HPLC is somewhat similar to the selection of columns in G.C, in the sense that, in the adsorption and partition modes, the separation mechanism is based on inductive forces, dipole-dipole interactions and hydrogen bond formation. In case of ion-exchange chromatography, the separation is based on the differences in the charge, size of the ions generated by the sample molecules and the nature of ionisable group on the stationary phase. In the case of size-exclusion chromatography the selection of the column is based on the molecular weight and size of the sample components.

Choice of the operating conditions to obtain the adequate resolution of the mixture

Most of the drugs come under the category of regular samples. Regular samples mean typical mixtures of small molecules that can be separated using more or less standardized starting conditions. Regular samples can be further classified as neutral or ionic. Samples classified as

ionic include acids, bases, amphoteric compounds and organic salts. If the sample is neutral buffers or additives are generally not required in the mobile phase. Acids or bases usually require the addition of a buffer to the mobile phase. For basic or cationic samples, less acidic reverse phase columns are recommended. Based on recommendations of the conditions, the first exploratory run is carried and then improved systematically. On the basis of the initial exploratory run isocratic or gradient elution can be selected as most suitable. If typical reverse-phase conditions provided inadequate sample retention it suggests the use of either ion-pair or normal phase HPLC. Alternatively the sample may be strongly retained with 100% acetonitrile as mobile phase suggesting the use of non-aqueous reverse phase chromatography or normal phase HPLC.

Getting Started on Method Development

One approach is to use an isocratic mobile phase of some average organic solvent strength (50%). A better alternative is to use a very strong mobile phase first (80-100%) then reduce %B as necessary. The initial separation with 100% B results in rapid elution of the entire sample but few groups will separate. Decreasing the solvent strength shows the rapid separation of all components with a much longer run time, with a broadening of latter bands and reduced retention sensitivity. Goals that are to be achieved in method development are briefly summarized.

Separation or resolution is a primary requirement in quantitative HPLC. The resolution (R_s) value should be maximum (R_s>2) favors maximum precision. Resolution usually degrades during the life of the column and can vary from day to day with minor fluctuations in separation conditions. Therefore values of R_s=2 or greater should be the goal during method development for sample mixtures. Such resolution will favor both improved assay precision and greater method ruggedness. Some HPLC assays do not require base line separation of the compounds of interest (qualitative analysis). In such cases only enough separation of individual components is required to provide characteristic retention times for peak identification. The time required for a separation (runtime = retention time for base band) should be as short as possible and the total time spent on method development is reasonable (runtimes 5 to 10 minutes are desirable).

Repeatable separation

As the experimental runs described above are being carried out, it is important to confirm that each chromatogram can be repeated. When we change conditions (mobile phase, column, and

temperature) between method development experiments, enough time must elapse for the column to come into equilibrium with the new mobile phase and temperature.

Usually column equilibration is achieved after passage of 10 to 20 volumes of the new mobile phase through the column. However this should be confirmed by repeating the experiment under the same conditions. When constant retention times are observed in two such back-to-back repeat experiments (± 0.5% or better), it can be assumed that the column is equilibrated and the experiments are repeatable.

Optimization of HPLC method

During the optimization stage, the initial sets of conditions that have evolved from the first stages of development are improved or maximized in terms of resolution and peak shape, plate counts, asymmetry, capacity factor, elution time, detection limits, limit of quantitation and overall ability to quantify the specific analyte of interest.

The various parameters that include to be optimized during method development are

- Selection of mode of separation.
- Selection of stationary phase.
- Selection of mobile phase.
- Selection of detector.

Selection of mode of separation

In reverse phase mode, the mobile phase is comparatively more polar than the stationary phase. For the separation of polar or moderately polar compounds the most preferred mode is reverse phase. The nature of the analyte is the primary factor in the selection of the mode of separation. A useful and practical measurement of peak shape is peak asymmetry factor and peak tailing factor. Peak asymmetry is measured at 10% of full peak height and peak tailing factor at 5%. Reproducibility of retention times and capacity factor is important for developing a rugged and repeatable method.

Buffers and buffer capacity

Buffer and its strength play an important role in deciding the peak symmetries and separations. Some of the most commonly employed buffers are phosphate buffers.

Table No. 7 Table showing buffers and buffer capacity

Buffer	pK_a (25°C)	Maximum Buffer Range	UV Cut-off (nm)
TFA	0.3	--	210 (0.1%)
Phosphate, H_2PO_4 pK_1	2.1	1.1-3.1	< 200
Phosphate, pK_2 HPO_4^{2-}	7.2	6.2-8.2	< 200
Phosphate, pK_3 PO_4^{3-}	12.3	11.3-13.3	< 200
Citrate, pK_1 $C_3H_5O(CO_2H)_2(CO_2-)_1$	3.1	2.1-4.1	230
Citrate, pK_2 $C_3H_5O(CO_2H)_1(CO_2-)_2$	4.7	3.7-5.7	230
Citrate, pK_3 $C_3H_5O(CO_2-)_3$	6.4	4.4-6.4	230
Carbonate, pK_1 HCO_3^{2-}	6.1	5.1-7.1	< 200
Carbonate, pK_2 CO_3^{2-}	10.3	9.3-11.3	> 200
Formate	3.8	2.8-4.8	210 (10nM)
Acetate	4.8	3.8-5.8	210 (10nM.)
Ammonia	9.3	8.3-10.3	200 (10nM)
Borate	9.2	8.2-10.2	N / A
TEA	10.8	9.8-11.8	< 200

Mobile Phase Composition

Most chromatographic separations can be achieved by choosing the optimum mobile phase composition. This is due to the fact that fairly large amount of selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Most widely used solvents in reverse phase chromatography are methanol and acetonitrile. Experiments should be conducted with mobile phases having buffers with different pH and different organic phases to check for the best separations of analyte peak. A mobile phase

which gives separation of analyte peak and which is rugged for variation of both aqueous and organic phase by at least ± 0.2% of the selected mobile phase composition should be used.

Selection of Detector

The detector was chosen depending upon some characteristic property of the analyte like UV absorbance, florescence, conductance, oxidation, reduction etc. The characteristics that are to be fulfilled by a detector to be used in HPLC determination are,

- High sensitivity facilitating trace analysis.
- Negligible baseline noise to facilitate lower detection, Low dead volume.

Pharmaceutical ingredients do not absorb all UV light equally, so that selection of detection wavelength is important. An understanding of the UV light absorptive properties of the organic impurities and the active pharmaceutical ingredient is very helpful. For the greatest sensitivity λ_{max} should be used. Ultra violet wavelengths below 200nm should be avoided because detector noise increases in this region. Higher wavelengths give greater selectivity.

1.6 ANALYTICAL METHOD VALIDATION

Method validation can be defined as per ICH "Establishing documented evidence which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics".

ICH Method validation parameters[18-19]

For chromatographic methods used in analytical applications there is more consistency in validation. Related substances are commonly present in the pharmaceutical products but those are always within the limits as specified in ICH (Q2B).

- Specificity
- Linearity
- Accuracy
- Precision
- Limit of Detection
- Limit of Quantitation
- Robustness
- System suitability

1.5.1 Specificity/Selectivity

Specificity is ability to assess unequivocally the analyte in the presence of components that may be expected to be present. The terms selectivity and specificity are often used interchangeably. According to ICH the term specific generally refers to a method that produces a response for a single analyte only while the term selectivity refers to a method that provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate.

Specificity is the ability of a method to discriminate between the analytes of interest and other components that are present in the sample. Studies are designed to evaluate the degree of interference, if any which can be attributed to other analytes, impurities, degradation products, reagent "blanks" and excipients. This provides the analyst with a degree of certainty that the response observed is due to the single analyte of interest. The degree of specificity testing varies depending on the method type and the stage of validation. Specificity should be evaluated continually through the drug development process. Specificity is sometimes used interchangeably with the term "selectivity". The argument over which term is more correct is one of semantics. Although there is some dissention, the term "specificity" has been adopted by the regulatory guidance documents and should be used to prevent further confusion.

- Blank solution to show no interference with any HPLC system.
- Placebo to demonstrate the lack of interference from excipients.
- Drug substance to show that all significant related substances are resolved from the drug substance.
- Authentic samples of critical related substances to show that all known related substances are resolved from each other.

1.5.2 Accuracy

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

Accuracy may be inferred once precision, linearity and specificity have been established. Accuracy for the area percent method should be established from 50% of the ICH reporting limit to the nominal concentration of drug substance in the sample solution. For the high–low

and external standard methods, determine accuracy from 50% of the ICH reporting level to 150% of the proposed shelf life specification of the related substances. In addition for the area percent and high–low methods, it is necessary to determine the accuracy of the related substances and the drug substance. For the external standard method only the accuracy of related substances is required. Since the response of the drug substance in the sample solution is not used in the external standard calculation it is not necessary to determine accuracy for the drug substance. Typically known amounts of related substances and the drug substance in placebo are spiked to prepare an accuracy sample of known concentration of related substance. According to the ICH accuracy should be determined using a minimum of nine determinations over a minimum of three concentration levels covering the range.

1.5.3 Precision

ICH defines the precision of an analytical procedure as the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. ICH has defined precision to contain three components: repeatability, intermediate precision and reproducibility. Ruggedness as defined in USP XXII <1225>, 1990 incorporates the concepts described under the terms *"intermediate precision", "reproducibility*" and *"repeatability*" of this guide.

1.5.4 Linearity

Linearity of an analytical procedure as its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample.

Concentration Range

The concentration range used for linearity should be large enough to encompass the desired range of the method. A minimum of five concentration ranges should be investigated and a plot of the detector response vs. the sample concentration should be generated. It is important that the concentration ranges selected for the linearity study are relatively equally spaced throughout the range of the method (e.g., 25%, 50%, 75%, 100%, 125% and 150%), and not clustered, as this will provide a skewed estimation of linearity.

Acceptance Criteria

Solutions of known concentrations are used to determine the linearity. A plot of peak area versus concentration (in percent related substance) is used to demonstrate the linearity. Authentic samples of related substances with known purity are used to prepare these solutions. In most cases, for the linearity of a drug product, spiking the related substance authentic sample into excipients is not necessary, as the matrix effect should be investigated in method accuracy. Visual inspection is the most sensitive method for detecting nonlinearity. Therefore, the plot has to be linear by visual inspection. In addition, according to ICH guidelines, the following results should be reported: slope, correlation coefficient, y-intercept, and residual sum of squares. Under most circumstances, regression coefficient (r^2) is 0.999. Intercept and slope should be indicated.

