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# HPLC Method Development and Validation Process of Drug Analysis and Applications

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**Abstract**: *High-Performance Liquid Chromatography (HPLC) is a widely used analytical technique in pharmaceutical analysis due to its accuracy, sensitivity, and reproducibility. This project focuses on the development and validation of an HPLC method for the qualitative and quantitative analysis of a selected pharmaceutical drug compound. The objective is to establish a reliable, efficient, and robust method that can be applied in quality control and regulatory compliance.*

*The method development phase involves the selection of optimal chromatographic conditions, including mobile phase composition, flow rate, detection wavelength, and stationary phase, to achieve precise separation and resolution of the drug from its impurities and degradation products. Method validation is carried out according to ICH g parameters such as es, assessing urity, accuracy,parameters such as linearity, accuracy, precision, specificity, limit of detection (LOD), limit of quantification (LOQ), robustness, and system suitability.*

**Keywords**: HPLC

## I. INTRODUCTION

High-performance liquid chromatography (HPLC) is a type of column chromatography used in biochemistry and analysis to separate, identify, and quantify active compounds [1]. It consists of a stationary phase column, a pump that moves the mobile phase(s), and a detector that measures the retention times of molecules. Retention time varies depending on the sample.[2]The sample to be analyzed is introduced in small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase. The amount of retardation depends on the nature of the analyte and composition of both stationary and mobile phase. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time. Common solvents used include anymiscible combinations of water or organic liquids (the most common are methanol and acetonitrile). [3]

Sepration has been done to vary the mobile phase composition during the analysis; this is known as gradient elution. [4] The gradient separates the analyte mixtures as a function of the affinity of the analyte for the current mobile phase. The choice of solvents, additives and gradient depend on the nature of the stationary phase and the analyte. Analytical chemistry is used to determining the qualitative and quantitative composition of material under study. Both these aspects are necessary to understand the sample material. Analytical chemistry is divided into two branches quantitative and qualitative.

A qualitative analysis gives us the information about the nature of sample by knowing about the presence or absence of certain components. A quantitative analysis provides numerical information as to the relative amount of one or more of this component. For analyzing the drug samples in bulk, pharmaceutical formulations and biological fluids, different analytical methods are routinely being used.In non-instrumental, the conventional and physicochemical property are use to analyze the sample. The instrumental methods of analysis are based upon the measurements ofsome physical property of substance using instrument to determine its chemical composition [5]

**Chromatography :-**Chromatography is a technique used for separation of the components of mixture by continuous distribution of the component between two phases. One phase moves (mobile phase) over the other phase (stationary phase) in a continuous manner.Chromatography according to USP can be defined as a procedure by which solute are separated by a differential migration process in a system consisting of two or more phases, one of which move continuously in a given direction [6].

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**Principles of method development in HPLC**

To understand the principles of method development in hplc one needs to know the basic operation, elution processes and physical and chemical parameters of the technique and the selection of detectors for detecting the analyte. In this review article, we will discuss the above mentioned principles and suggest the most optimum modifications for method development in HPLC. [7]

**Principle of chromatography**

**Adsorption Chromatography**: When the stationary phase is a solid and mobile phase is liquid or gaseous phase, it is called Adsorption Chromatography. Examples: Thin layer chromatography, Column Chromatography, Gas-solid chromatography.

**Partition Chromatography**: When the stationary phase and mobile phase are liquid, it is called Partition Chromatography . Example: Paper partition chromatography, Gas-liquid chromatography.

**Theory of Chromatography**

Two theoretical approaches have been developed to describe the processes involved in the passage of solutes through a chromatographic system.

**The Plate Theory**

According to Martin and Synge, a chromatographic system consists of discrete layers of theoretical plates. At each of these, equilibration of the solute between the mobile and stationary phases occurs. The movement of solute is considered as a series of stepwise transfers from plate to plate.

**The Rate Theory**

This theory considers the dynamics of the solute particles as it passes through the void space between the stationary phase particles in the system as well its kinetic as it is transferred to and from the stationary phase. Phases of Chromatography

**Normal Phase Chromatography:**

In Normal Phase mode the stationary phase is polar and the mobile phase is non polar in nature. In this technique, non polar compounds travel faster and are eluted first. This is because of the lower affinity between the non polar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore take more times to elute. Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute. [8]

**Reversed Phase Chromatography:** It is the most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this modethe stationary phase is non polar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. The polar compound gets eluted first in this mode and non polar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are Octa Decyl Silane (ODS) or C18, C8,C4, (in the order of increasing polarity of the stationary phase). An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity.[9]

**Ion Exchange Chromatography**: The stationary phase contains ionic groups like NR3+, SO3-which interact with the ionic groups of the sample molecules. This is suitable for the separation of charged molecules only. Changing the pH and salt concentration can modulate the retention. Ion Pair Chromatography: This technique is also referred to as Reversed Phase Ion Pair Chromatography or Soap Chromatography. It may be used for the separation of ionic compounds and this method can also substitute for Ion Exchange Chromatography. Strong acidic and basic compounds may be separated by reversed phase mode by forming ion pairs (coulumbic association species formed between two ions of opposite electric charge) with suitable counter ions.