Table No. 8 Table showing acceptance criteria

Test	Level	Range	Acceptance criteria
Assay	5	50% to 150%	R> 0.999,
Dissolution	5-8	10% to 150%	R > 0.99,
Impurity	5	LOQ to 2%	R > 0.98

1.5.5. Limit of Detection (LOD)

Limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected, but not necessarily qualtitated, under the stated experimental conditions. With UV detectors, it is difficult to assure the detection precision of low level compounds due to potential gradual loss of sensitivity of detector lamps with age or noise level variation by detector manufacturer. At low levels, assurance is needed that the LOD and LOQ limits are achievable with the test method each time. With no reference standard for a given impurity or means to assure detectability, extraneous peak(s) could "disappear / appear." A crude method to evaluate the feasibility of the extraneous peak detection is to use the percentage claimed for LOD from the area counts of the analyte. Several approaches for determining the LOD are possible, depending on whether the procedure is a non-instrumental or instrumental.

- Based on visual evaluation
- Based on signal-to-noise
- Based on the standard deviation of the response and the slope

The LOD may be expressed as:

$$\mathbf{LOD = 3.3\ \sigma / S}$$

Where,

σ = Standard deviation of Intercepts of calibration curves

S = Mean of slopes of the calibration curves

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

1.5.6. Limit of quantification

Limit of quantification (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. Several approaches for determining the LOQ are possible depending on whether the procedure is a non-instrumental or instrumental.

- Based on visual evaluation
- Based on signal-to-noise Approach
- Based on the standard deviation of the response and the slope

The LOQ may be expressed as

$$\mathbf{LOQ = 10\ \sigma / S}$$

Where,

σ = Standard deviation of Intercepts of calibration curves

S = Mean of slopes of the calibration curves

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

1.5.7 Robustness

The robustness of an analytical procedure is defined as a measure of its capacity to obtain comparable and acceptable results when perturbed by small but deliberate variations in specified experimental conditions. Robustness provides an indication of the test method's suitability and reliability during normal use. During a robustness study, conditions are intentionally varied to see if the method results are affected. The key word in the definition is deliberate. Example HPLC variations are illustrated for isocratic and gradient methods, respectively.

Examples of typical variations are:

- Stability of analytical solutions
- Extraction time

In the case of liquid chromatography, examples of typical variations are

- Influence of variations of pH in a mobile phase
- Influence of variations in mobile phase composition
- Different columns
- Temperature
- Flow rate.
-

1.5.8 System Suitability

According to the USP, system suitability tests are an integral part of chromatographic methods. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. The purpose of the system suitability test is to ensure that the complete testing system is suitable for the intended application. Similar to the analytical method development, the system suitability test strategy should be revised as the analysts develop more experience with the assay. In general consistency of system performance, and chromatographic suitability. (Tailing factor, column efficiency and resolution of the critical pair, detector sensitivity) are the main components of system suitability.

Table No. 9 Showing system suitability parameters and recommendations

Parameter	Recommendation
Capacity Factor (k')	The peak should be well-resolved from other peaks and the void volume, generally k' 1 to 20
Repeatability	RSD $\leq$ 1% for N $\geq$ 5 is desirable.
Relative retention	Not essential as long as the resolution is stated.
Resolution (R_s)	R_s of > 2 between the peak of interest and the closest eluting potential interfering (impurity, excipient, degradation product, internal standard, etc.)
Tailing Factor (T)	T of >0.5 and $\leq$ 2
Theoretical Plates (N)	N < 2000

DRUG PROFILE

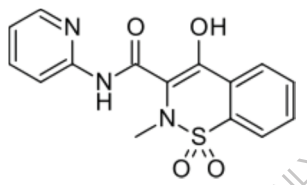
PIROXICAM

Synonym **:** Piroxicamum

Drug category : Anti-Inflammatory Agents

Brand name (Single Drug) : Roxam

Structure:



Chemical name/ Nomenclature / IUPAC Name: 4-Hydroxy-2-methyl—N-(2-pyridinyl)-2H-1,2-benzothiazine-3-carboxamine 1,1-dioxide

Molecular Formula : $C_{15}H_{13}N_3O_4S$

Molecular Weight : 331.348gm/mole

Official Pharmacopoeia **:** European pharmacopoeia

PHYSICOCHEMICAL PROPERTIES

Description (Physical State) **:** solid

Solubility **:** water

Stability : 23mg/L (at22 °C

StorageConditions **:** Store it at room temperature

Dosage **:** 20 mg

Melting point **:** 198-200 °C

pKa(strongest acidic **:** 6.3

Log P **:** 3.06

PHARMACOKINETIC PROPERTIES:

Bioavailability:

Half-life : 50hours

Absorption : Well absorbed from the GIT (oral)

Volume of Distribution : Detected in breast milk. Protein-binding: 99%

Protein binding : 99%

Metabolism: Metabolism: Metabolism of piroxicam occurs by hydroxylation at the 5 position of the pyridyl side chain and conjugation of this product; by cyclodehydration; and by a sequence of reactions involving hydrolysis of the amide linkage, decarboxylation, ring contraction and N-demethylation. The biotransformation products of piroxicam metabolism are reported to not have any anti-inflammatory activity.

Excretion: Mainly in urine (as metabolites and unchanged drug), faeces.

Adverse Effects: GI disturbances, peptic ulcer, GI bleeding, headache, dizziness, blurred vision, tinnitus, skin rashes and pruritus. Haematological changes and photosensitivity. Potentially Fatal: Thrombocytopaenia and acute nephropathy. Toxic epidermal necrolysis and Stevens-Johnson syndrome.

PHARMACODYNAMICS : Piroxicam capsules are a non-steroidal anti-inflammatory drug (NSAID) that exhibits anti-inflammatory, analgesic, and antipyretic activities in animal models. The mechanism of action of Piroxicam, like that of other NSAIDs, is not completely understood but may be related to prostaglandin synthetase inhibition.

Mechanism of Action: Piroxicam is an NSAID and, as such, is a non-selective COX inhibitor possessing both analgesic and antipyretic properties.[3]

Adverse Reactions: As with other NSAIDs the principal side effects include: digestive complaints like nausea, discomfort, diarrhoea and bleeds or ulceration of the stomach, as well as headache, dizziness, nervousness, depression, drowsiness, insomnia, vertigo, hearing disturbances (such as tinnitus), high blood pressure, oedema, light sensitivity, skin reactions

(including, albeit rarely, Stevens-Johnson syndrome and toxic epidermal necrolysis) and rarely, kidney failure, pancreatitis, liver damage, visual disturbances, pulmonary eosinophilia and alveolitis. Compared to other NSAIDs it is more prone to causing GI disturbances and serious skin reactions.

Contraindications: Active peptic ulcer, hypersensitivity to aspirin (acetylsalicylic acid) or other anti-inflammatory agents.

INTERACTIONS: Increased risk of hyperkalaemia when used with ACE inhibitors and potassium-sparing diuretics. Convulsions may occur when used with quinolones. Effects of phenytoin and sulfonylureas may be enhanced. Antihypertensive effects of ACE inhibitors, β-blockers and diuretics may be reduced. Increased risk of GI bleeding and ulceration when used with corticosteroids, SSRIs or antiplatelet agents. Increased risk of haematotoxicity when used with zidovudine. ACE inhibitors, ciclosporin, tacrolimus or diuretics may increase nephrotoxicity.

Potentially Fatal: May potentiate anticoagulants, increased levels of lithium, methotrexate and cardiac glycosides.

Drug Interactions: piroxicam reduce renal blood flow and thereby decrease the efficacy of diuretics, and inhibit the elimination of lithium and methotrexate

Food Interaction and Precautions: Take with food, avoid alcohol.

LITERATURE REVIEW

Surya N. Singh *et al.,*(2014) Estimation of Non micronized Piroxicam in SEDDS Formulation by HPLC Method retention time of Piroxicam was about 12 minutes. The method was validated for its specificity, accuracy, precision, and linearity, limit of detection (LOD), limit of quantification(LOQ), robustness and stability parameters. The linear regression analysis data for the calibration plots shows a good linear relationship over the concentration range of 5-150 mg/mL. The method showed good recoveries (98.0 – 99.8%) and has been applied to formulation without interference of excipients in the formulation. The result of method was reproducible and within official limits. The HPLC method has been proved more authentic as it can be used for the quantitative and entrapment efficient to determine non micronized Piroxicam in SEDDS formulation in hard gelatin capsules.[28]

Abdulkarim *et al.,*(2011) Modification and Validation of an HPLC Method for Quantification of Piroxicam is a NSAID that is widely used in the treatment of joint pain and osteoarthritis. The objectives of the study were to modify and validate HPLC method so as to obtain an accurate, sensitive and precise method to quantify piroxicam concentrations without interference from the other ingredients presence in the formulation. The method published by Owen et al. was adapted and modified to suit the above requirements. The modification was carried out on the mobile phase as the mobile phase used by the authors was not able to separate the drug peak from the interference of the formulation excipients. The modified mobile phase consisted of 5 mM of disodium hydrogen phosphate adjusted to pH 3 with concentrated ortho phosphoric acid, methanol, Acetonitrile and glacial acetic acid at ratios of 27:20:52:1 respectively. The method was validated and found to be specific, precise, accurate and reproducible even when run at different times of the same day or on different times on different days. The limit of detection and quantification were determined to be 0.035 Î¼g/ml and 0.0625 Î¼g/ml respectively. It could be concluded that this method could be used to determine piroxicam concentration in the samples collected from in vitro study of permeability through the synthetic membrane and excised rat skin.[29]

Madhukar A *et al.,*(2011), Rapid analytical method development and validation of Piroxicam by RP-HPLC. This paper describes the analytical method suitable for validation of Piroxicam by reversed Phase High Performance liquid chromatography (RP-HPLC) method. The method utilized RPHPLC (Younglin with UV-detector) model and a column, 150mm ´

4.6 mm, 5m (Symmetry, ODS- 3V, 150mm ´ 4.6mm, 5m). The mobile phases were comprised of Methanol and Water pH 3.2 (55:45v/v). Validation experiments were performed to demonstrate System suitability, precision, linearity and Range, Accuracy study, stability of analytical solution and robustness. The method was linear over the concentration range of 1-200 mg/ML-1. The method showed good recoveries (99.8 – 102.9%). [30]

Vijay Kumar. R *et al.,* (2010) Analytical method development and validation of Piroxicam by RP-HPLC. This paper describes the analytical method suitable for validation of Piroxicam by reversed Phase High Performance liquid chromatography (RP-HPLC) method. The method utilized RP-HPLC (Water 2695 with PDA detector) model and a column, 150mm × 4.6 mm, 5µ (Inertsil, ODS- 3V, 150mm × 4.6mm, 5µ). The mobile phases were comprised of A, B of Methanol and Buffer pH 3.0 (55:45v/v). Validation experiments were performed to demonstrate System suitability, precision, linearity and Range, Accuracy study, stability of analytical solution and robustness. The method was linear over the concentration range of 5-150 µg/ML-1. The method showed good recoveries (98.0 – 99.8%)[31]