**Affinity Chromatography:** This technique uses highly specific biochemical interactions for separation. The stationary phase contains specific groups of molecules which can absorb the sample if certain steric and charge related conditions are satisfied. This technique can be used to isolate proteins, enzymes as well as antibodies from complex mixtures.

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**Size Exclusion Chromatography:** It separates molecules according to their molecular mass. Largest molecules are eluted first and the smallest molecules last. This method is generally used when a mixture contains compounds with a molecular mass difference of at least 10%.

## TYPES OF HPLC

Types of HPLC generally depend on phase system used in the process. Following types of HPLC generally used in analysis.[10]

**Normal phase chromatography:** Also known Normal phase HPLC (NP-HPLC), this method separates analytes based on polarity. NP-HPLC uses a polar stationary phase and a non-polar mobile phase. The polar analyte interacted with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase increases the elution time.

**Reversed phase chromatography:** Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. 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.

**Size exclusion chromatography:** Size exclusion chromatography (SEC), also called as gel permeation chromatography or gel filtration chromatography mainly separates particles on the basis of size. It is also useful for determining the tertiary structure and quaternary structure of proteins and amino acids. This technique is widely used for the molecular weight determination of polysaccharides. Ion exchange chromatography: In Ionexchange chromatography, retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Ions of the same charge are excluded. This form of chromatography is widely used in purifying water, Ligandexchange chromatography, Ion-exchange chromatography of proteins, High-pH anion-exchange chromatography of carbohydrates and oligosaccharides, etc. [11,12]

**Bio-affinity chromatography:** Separation based on specific reversible interaction of proteins with ligands. Ligands are covalently attached to solid support on a bio-affinity matrix, retains proteins with interaction to the column-bound ligands. Proteins bound to a bioaffinity column can be eluted in two ways:

## INSTRUMENTATION

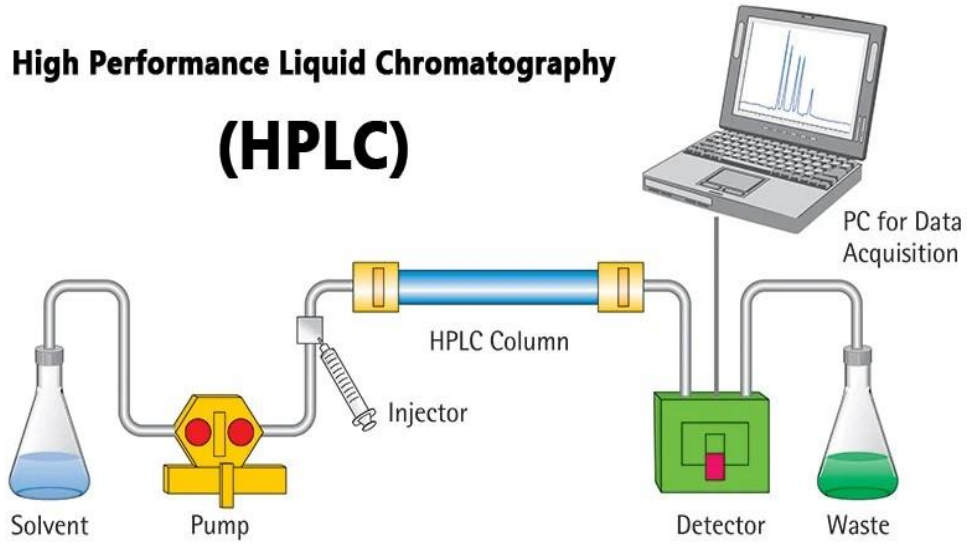


**Figure1**: A Basic instrumentation of HPLC

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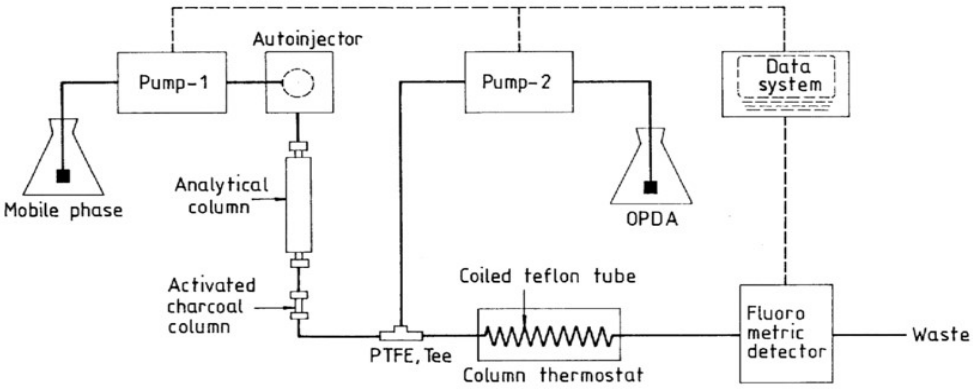


**Figure 2**: B flow scheme for HPLC

**Instrumentation Of HPLC**

- Pump
- Mixing unit
- Solvent degassing
- Injector
- Column
- Detectors

**1.pump**

The role of the pump is to force a liquid ( called the mobile phase) through the liquid chromatography at a specific flow rate, expressed in milliliters per min (mL/min) Normal flow rates in HPLC are in the 1-2mL/min range. During the chromatographic experiment, a pump can deliver a constant mobile phase composition (isocratic) or an increasing mobile phase composition (gradient). Best for the analysis of complex samples.

**Types of pumps:** Mainly three types:

Constant flow reciprocating pump

Syringe type pump

Pneumatic pump

Constant flow reciprocating pump : The term reciprocating describes any continuously repeated backwards and forwards motion. Widely used type of pump. Solvent is sucked during back stroke and gets deliver to the column in forward stroke.

**Syringe or displacement type pump :** Consists of large syringe like chamber. Suitable for small bore column.

**Pneumatic pump** : Gas is used to pressurize the mobile phase present in a collapsible solvent container.

**2. Mixing Unit :** Mixing unitt is used to mix solvents in different proportions and pass through the column. There are two types of mixing units. - they are low pressure mixing chamber which uses helium for degassing solvents. - high pressure mixing chamber does not require helium for degassing solvents . Mixing of solvent is done either with a static mixer which is packed with beads or a dynamic mixer which uses magnetic stirrer and operates under high pressure.

**3. Solvent Degassing :** Several gases are soluble in organic solvents. When solvents are pumped under high pressure, gas bubbles are formed which will interfere with the separation process, steady baseline and the shape of the peak. Hence degassing is necessary.

**This can be done by using following techniques**:

Vacuum filtration:- which can remove air bubbles, but it is not always reliable and complete. ¬ Helium purging:- By passing helium through the solvent. This is very effective but expensive.

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Ultrasonification:- By using Ultrasonicator, which converts ultra high frequency to mechanical vibrations. This causes the removal of air bubbles.

**4.Injector :** The injector serves to introduce the liquid sample into the flow stear the mobile phase. Typical sample volumes are 5-20microliters The injector must also be able to withstand the high pressure of the liquid system. An auto sampler is the automatic version for when the user has many samples to analyze or when manual injection is not practical.

Type of injectors septum injectors : 1) For injecting the sample through a rubber septum. This is not common, since the septum has to withstand high pressure.

2) Stop flow: In which the flow of mobile phase is stopped for a while and the sample is injected through a valve device.

3) Rheodyne injector: It is the most popular injector. This has a fixed volume loop like 20µL or 50µL or more. Injector has 2 modes, i.e., load position when the sample is loaded in the loop and inject mode when the sample is injected.

**5.column :** It is the heart of the chromatograph Column length: varies from 5cm to 30cm Column diameter: ranges from 2mm to 50mm Particle size: from 1µ to 20µ Particle nature: spherical, uniform sized, porous materials are used.

**Materials of construction for the tubing:**

Stainless steel ( the most popular, gives high pressure capabilities)

Glass (mostly for biomolecules)

PEEK (poly ether ether ketone) polymer (biocompatibility and chemically inert to most solvents).

**Packing material:**

The packing material is prepared from SILICA particle, ALUMINA particle and ion exchange RESIN.

Porous plug of stainless steel or Teflon are used in the end of the columns to retain the packing material.

**6.Detector**

**HPLC Detector types** :

Based on the principle used in detection the detectors available are UV detectors, Fluorescent detectors, Electrochemical detectors and Photo diode-array detectors (PDA) and Refractive index detectors.

**UV detectors**

The sample detection depends on absorption of UV ray energy by the analyte. The detector comprises of accessories in order as UV source, grating (for light defraction), sample passing through a tubing exposed to rays, photo cell, charge conductor etc. When the UV rays emitted by lamp pass through gratings, rays split into different wavelengths. One specific wavelength rays are passed through sample. Some amount of light is absorbed by sample and the unabsorbed rays which fall on photo cell. These rays on collision on photo cells produce electrons whose current is recorded. This is indicative of nature and quantity of sample. This UV wavelength range of absorption is specific for sample. These are the HPLC detectors used in general, unless there is requirement for analysis of special compounds. They are capable to detect very wide range of compounds. The sensitivity ranges till microgram quantity of estimation.

**PDA detectors**

These are detectors which follow principle similar to UV detectors but the range of detection extends from UV, visible and to some extent to IR region. Thus, the advantages are higher sensitivity and it measures the entire absorption range i.e it gives the scan of the entire spectrum.

**Fluorescence detector**

In this detector the fluorescence rays emitted by sample after absorbing incident light is measured as a function of quality and quantity of the sample. The equipment comprises of accessories in order as light source, sample passing through a tubing exposed to rays, grating (for light defraction), photo cell, charge conductor etc. Xenon arc lamp is used to produce light for excitation of sample molecules. These light rays excite the sample molecules. These excited molecules emit florescence, which pass through gratings. These gratings pass the florescence at specific wavelength to photo cell which is recorded. The detector is suitable for compounds which can produce florescence. Some compounds are chemically altered to produce fluorescence by chemical derivatization to estimate by this detector. These detectors have high precision and sensitivity (with less noise in data). Compounds are measurable till nanogram quantities.

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**Electrochemical detectors**

This detector is especially suitable to estimate oxidisable & reducible compounds. The principle is that when compound is either oxidized or reduced, the chemical reaction produces electron flow. This flow is measured as current which is the function of type and quantity of compound. The system has electrodes like working electrode where oxidation or reduction takes place and reference electrode which acts to subtract conductivity of mobile phase to that of sample. This electrode is suitable for compounds which can't be assayed by UV detector especially due to their similarities in light absorption properties ex: monoamines. This detector has super sensitivity which ranges till picograms measurement. So, very minute quantities of compounds that are present in the sample can be measured. This electrochemical detector produces severe noise or fluctuations in peaks. So it is difficult to work with when compared to other detectors. As such all types of HPLC detectors are used based on the requirement of labs.

**Refractive Index detectors**

These are detectors which measure the change of refractive index of the eluant from the column with respect to pure mobile phase. They have several disadvantages like lack of high sensitivity, non suitability for gradient elution, and also require strict temperature control ±0.001oC to operate at their highest sensitivity.

## PARAMETERS

For the accurate analysis of a compound, there are some parameters which are used as a standard for a particular compound. If there is a change occurs in the parameters the result may be affected greatly. The most commonly used parameters are internal diameter, particle size, pore size, pump pressure. For different compounds the parameters can be changed according to their nature and chemical properties.

**Internal diameter:** The internal diameter (ID) of an HPLC column is a critical aspect that determines quantity of analyte that can be loaded onto the column and also influences sensitivity. Larger columns are usually seen in industrial applications such as the purification of a drug product for later use. Low ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity**.**

**Particle size:** Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). Smaller particles generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter squared.

**Pore size:** Many stationary phases are porous to provide greater surface area. Small pores provide greater surface area while larger pore size has better kinetics especially for larger analytes. Pore size defines an ability of the analyte molecules to penetrate inside the particle and interact with its inner surface. This is especially important because the ratio of the outer particle surface to its inner one is about 1:1000. The surface molecular interaction mainly occurs on the inner particle surface.

**Pump pressure:** Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible flow rate. Modern HPLC systems have been improved to work at much higher pressures, and therefore be able to use much smaller particle sizes in the columns (< 2 micrometres)

**Solvent Selectivity** :The elution strength of a given solvent is determined by its hydrophobicity but the selectivity of a solvent is determined by its polar characteristics. Heptane and hexane have the same elution strength but different selectivity. For example, Methanol is a strong proton donor and a strong proton acceptor in hydrogen bonding. Acetonitrile has a dipole moment but is only a very weak proton acceptor in hydrogen bonding. Tetrahyrofuran accepts a proton in hydrogen bonding but cannot donate a proton [20].

**Effect of pH on analyte**: ionization The primary mechanism of retention in RP chromatography is hydrophobic interaction. Ionizing compounds will cause them to behave as more polar species, and reduce their hydrophobic interaction with the stationary phase, leading to decreased retention.The ionization state of a molecule will be determined by the pH of the mobile phase and therefore pH of mobile pnase will dictate the retention behavior of analytes with ionizable functional groups.