M.Saeed Arayne *et al* (2005) Determination and quantification of piroxicam in tablets by RP-HPLC. A simple and rapid high performance liquid chromatographic(HPLC) method for the determination and quantification of piroxicam has been developed and validated; meloxicam was used as internal standard. Methanol/water (70:30 v/v) was used as mobile phase with flow rate 2ml/min. p^H was adjusted to 2.6 with phosphoric acid. UV detection was performed at 230 nm .Recovery of piroxicam (in Feldene flash tablets 20mg tablets) was from 99.49 to 100.29 %[32]

AIM AND OBJECTIVES

- Review of literature for piroxicam gave information regarding its physical and chemical properties, various analytical methods that were conducted alone and in combination with other drugs.
- Literature survey reveals that certain chromatographic methods were reported for the estimation of Piroxicam and single method is available for such estimation by RP-HPLC.
- In view of the need for a suitable RP-HPLC method for routine analysis of piroxicam in formulations, attempts were made to develop simple, precise and accurate analytical method for the estimation of Piroxicam and extend it for their determination in formulation.
- Validation is a necessary and important step in both framing and documenting the capabilities of the developed method.
- The utility of the developed method to determine the content of drug in commercial formulation was also demonstrated. Validation of the method was done in accordance with USP and ICH guideline for the assay of active ingredient. The method was validated for parameters like system suitability, linearity, precision, accuracy, specificity, ruggedness, robustness, limit of detection and limit of quantification. This method provides means to quantify the component. This proposed method was suitable for the analysis of Pharmaceutical dosage forms.

PRIMARY OBJECTIVE OF PROPOSED WORK

- ✓ To develop new simple, sensitive, accurate and economical analytical method for the estimation of Piroxicam.
- ✓ To validate the proposed method in accordance with USP and ICH guidelines for the intended analytical application i.e., to apply the proposed method for analysis of the piroxicam in dosage form.

MATERIALS AND METOD

EXPERIMENTAL WORK

INSTRUMENTS USED

Table: Instruments used

S.No	Instruments And Glassware	Model
1	HPLC	WATERS, software: Empower 2, Alliance 2695 separation module. 996 PDA detector.
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Enertech

CHEMICALS USED:

S.No	Chemical	Brand names
1	Piroxicam	Sura labs
2	Water and Methanol for HPLC	LICHROSOLV (MERCK)
3	Acetonitrile for HPLC	Merck

HPLC METHOD DEVELOPMENT:

TRAILS

Preparation of standard solution:

Accurately weigh and transfer 10 mg of piroxicam working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

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

Procedure:

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Water and Water: Acetonitrile with varying proportions. Finally, the mobile phase was optimized to Acetonitrile, Water in proportion 40:60 v/v respectively.

Optimization of Column:

The method was performed with various columns like C18 column, X- bridge column, Xterra, and C18 column. X-Terra C18 (4.6 x 150mm, 5µm, Make: X terra) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Instrument used : Waters HPLC with auto sampler and PDA Detector 996 model.

Column Temperature : 30ºC

Mobile phase : Acetonitrile: Water (40:60)

Stationary Phase : X-Terra C18 (4.6×250mm) 5µ

Flow rate : 0.9ml/min

Wavelength : 227nm

Injection volume : 10µl

Run time : 10min

VALIDATION

PREPARATION OF MOBILE PHASE:

Preparation of Mobile Phase:

Accurately measured 400 ml (40%) of Acetonitrile and 600 ml of Water (60%) were mixed and degassed in an digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

VALIDATION PARAMETERS

SPECIFICITY STUDY OF DRUG:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of piroxicam working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

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

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

Preparation of Sample Solution:

Take average weight of Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight of piroxicam sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

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

Procedure:

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

PREPARATION OF DRUG SOLUTIONS FOR LINEARITY:

Accurately weigh and transfer 10 mg of piroxicam working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (10 ppm of piroxicam):

Further pipette 0.1ml of the above piroxicam solution into a 10ml of volumetric flask and dilute up to the mark with diluent.

Preparation of Level – II (20 ppm of piroxicam):

Further pipette 0.2 ml of the above Piroxicam solution into a 10ml of volumetric flask and dilute up to the mark with the diluents.

Preparation of Level – III (30 ppm of piroxicam):

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

Preparation of Level – IV (40 ppm of piroxicam):

Further pipette 0.4ml of the above Piroxicam solution into a 10ml of volumetric flask and dilute up to mark with the diluents

Preparation of Level – V (50 ppm of piroxicam):

Further pipette 0.5ml of the above Piroxicam solution into a 10ml of volumetric flask and dilute up to mark with the diluents

Procedure:

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

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

PRECISION

REPEATABILITY

Preparation Of piroxicam Product Solution for Precision:

Accurately weigh and transfer 10 mg of piroxicam working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

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

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

INTERMEDIATE PRECISION:

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

Procedure:

DAY 1:

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

DAY 2:

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

Accuracy:

For preparation of 50% Standard stock solution:

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

Further pipette 0.15ml of the above piroxicam stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

For preparation of 100% Standard stock solution:

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

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

For preparation of 150% Standard stock solution:

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

Further pipette 0.45ml of the above piroxicam stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:

Inject the Three replicate injections of individual concentrations (50%,100%,150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for piroxicam and calculate the individual recovery and mean recovery values.

ROBUSTNESS:

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results. .

For preparation of Standard solution:

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

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

Effect of Variation of flow conditions:

The sample was analyzed at 0.8 ml/min and 1.0 ml/min instead of 0.9ml/min, remaining conditions are same. 10µl of the above sample was injected twice and chromatograms were recorded

Effect of Variation of mobile phase organic composition:

The sample was analyzed by variation of mobile phase i.e. ACN: Water was taken in the ratio and 35:65, 45:55 instead of 40:60, remaining conditions are same. 10µl of the above sample was injected twice and chromatograms were recorded.

RESULTS AND DISCUSSION

Trails

Trail 1:

Column : Inertsil C18 (4.6×250mm) 5µ

Column temperature : 35ºC

Wavelength : 227 nm

Mobile phase ratio : Methanol: Water (15:85v/v)

Flow rate : 0.7ml/min

Injection volume : 10µl

Run time : 10min

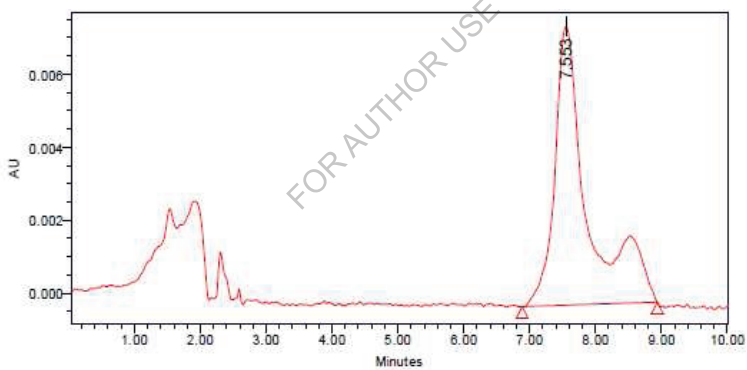


Figure: chromatogram for trail 1

Table: Peak Results for Trail 1

S.No	Peak Name	R_t	Area	Height	USP Tailing	USP Plate count
1	Piroxicam	7.553	263569	7627	1.87	903

OBSERVATION:

In this trial it shows less plate count and improper separation of peak in the chromatogram. so its required more trials to obtain good peaks.

Trail 2:

Column : Inertsil C18 (4.6×250mm)5µ

Column temperature : 35ºC

Wavelength : 227nm

Mobile phase ratio : Methanol: Water (80:20)

Flow rate : 1.2ml/min

Injection volume : 10µ1

Run time : 8min

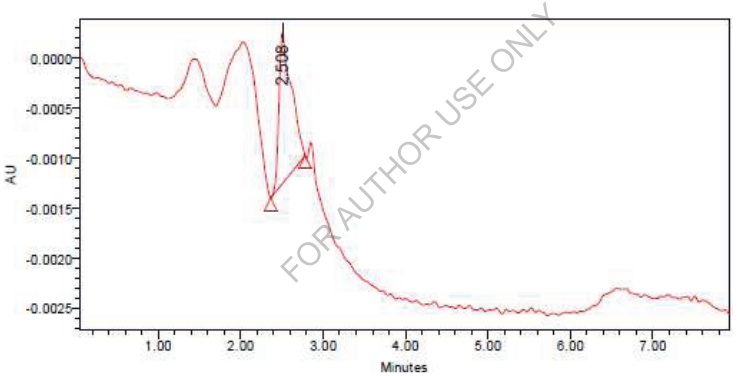


Figure: chromatogram for trail 2

Table: peak results for trail 2

S. No	Peak name	R_t	Area	Height	USP Tailing	USP plate count
1	Piroxicam	2.508	15742	1523	1.73	570

Observation: This trial shows improper baseline and less plate count in the chromatogram. so it required more trials to obtain good peaks

Trail 3:

Column	: ODS C18 (4.6×250mm)5µ
Column temperature	: 30ºC
Wavelength	: 227nm
Mobile phase ratio	: Water: Methanol (40:60) V/V
Flow rate	: 1.2ml/min
Injection volume	: 10µl
Run time	: 10min

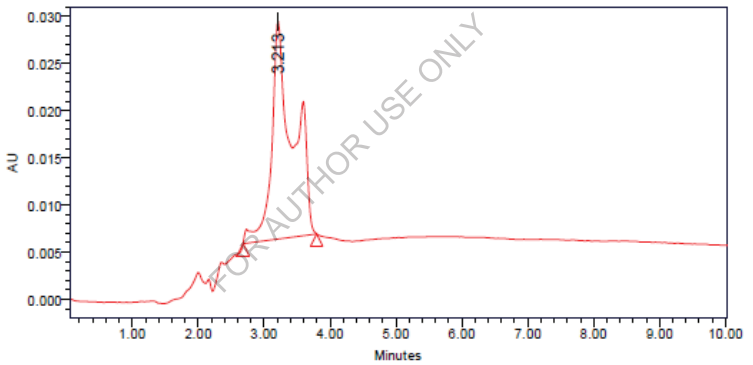


Figure- chromatogram for trail 3

Table: - Peak Results for Trail 3

S. No	Peak name	R_t	Area	Height	USP Tailing	USP plate count
1	Piroxicam	3.213	510928	23107	2.5	1238

Observation: In this trial it shows less plate count, more tailing and improper baseline in the chromatogram. so it required more trials to obtain good peaks

Trail 4:

Column : ODS C18 (4.6×250mm)5µ

Column temperature : 30ºC

Wavelength : 227nm

Mobile phase ratio : ACN: Water (50:50) V/V

Flow rate : 1.0ml/min

Injection volume : 10µl

Run time : 10min

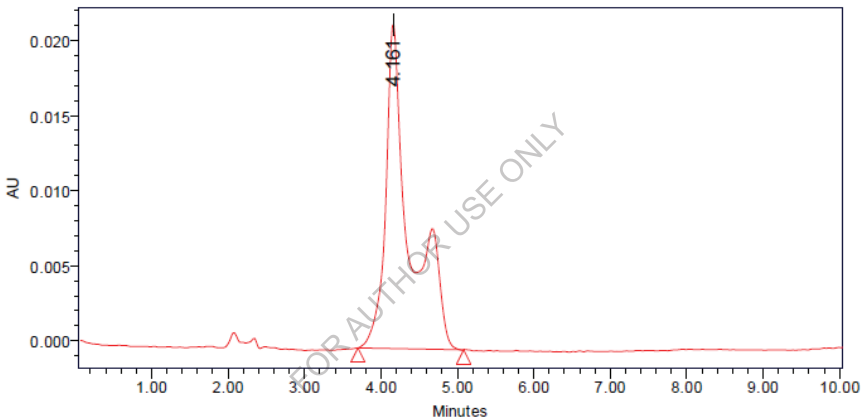


Figure: chromatogram for trail 4

Table: peak results for trail 4

S.No	Peak Name	R_t	Area	Height	USP Tailing	USP plate count
1	Piroxicam	4.161	471902	21641	1.69	1976

Observation: In this trial it shows less plate count and more tailing in the chromatogram. so it required more trials to obtain good peaks

Optimized Chromatogram (Standard)

Mobile phase ratio : Acetonitrile: Water (40:60)

Column : X-Terra C18 (4.6×250mm) 5µ

Column temperature : 30ºC

Wavelength : 227 nm

Flow rate : 0.9 ml/min

Injection volume : 10µl

Run time : 10 min

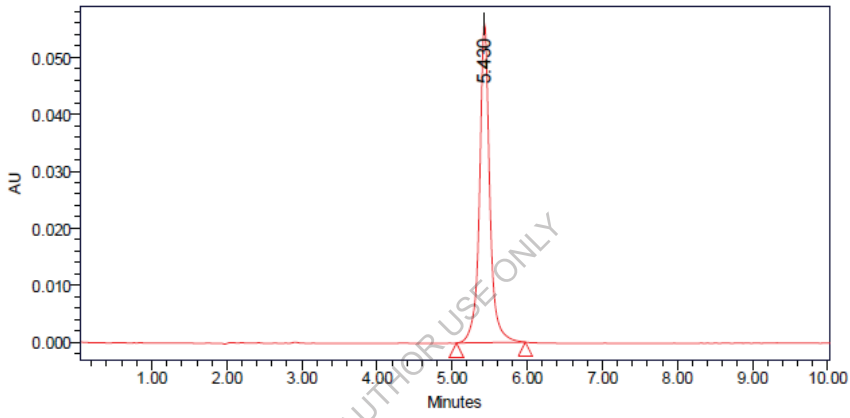


Figure: Optimized Chromatogram (Standard)

Table: Optimized Chromatogram (Standard)

S.no	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	piroxicam	5.430	531649	56299	1.05	9364

Observation: This trial shows good peak, proper plate count and less tailing in the chromatogram. And it's Passes the all system suitability parameters. So it's optimized chromatogram.

Optimized Chromatogram (Sample)

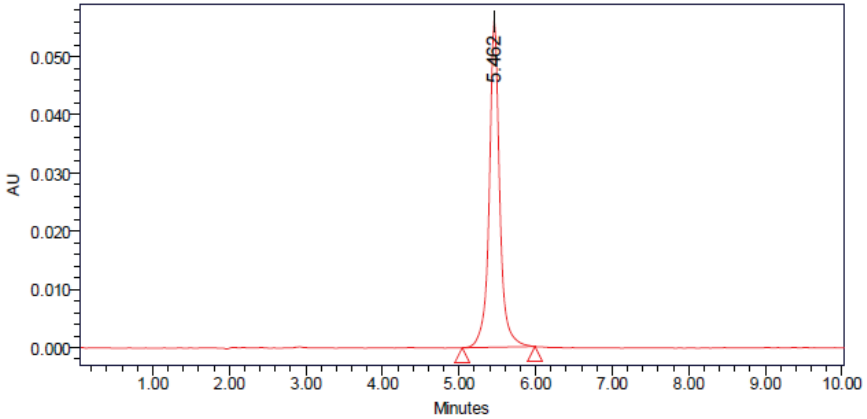


Figure: Optimized Chromatogram (Sample)

Table: Optimized Chromatogram (Sample)

S.no	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Piroxicam	5.462	532954	56050	1.03	9207

Acceptance criteria:

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

VALIDATION

Blank:

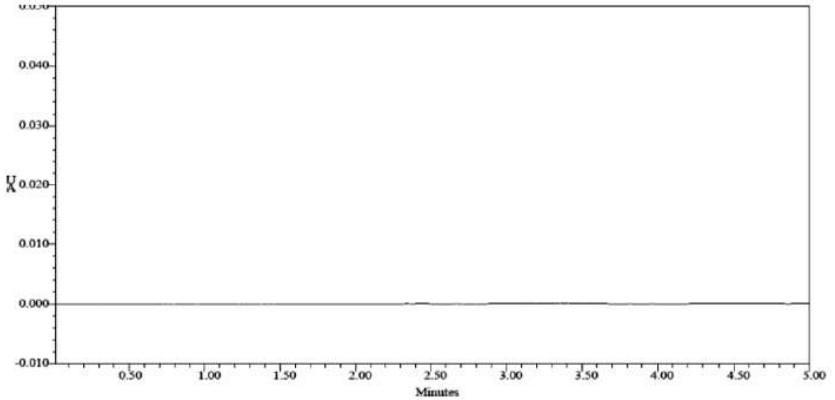


Fig: Chromatogram showing blank (mobile phase preparation)

SPECIFICITY

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

Analytical method was tested for specificity to measure accurately quantitate piroxicam in drug product.

Assay (Standard) :

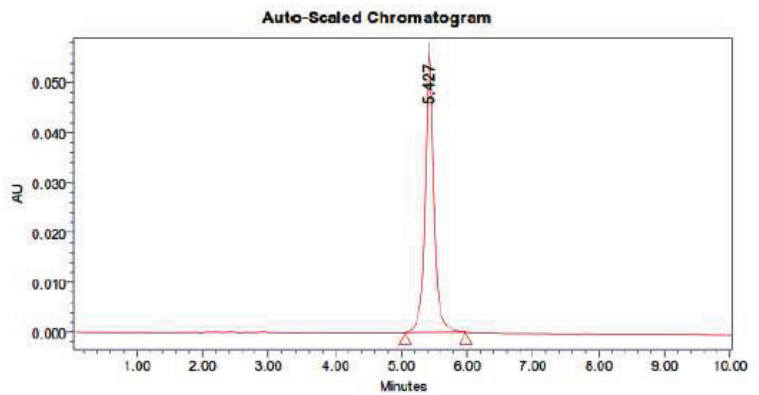


Fig: Chromatogram showing injection -1

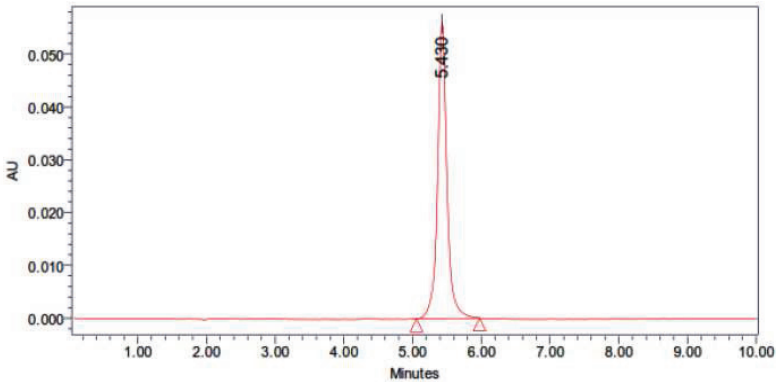


Fig: Chromatogram showing injection -2

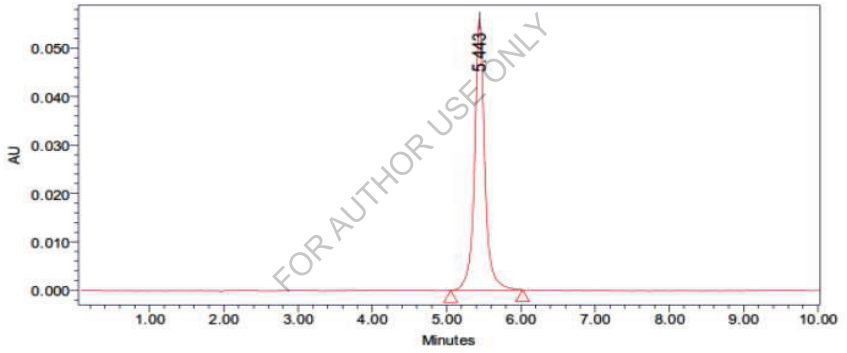


Fig: Chromatogram showing injection -3

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Piroxicam	5.427	513023	56127	9118	1.03
2	Piroxicam	5.430	513649	56299	9364	1.05
3	Piroxicam	5.443	513969	55991	9186	1.05

Acceptance criteria:

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

Assay (Sample):