**Effect of temperature Temperature**: conditions in HPLC method development present a challenge because it can have unpredictable effects on selectivity. The use of elevated temperatures will:

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Reduce mobile phase viscosity and back-pressure. This can allow you to operate at higher flow rates or to use longer columns/smaller particle sizes.
Reduce elution time.
Improve method reproducibility (as opposed to operating at room temperature). However, it is impossible to determine if the use of elevated temperatures will help or hinder a specific separation because for complex separations, improvements in one portion of the chromatogram are almost always accompanied by disimprovement in another part of the same chromatogram [22].

**Retention time** : The time taken for a particular compound to travel through the column to the detector is known as its retention time. Retention time is measured from the time at which the sample is injected into the system to the point at which the display shows a maximum peak height for that compound. Different compounds have different retention times. The retention time for a particular compound will vary depending on:
the pressure used (because that affects the flow rate of the solvent)
the nature of the stationary phase (material and particle size)
the exact composition of the solvent
the temperature of the column
If you are using retention times as a way of identifying compounds conditions have to be carefully controlled.

**Buffer Selection**

Choice of buffer is governed by the pH that is desired. The typical pH range for reversed phase on silica based packing is pH 2 to 8. It is important that the buffer has a pKa close to the desired pH since buffer controls pH best at their pKa. A rule is to choose a buffer with a pKa value.

**General contemplations during buffer selection:**

Phosphate is more soluble in methanol/water than in acetonitrile/water or THF/water.
Some salt buffers are hygroscopic and this may lead to changes in the chromatography like increased tailing of basic compounds and possibly selectivity differences.
Ammonium salts are generally more soluble in organic/water mobile phases·
Trifluoroacetic acid can degrade with time. It is volatile and absorbs at low UV wavelengths.
Microbial growth can quickly occur in buffered mobile phases that contain little or no organic modifier at all. The growth accumulates on column inlets and can damage chromatographic performance.
At pH greater than 7, phosphate buffer accelerates the dissolution of silica and severely shortens the lifetime of silica-based HPLC columns. If possible, organic buffers should be used at pH greater than 7.
Ammonium bicarbonate buffers usually are prone to pH changes and are usually stable for only 24 - 48 hrs. The pH of this mobile phase tends to become more basic due to the release of carbon dioxide.
After buffers are prepared, they should be filtered through a 0.2-µm filter.
Mobile phases should be degassed.
Buffer Concentration Generally, a buffer concentration of 10-50 mM is adequate for small molecules. Generally, no more than 50% organic should be used with a buffer. This will depend on the specific buffer as well as its concentration. Phosphoric acid and its sodium or potassium salts are the most common buffer systems for reversed-phase HPLC. Sulfonate buffers can replace phosphonate buffers when analyzing organophosphate compounds . Selection of detectors HPLC detectors are important accessories of the HPLC instrument. This part of HPLC helps in detection and identification of compounds in the sample injected. The detectors are designed to have certain properties like
They should be inert (non-reactive) to the samples injected and the mobile phases passing through.
They should be preferably non-destructive to the sample.
Should be able to produce quick and quantitative response.
Reliable, uniform and reproducible detection and analytic data.
Compatible with all types of compounds under testing.
All types of HPLC detectors fulfill most of the above properties [23].

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## METHOD OPTIMIZATION

The experimental conditions should be optimized to get desired separations and sensitivity after getting appropriate separations. Stability indicating assay experimental conditions will be achieved through planned/systemic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, temperature, sample amounts, Injection volume and diluents solvent type.

### Validation of method

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use. The methods validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to support analytical procedure [24-25]. All analytical methods that are intended to be used for analyzing any clinical samples will need to be validated. The validation of analytical methods is done as per ICH guidelines.

Components of method validation The following are typical analytical performance characteristics which may be tested during methods validation:

- Accuracy
- Precision
- Repeatability
- Intermediate precision
- Linearity
- Detection limit
- Quantitation limit
- Specificity
- Range
- Robustness
- System suitability determination
- Forced degradation studies
- Solution stability studies

**Accuracy** is the nearness of a measured value to the true or accepted value. Accuracy indicates the deviation between the mean value found and the true value. lt is determined by applying the method to samples to which known amounts of analyte have been added. These should be analysed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay. It may often be expressed as the recovery by the assay of known, added amounts of analyte [26]

**The precision** of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample. Precision is a measure of the reproducibility of the whole analytical method [27]. It consists of two components: repeatability and intermediate precision. Intermediate precision is the variation within a laboratory such as different days, with different instruments, and by different analysts [28-29] The precision is then expressed as the relative standard deviation. Accuracy and precision are not the same. A method can have good precision and yet not be accurate.

**Repeatability**: is the variation experienced by a single analyst on a single instrument. It does not distinguish between variation from the instrument or system alone and from the sample preparation process. During validation, repeatability is performed by analyzing multiple replicates of an assay composite sample by using the analytical method. The recovery value is calculated.

**Linearity:** is the ability of analytical procedure to obtain a response that is directly proportional to the concentration (amount) of analyte in the sample. If the method is linear, the test results are directly or by well-defined mathematical transformation proportional to concentration of analyte in samples within a given range. Linearity is usually expressed as the confidence limit around the slope of the regression line.

**Detection Limit**: The detection limit (DL) or limit of detection (LOD) of an individual procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. In analytical procedures

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that exhibit baseline noise, the LOD can be based on a signal-to-noise (S/N) ratio (3:1), which is usually expressed as the concentration of analyte in the sample.(book) The signal-to-noise ratio is determined by: s = H/h Where H = height of the peak corresponding to the component. h = absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.

**Quantitation Limit**: The limit of Quantitation (LOQ) or Quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. For analytical procedures such as HPLC that exhibit baseline noise, the LOQ is generally estimated from a determination of S/N ratio (10:1) and is usually confirmed by injecting standards which give this S/N ratio and have an acceptable percent relative standard deviation as well.

**Specificity**: is 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 excipients. Specificity measures only the desired component without interference from other species that might be present; separation is not necessarily required.

**Range**: is defined as the interval between the upper and lower concentrations of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity.

**Robustness**: is defined as the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters (e.g. pH, mobile phase composition, temperature and instrumental settings) and provides an indication of its reliability during normal usage. Determination of robustness is a systematic process of varying a parameter and measuring the effect on the method by monitoring system suitability and/or the analysis of samples.

**System Suitability Determination** : is the evaluation of the components of an analytical system to show that the performance of a system meets the standards required by a method [29]. These parameters can be calculated experimentally to provide a quantitative system suitability test report: number of theoretical plates (efficiency), capacity factor, separation (relative retention), resolution, tailing factor, relative standard deviation (precision). These are measured on a peak or peaks of known retention time and peak width [30].

**Forced Degradation Studies** : Forced degradation or stress studies are considered to deliberately degrade the sample. These studies have been used to evaluate an analytical method's ability to measure an active ingredient and its degradation products, without interference, by generating potential degradation products. During validation of the method, drug substance have been exposed to acid, base, heat, light and oxidizing agent to produce approximately 10% to 30% degradation of active substance. The studies may also provide information about the degradation pathways and degradation products that could form during storage. These studies may also help in the formulation development, manufacturing, and packaging to improve drug product stability.[31-32].

**Aim and objectives**

**Aim**:

HPLC Method Development And Validation Process Of Drug Analysis And Applications.

**Objectives**

To develop a robust and reliable HPLC method for separating and quantifying compound

To validate the HPLC method according to regulatory guidelines, ensuring its accuracy, precision, specificity, and sensitivity.

To evaluate the performance of the HPLC method in terms of its limit of detection (LOD), limit of quantification (LOQ), and robustness.

To demonstrate the applicability of the HPLC method for routine analysis and quality control.

**Literature survey**

1.Mustafa Çelebier, Tuba Reçber, RP-HPLC method development and validation for estimation of rivaroxaban in pharmaceutical dosage forms, Brazilian Journal of Pharmaceutical Sciences 49, 359-366, 2013

**Abstract**: Rivaroxaban, an anti-clotting medication, acts at a crucial point in the blood-clotting process and stops the formation of blood clots. In this study, RP-HPLC method was developed for the determination of rivaroxaban in tablets

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(Xarelto® (10 mg)). Phenomenex Luna 5 µm C18 100 Å LC Column (250 x 4.6 mm) was used at 40 ºC. Isocratic elution was performed with ACN:Water (55:45 v/v) mixture. The flow rate was 1.2 mL min-1 and UV detection was at 249 nm. Internal standard (Caffeine) and rivaroxaban were eluted within 2.21 and 3.37 minutes, respectively. The developed method was validated according to the ICH guidelines and found to be linear within the range 0.005 - 40.0 µg mL-1. The method was accurate, precise, robust and rapid. Thus, it was applied successfully for the quality control assay of rivaroxaban in tablet dosage form.