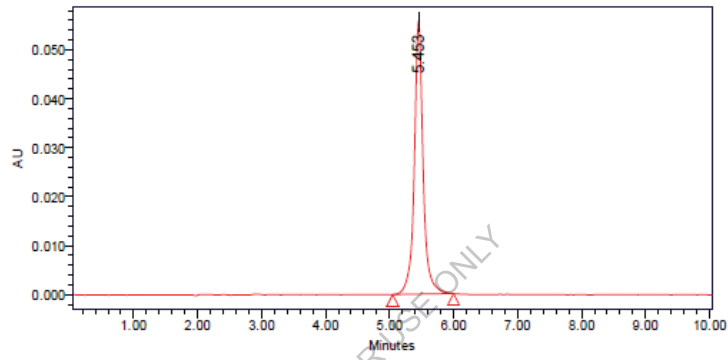


Fig: Chromatogram showing assay of sample injection-1

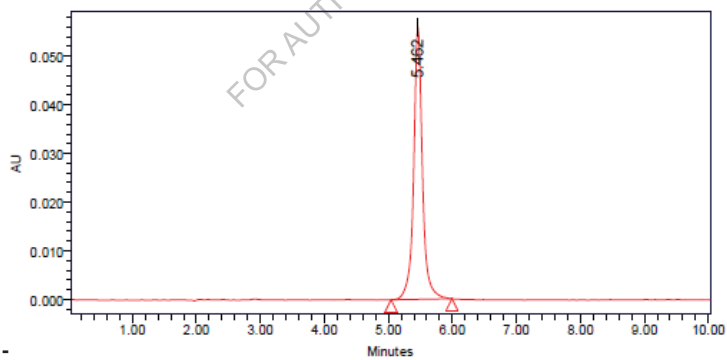


Fig. Chromatogram showing assay of sample injection-2

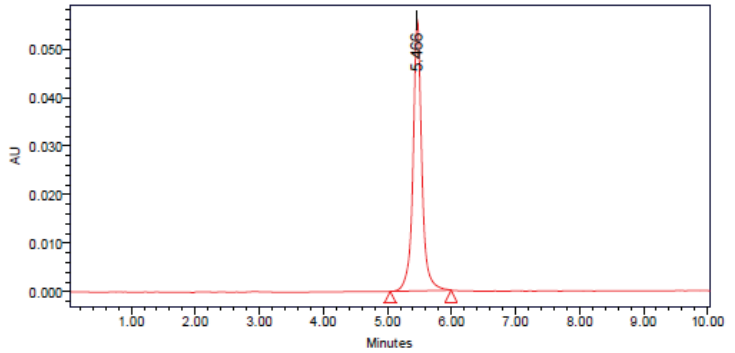


Fig: Chromatogram showing assay of sample injection-3

Table: Peak results for Assay sample

S.No	Name	RT	Area	Height	USP	USP Plate	Injectio
1	Piroxicam	5.453	514995	55722	1.05	9124	1
2	Piroxicam	5.462	512954	56050	1.03	9207	2
3	Piroxicam	5.466	513577	56095	1.03	9235	3

%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

=513842/513547×10/30×30/0.2912×99.7/100×0.5825/20×100

=99.77%

The % purity of piroxicam in pharmaceutical dosage form was found to be 99.77 %.

LINEARITY

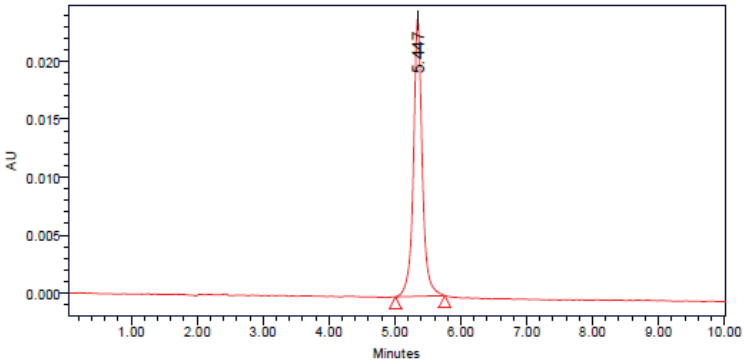


Fig. Chromatogram showing linearity level-1

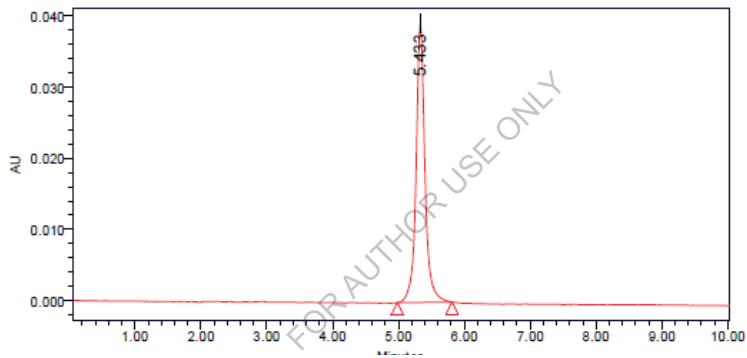


Fig. Chromatogram showing linearity level-2

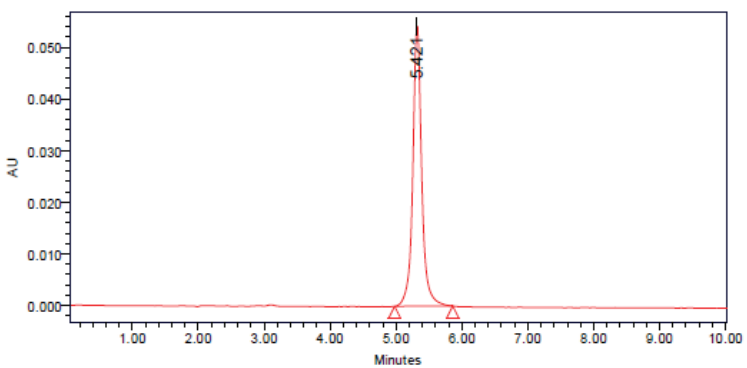


Fig. Chromatogram showing linearity level-3

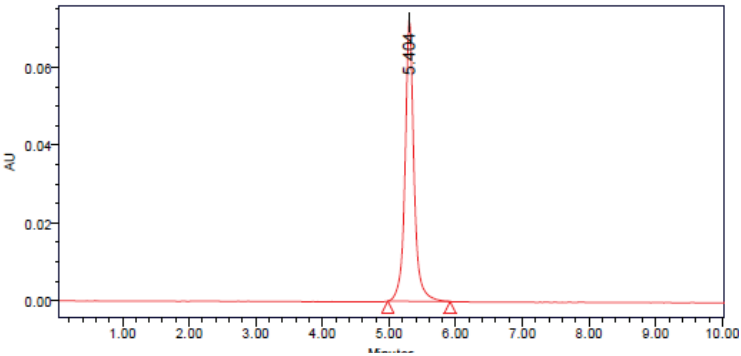


Fig. Chromatogram showing linearity level-4

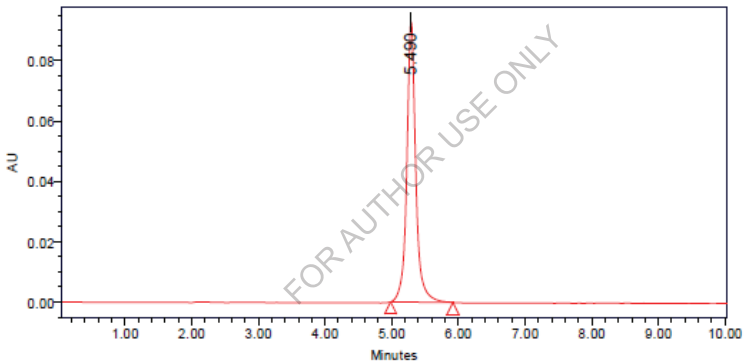
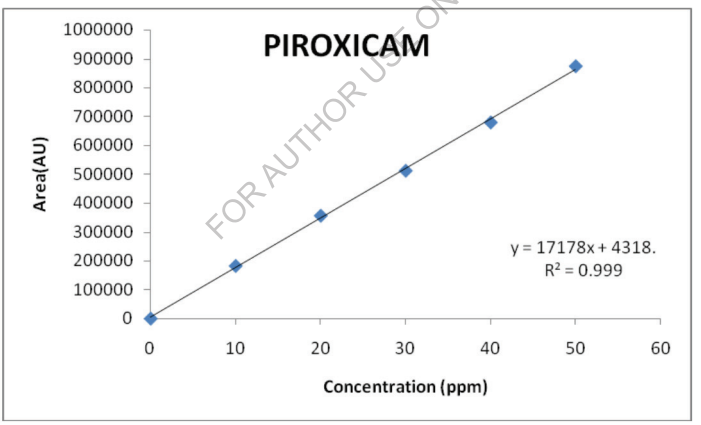


Fig. Chromatogram showing linearity level-5

CHROMATOGRAPHIC DATA FOR LINEARITY STUDY:

Concentration Level (%)	Concentration µg/ml	Average Peak Area
60	10	182423
80	20	356108
100	30	511715
120	40	678851
140	50	873452



LINEARITY PLOT:

The plot of Concentration (x) versus the Average Peak Area (y) data of DRUG is a straight line.

Y = mx + c

Slope (m) = 17178

Intercept (c) = 4318

Correlation Coefficient (r) = 0.99

VALIDATION CRITERIA: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

CONCLUSION: Correlation Coefficient (r) is 0.99, and the intercept is 4318. These values meet the validation criteria.

Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

REPEATABILITY

Obtained Five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

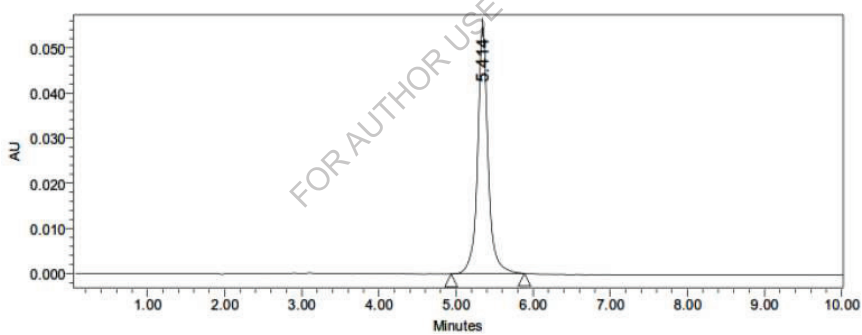


Fig. Chromatogram showing precision injection -1

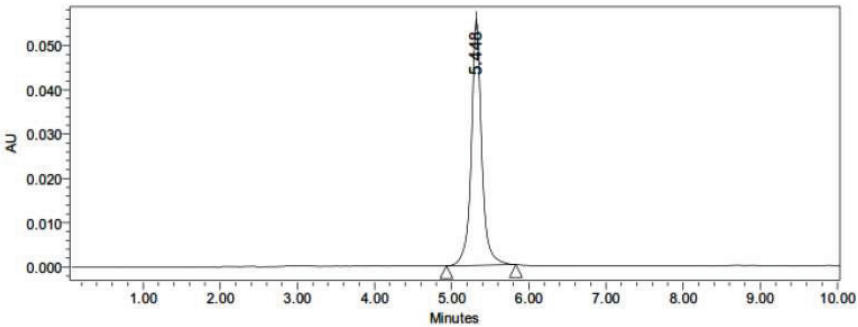


Fig. Chromatogram showing precision injection -2

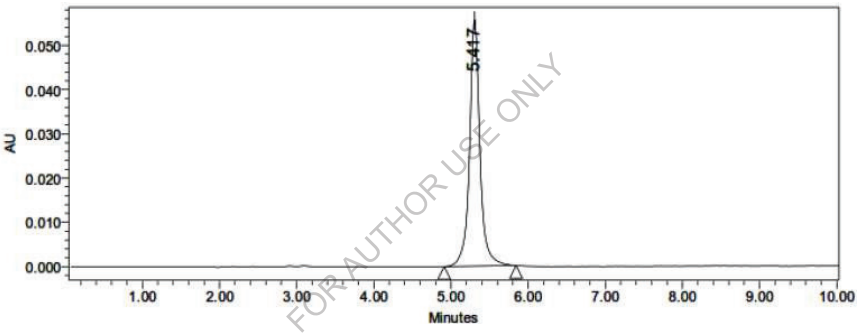


Fig. Chromatogram showing precision injection -3

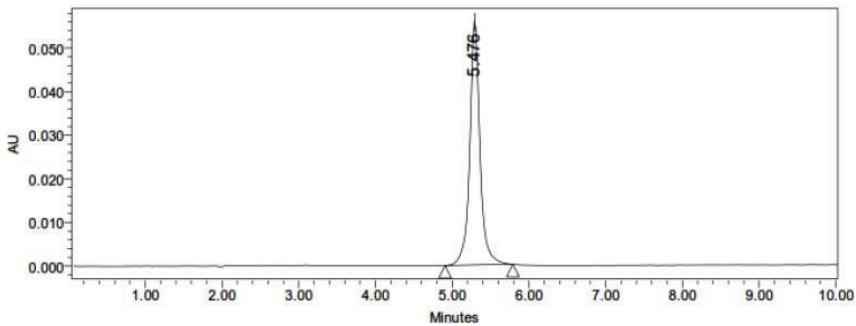


Fig. Chromatogram showing precision injection -4

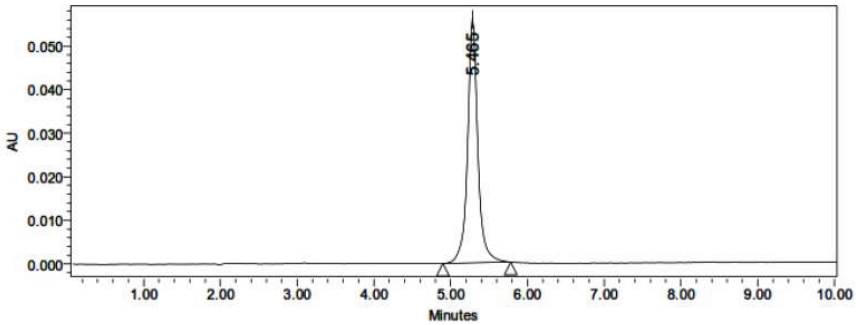


Fig. Chromatogram showing precision injection -5

Table: Results of Repeatability for Piroxicam:

S. No	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Piroxicam	5.414	517041	449653	5806	1.55
2	Piroxicam	5.448	504537	444489	5803	1.60
3	Piroxicam	5.417	512545	440821	5785	1.60
4	Piroxicam	5.476	505013	447677	5778	1.56
5	Piroxicam	5.465	506855	433328	5777	1.60
Mean			509198.2			
Std.dev			5418.576			
%RSD			1.06			

ACCEPTANCE CRITERIA:

- %RSD for sample should be NMT 2

- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Intermediate precision:

Day 1:

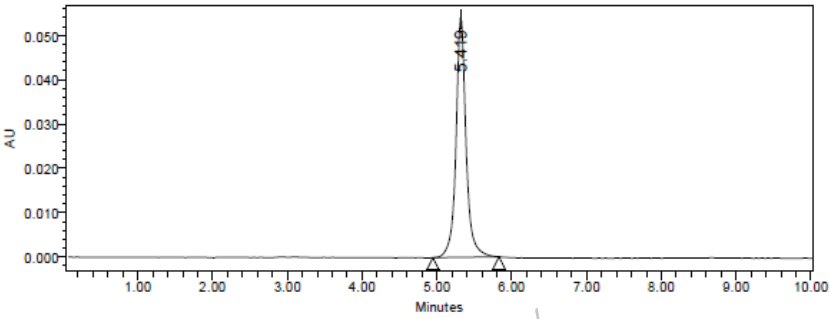


Fig: Chromatogram showing Day1 injection -1

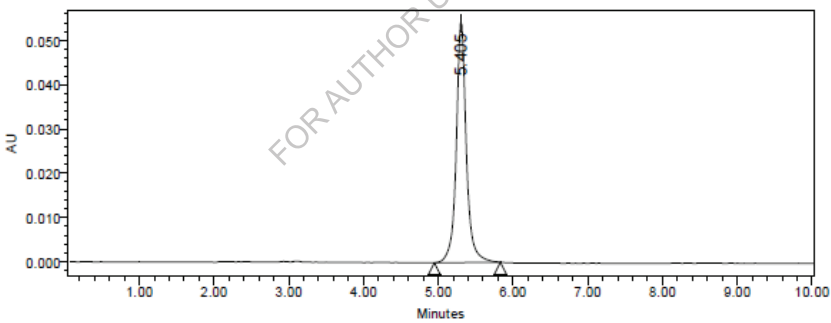


Fig: Chromatogram showing Day1 injection -2

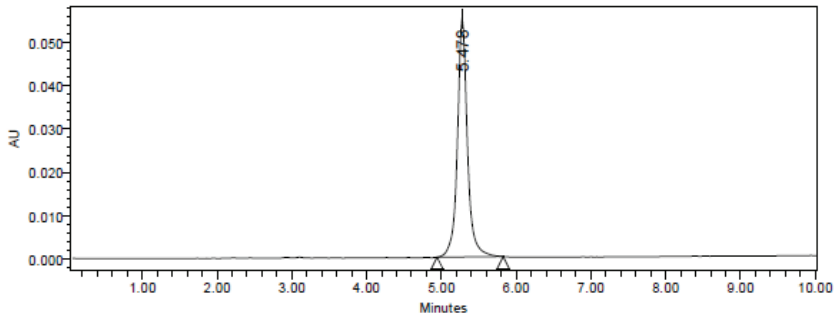


Fig: Chromatogram showing Day1 injection -3

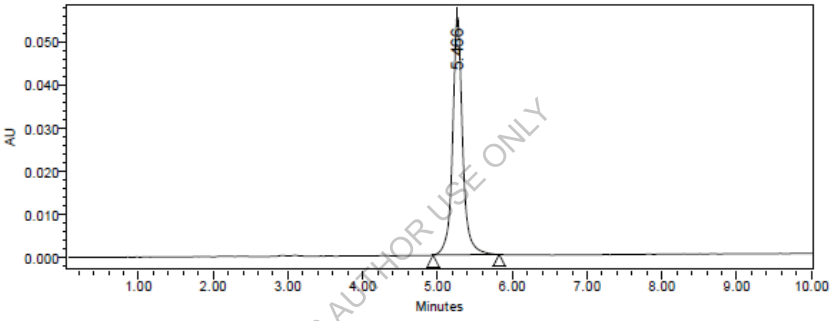


Fig: Chromatogram showing Day1 injection -4

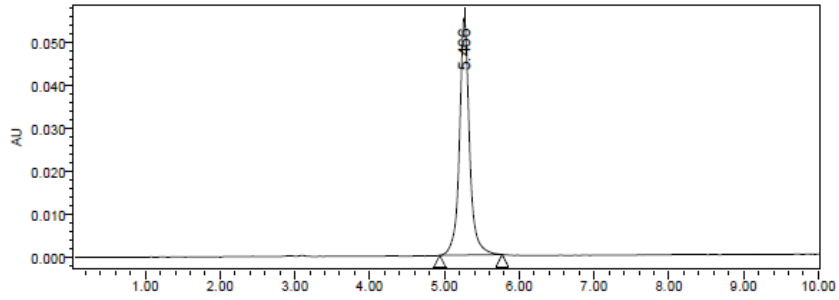


Fig: Chromatogram showing Day1 injection -5

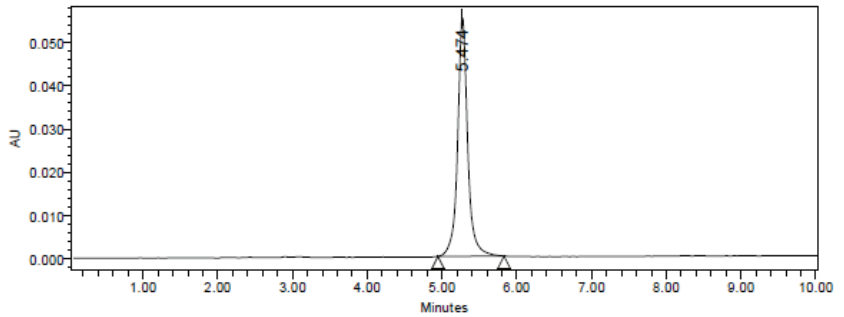


Fig: Chromatogram showing Day1 injection -6

Table: Results of Intermediate precision for piroxicam

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Piroxicam	5.419	507837	54219	8931.7	1.1
2	Piroxicam	5.405	510468	54508	8957.7	1.1
3	Piroxicam	5.478	514561	55259	8764.6	1.1
4	Piroxicam	5.466	515381	55552	9037.7	1.1
5	Piroxicam	5.466	516416	55506	8972.4	1.1
6	Piroxicam	5.474	518217	55506	8953.2	1.1
Mean			513813.4			
Std. Dev.			3901.1			
% RSD			0.8			

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2

Day 2:

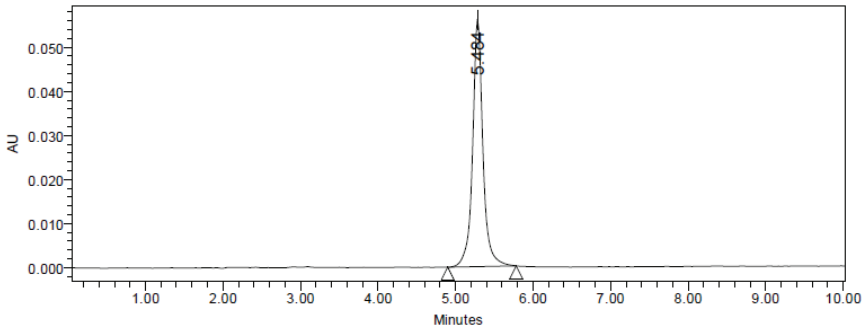


Fig: Chromatogram showing Day 2 injection -1

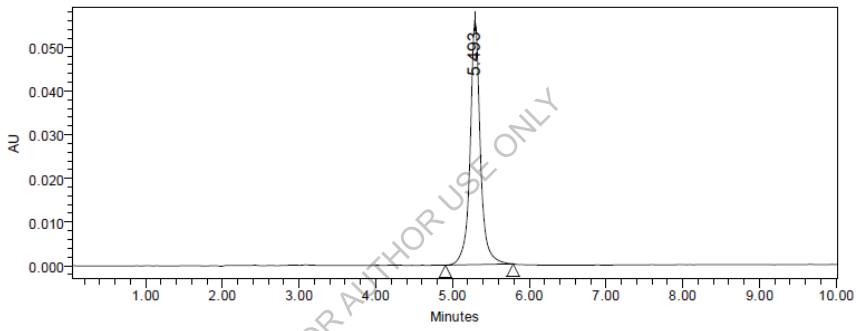


Fig: Chromatogram showing Day 2 injection -2

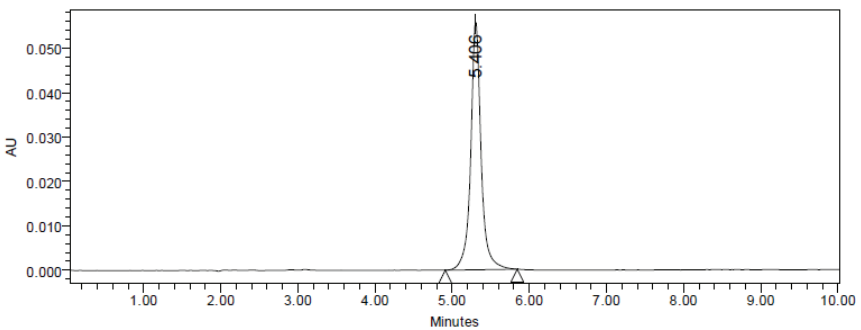


Fig: Chromatogram showing Day 2 injection -3

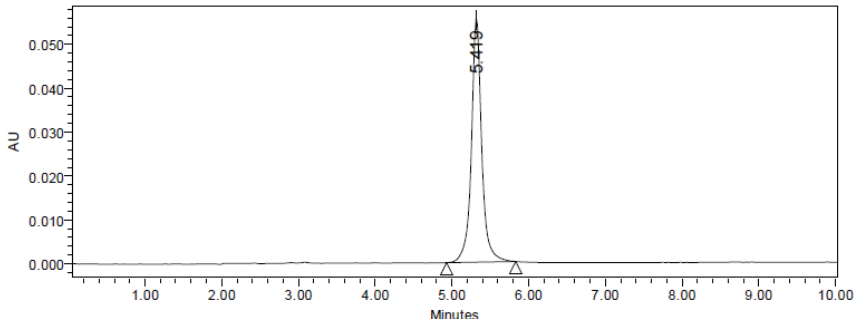


Fig: Chromatogram showing Day 2 injection -4

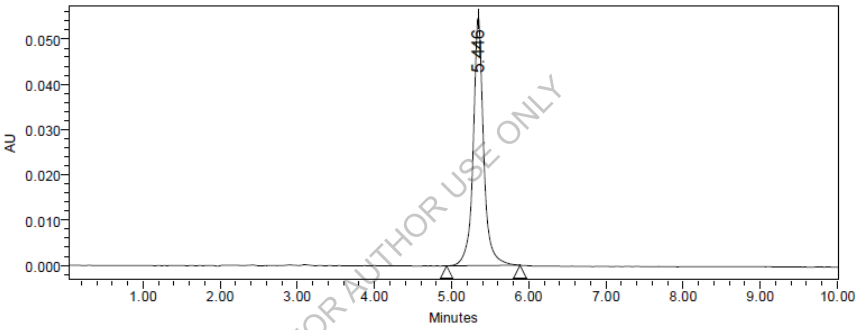


Fig: Chromatogram showing Day 2 injection -5

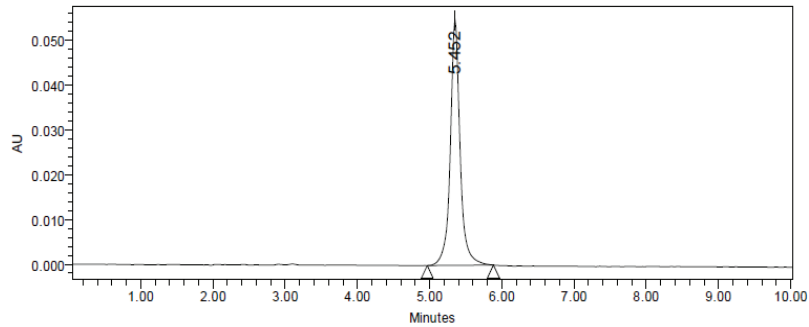


Fig: Chromatogram showing Day 2 injection -6

Table: Results of Intermediate precision Day 2 for piroxicam

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Piroxicam	5.452	516091	54804	9009.0	1.1
2	Piroxicam	5.446	518221	54903	9131.5	1.1
3	Piroxicam	5.493	519536	55996	9071.7	1.0
4	piroxicam	5.484	519881	56102	9015.7	1.0
5	piroxicam	5.419	519895	55577	8987.3	1.0
6	piroxicam	5.406	522826	55808	9070.5	1.0
Mean			519408.3			
Std. Dev.			2216.8			
% RSD			0.4			

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2

6.3.4: ACCURACY:

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

Accuracy50%:

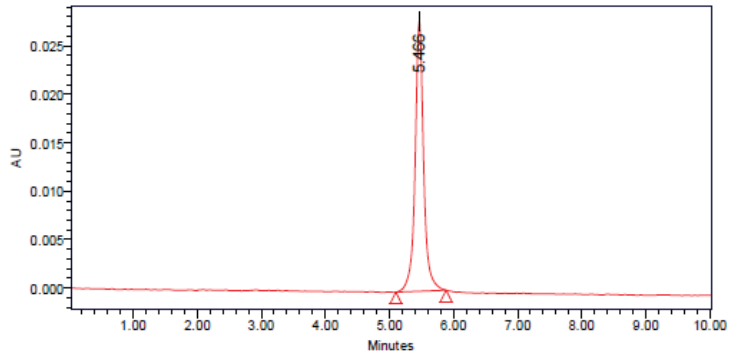


Fig. Chromatogram showing accuracy-50% injection-1

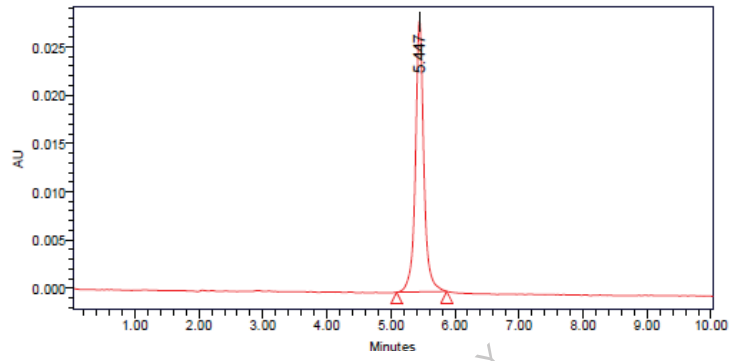


Fig. Chromatogram showing accuracy-50% injection-2

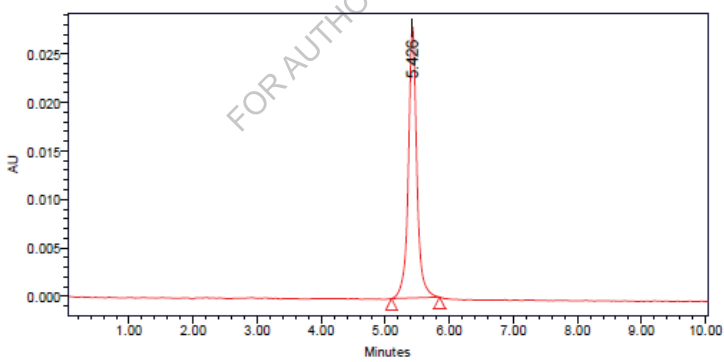


Fig. Chromatogram showing accuracy-50% injection-3

Table: Results of Accuracy for concentration-50%

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Piroxicam	5.466	261212	27932	1.03	9594	1
2	Piroxicam	5.447	261180	28124	1.04	10037	2
3	piroxicam	5.426	269152	28013	1.03	9677	3

Accuracy100%:

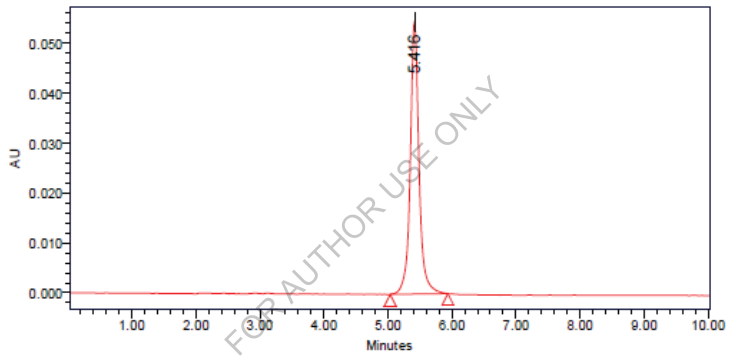


Fig Chromatogram showing accuracy-100% injection-1

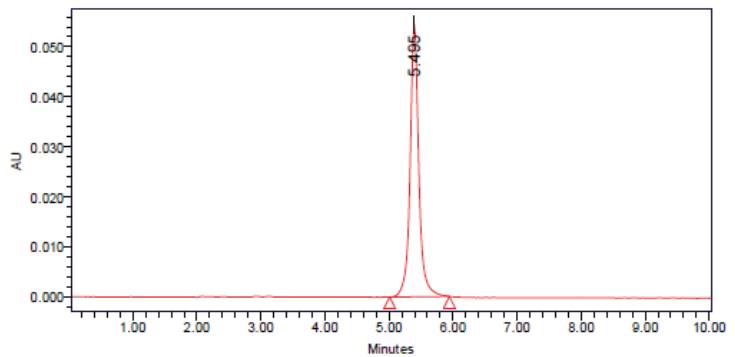


Fig: Chromatogram showing accuracy-100% injection-2

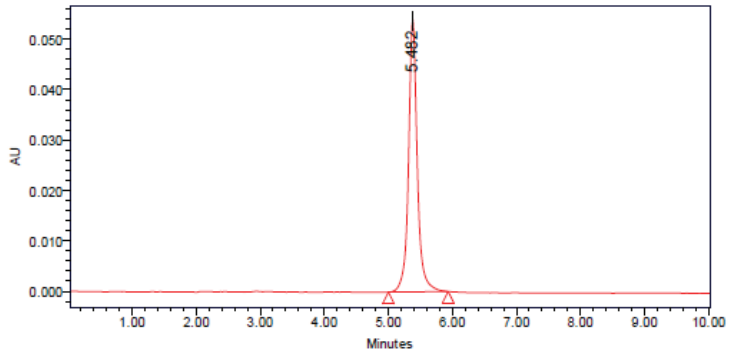


Fig: Chromatogram showing accuracy-100% injection-3

Table Results of Accuracy for concentration-100%

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Piroxicam	5.416	513814	54687	1.06	8971	1
2	Piroxicam	5.495	513719	54648	1.07	9011	2
3	piroxicam	5.482	513821	53975	1.07	8898	3