**2. V Ganjiwale, AP Dewani, AV Chandewar, A comprehensive overview of HPLC method development and validationJ Pharm Sci 2, 1, 2024**

**Abstract**: -Performance Liquid Chromatography (HPLC) is an analytical technique employed for the separation of solutes based on their differential rates of elution within a chromatographic column. This separation method hinges on the distribution of solutes between the mobile phase and the stationary phase. The HPLC instrumentation comprises eight fundamental components: a mobile phase reservoir, solvent delivery system, sample introduction device, column, detector, waste reservoir, connective tubing, and a computer, integrator, or recorder [1]. Achieving successful outcomes with HPLC involves optimizing various operating conditions, including the type of column packing and mobile phase, column length and diameter, mobile phase flow rate, column temperature, and sample size

**3.Pasha Latha E.and Sailaja B.Ranjit singh, Bioanalytical Method Development and Validation by HPLC: A Review,2014**

**Abstract:** The development of selective, sensitive and reliable bioanalytical methods for the quantitative evaluation of drugs and their metabolites in biological matrices is crucial for the successful drug development. The data obtained from these methods is required in the pharmacokinetic and toxicokinetic studies of investigational new drug applications (INDs), new drug applications (NDAs)and abbreviated new drug applications (ANDAs). The results of animal toxicokinetic studies and of human clinical trials, including bioavailability and bioequivalence studies requiring pharmacokinetic evaluation are used to make critical decisions supporting the safety and efficacy of a drug. Therefore, it is of paramount importance that the developed bioanalytical methods are well designed, adequately validated and documented to a satisfactory standard to apply in drug analysis in order to obtain reliable results. High pressure liquid chromatography is a versatile analytical tool useful in identification and quantitative estimation of low concentration of drugs and metabolites in biological matrices. So it is advantageous to develop and validate bioanalytical HPLC method for low dose drugs. This article reviews current progress in HPLC based bioanalytical method development and validation of different drugs. So, far drugs like Anti malarials, Omeprazole, Clofarabine, Palonosetron HCl, etc have been analyzed bioanalytically.

**4.HPLC method development and validation-an overview, Journal of Pharmaceutical Education & Research 4 (1), 2013**

**Abstract**: HPLC method development and validation play important role in the discovery, development and manufacture of pharmaceutical products. This article mainly focuses on the optimization of HPLC conditions and other important perspectives during method development and validation. Various critical steps related to analytical method development and validation is discussed. A sequence of events required for method development and analytical validation are described. The steps involved in developing a stability-indicating HPLC method influences the analysis of degradation products/impurities in stability study and its validation demonstrate the suitability for its intended purpose.

**5.N A espthein , validation of HPLC technique for pharmaceutical analysis, 14, Vol. 38, No. 4, 2014**

**Abstract**: Validation (evaluation of suitability) of an analyticaltechnique is a procedure aimed at obtaining experimentallyjustified evidence of the ability of this technique to give re-sults characterized by the required accuracy and All analytical techniques used for the developmentof pharmaceuticals and for the determination of their qualitycharacteristics have to be validated. In the case of usingmethods stipulated and described in the State Pharmaco-poeia, it is not necessary to evaluate their suitability, pro-vided that the analyses are conducted with strict observation of the text of each particular article. In most other cases, es-pecially in cases of modification of the drug composition, thescheme of synthesis, or the analytical procedure, it is neces-sary to re-evaluate the suitability of the analytical techniques.

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**6.SL Patwekar, RS Sakhare, NN Nalbalwar, HPLC method development and validation-A general Concept,International Journal of Chemical and Pharmaceutical Sciences 6 (1), 8-14, 2015**

**Abstract**: Chromatography, although primarily a separation technique, is mostly employed in chemical analysis. In which High-performance liquid chromatography (HPLC) is an extremely versatile technique where analytes are separated by passage through a column packed with micrometer-sized particles. Now a day reversed-phase chromatography is the most commonly used separation technique in HPLC. The reasons for this include the simplicity, versatility, and scope of the reversed-phase method as it is able to handle compounds of a diverse polarity and molecular mass. This article involves the strategies and the issues for designing HPLC method development and validation. The method development often follows the well established steps, like selection of buffer, selection mobile phase, selection of column. The method so developed should be as simple as possible, the best strategy being some blend of theoretical and empirical approach.

7.**pawan Tiwari, BK Singh, HPLC: a modern approach of development and validation World J Pharm Res 5, 1616-31, 2016**

**Abstract** : High performance liquid chromatography is the dominant analytical technique which is Proficient to separate, detect and quantify various drugs and its related degradants. HPLC methods development and validation play important roles in new discovery, development, manufacture of pharmaceutical drugs and various other studies related to humans and animals. Most of the drugs in multi component dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. A number of chromatographic parameters were evaluated in order to optimize the method.