Accuracy150%:

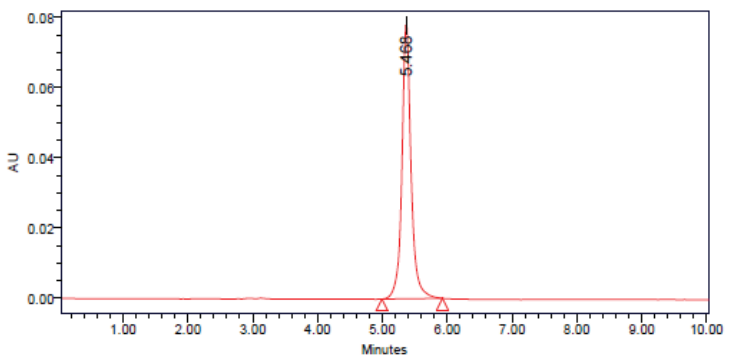


Fig Chromatogram showing accuracy-150% injection-1

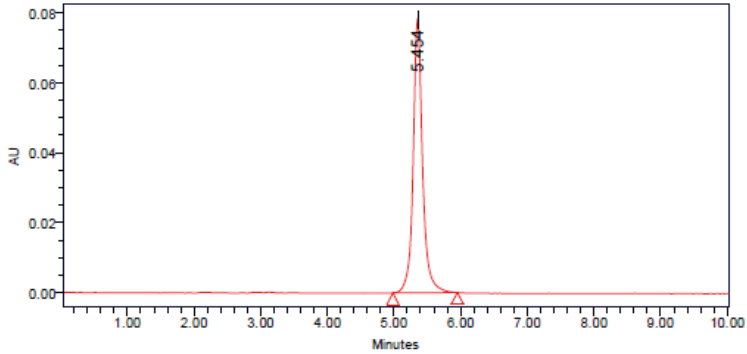


Fig. Chromatogram showing accuracy-150% injection-2

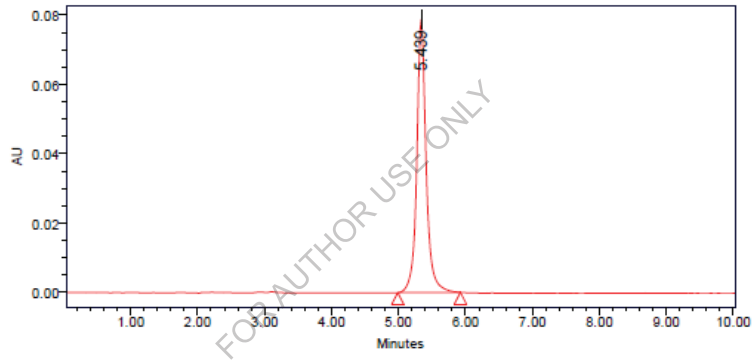


Fig. Chromatogram showing accuracy-150% injection-3

Table Results of Accuracy for concentration-150%

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Piroxicam	5.468	777819	78452	1.09	8134	1
2	Piroxicam	5.454	777920	78420	1.10	8194	2
3	piroxicam	5.439	777850	78658	1.10	8108	3

The accuracy results for piroxicam

% Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	263848	15	15.1	100.6	99.7%
100%	513784.7	30	29.6	98.6	
150%	777863	45	45.03	100.0	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

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

LIMIT OF DETECTION FOR Piroxicam

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

LOD= 3.3 × σ / s

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

= 3.3×11739.84501/17178

= 2.25µg/ml

Quantitation limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

LOQ=10×σ/S

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

= 10×11739.84501/17178

= 6.8µg/ml

Robustness

The robustness was performed for the flow rate variations from 0.8 ml/min to 1.0 ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for piroxicam. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ±5%. The standard and samples of piroxicam were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Variation in flow

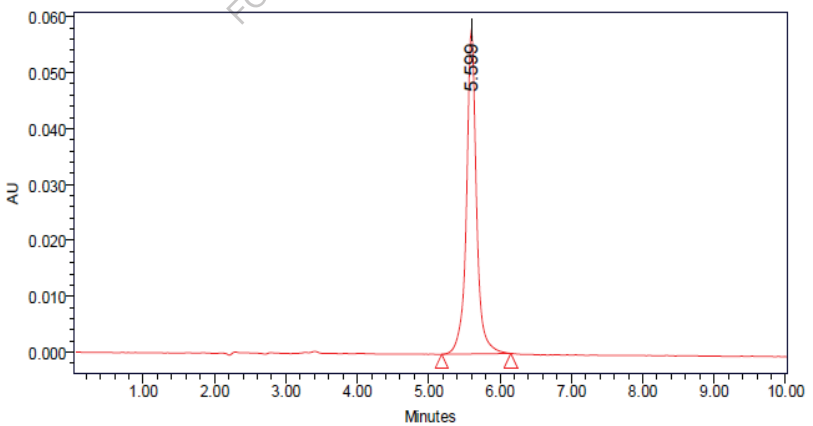


Figure: chromatogram showing less flow of 0.9ml/min

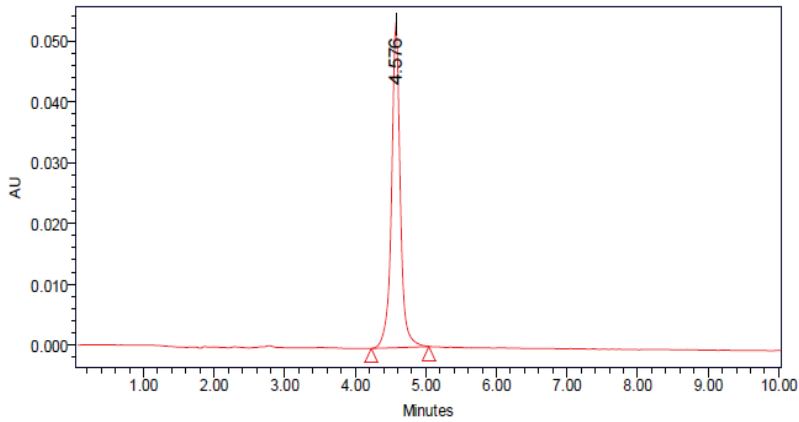


Figure: chromatogram showing more flow of 1.1 ml/min

Variation of mobile phase organic composition

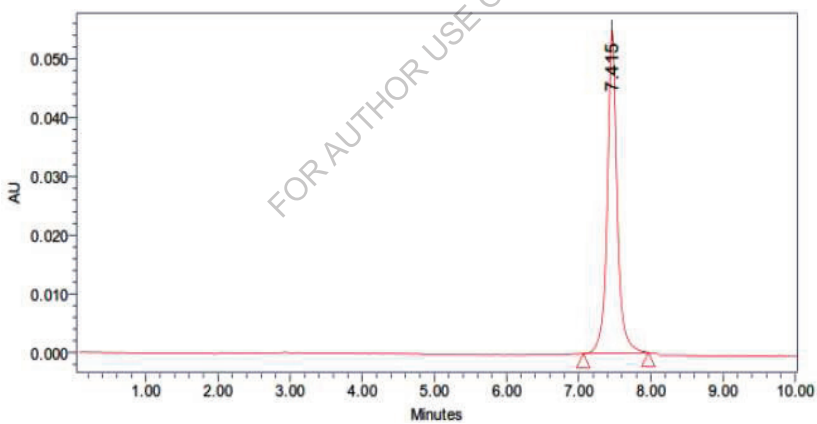


Figure: chromatogram showing less organic composition

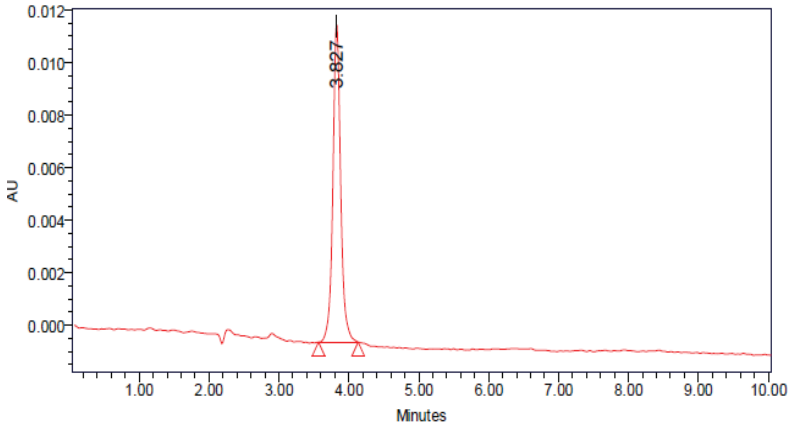


Figure: chromatogram showing more organic composition

Table: Results for Robustness

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Less Flow rate of 0.8 mL/min	56641	5.599	9364	1.02
Actual Flow rate of 0.9 mL/min	531649	5.430	9364	1.05
More Flow rate of 1.0 mL/min	459187	4.576	7559	0.98
Less Organic Phase	24366	7.415	12009	1.00
More Organic Phase	93382	3.827	6274	1.07

ACCEPTANCE CRITERIA:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

SUMMARY

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 227nm and the peak purity was excellent. Injection volume was selected to be 10µl which gave a good peak area. The column used for study was X-Terra C_{18} because it was giving good peak. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 0.9ml/min because of good peak area and satisfactory retention time.

Mobile phase Acetonitrile: Water (40:60) was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study.

Acetonitrile: Water (40:60) was selected because of maximum extraction sonication time was fixed to be 10min at which all the drug particles were completely soluble and showed good recovery.

Run time was selected to be 10 min because analyze gave peak around 5.4 and also to reduce the total run time.

The percent recovery was found to be 98.0-102 was linear and precise over the same range. Both system and method precision was found to be accurate and well within range.

The analytical method was found linearity over the range of 10-50ppm of the target concentration.

The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

CONCLUSION

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Piroxicam in bulk drug and pharmaceutical dosage forms. This method was simple, since diluted samples are directly used without any preliminary chemical derivatisation or purification steps.

Piroxicam was freely soluble in ethanol, methanol and sparingly soluble in water.

Acetonitrile: Water (40:60) was chosen as the mobile phase. The solvent system used in this method was economical. The %RSD values were within 2 and the method was found to be precise.

The results expressed in Tables for RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods.

This method can be used for the routine determination of Piroxicam in bulk drug and in Pharmaceutical dosage forms.

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