**8.Vidushi, Bharkatiya Meenakshi, M Bharkatiya, A review on HPLC method development and validation,Res J Life Sci, Bioinform, Pharm Chem Sci 2 (6), 178, 2017**

**Abstract**: is the dominant separation technique to detect, separate and quantify the drug. A number of chromatographic parameters were analyzed to optimize the method like sample pretreatment, choosing mobile phase, column, detector selection. The objective of this article is to review the method development, optimization and validation. HPLC method development depends on chemical structure of the molecules, synthetic route, solubility, polarity, pH and pKa values, and functional groups activity etc. Validation of HPLC method as per ICH Guidelines gives information regarding various stages and knowing characteristics like Accuracy, specificity, linearity limit of detection, limit of quantificationmar

**9.Nageswara Rao Ramisetti,,An overview of experimental designs in HPLC method development and validation, Journal of pharmaceutical and biomedical analysis 147, 590-611, 2018**

**Abstract** : Chemometric approaches have been increasingly viewed as precious complements to high performance liquid chromatographic practices, since a large number of variables can be simultaneously controlled to achieve the desired separations. Moreover, their applications may efficiently identify and optimize the significant factors to accomplish competent results through limited experimental trials. The present manuscript discusses usefulness of various chemometric approaches in high and ultra performance liquid chromatography for (i) methods development from dissolution studies and sample preparation to detection, considering the progressive substitution of traditional detectors with tandem mass spectrometry instruments and the importance of stability indicating assays (ii) method validation through screening and optimization designs.

10. Mohan Varma, Ashok Thulluru, KT Sunil Kumar, HPLC method development and validation: A review, World J Pharm Res 10, 405-426, 2021

**Abstract** : High performance liquid chromatography the most precise techniques commonly used for the qualitative and quantitative study of drug substance. Drug research, drug production, and pharmaceutical manufacturing all depend on the development and evaluation of analytical methods. It involves evaluating a drug substance's purity and toxicity. To refine the methods in the study of system production in HPLC, a variety of chromatographic parameters were evaluated. We create an effective mobile phase, column, column temperature, wavelength, and gradient.

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**PLAN OF WORK**

**Phase 1: Literature Review and Method Scouting**

**1.** Conduct a literature review of existing HPLC methods for the analysis of the drug.

2. Scout different HPLC columns and mobile phases to determine the most suitable conditions for the analysis.

**Phase 2: Method Development**

**1.** Optimize the HPLC conditions, such as column temperature, flow rate, and detection wavelength.

2. Evaluate the chromatographic performance, including resolution, tailing factor, and theoretical plates.

3. Refine the method as needed to achieve optimal performance.

**Phase 3: Method Validation**

**1.** Evaluate the specificity of the method by analyzing blank samples and samples with potential impurities.

2. Determine the linearity of the method by analyzing a range of concentrations.

3. Evaluate the accuracy of the method by analyzing samples with known concentrations.

4. Determine the precision of the method by analyzing replicate samples.

5. Evaluate the robustness of the method by varying parameters such as column temperature and flow rate.

**Phase 4: Documentation and Reporting**

1. Document the method development and validation procedures.

2. Prepare a detailed report of the results, including chromatograms and data analysis.

3. Discuss the implications of the results and potential future work.

**Phase 5: Implementation and Maintenance**

1. Implement the validated method in the laboratory.

2. Establish procedures for routine maintenance and troubleshooting of the HPLC system.

3. Ensure that personnel are trained in the use of the method and HPLC system**.**

**EXPERIMENTAL PART**

**INSTRUMENTS**

HPLC instrument: A high-performance liquid chromatograph with a suitable detector (e.g., UV-Vis, PDA, or MS).

2. Column: A suitable HPLC column (e.g., C18, C8) with a compatible stationary phase.

3. Autosampler: An autosampler for injecting samples into the HPLC instrument.

4. Detector: A detector suitable for sodium diclofenac analysis (e.g., UV-Vis detector).

**Additional Equipment**

**1.** Balance: An analytical balance for weighing samples and standards.

2. Pipettes: Pipettes for accurately measuring and transferring small volumes of liquids.

3. Volumetric flasks: Volumetric flasks for preparing standards and sample solutions.

**Chemicals**

**1.** Paracetamol standard- High-purity..

2. HPLC-grade solvents+High-quality solvents suitable for HPLC analysis, such as:
   - Acetonitrile
   - Methanol
   - Water

3. Mobile phase buffers- Buffers for preparing the mobile phase, such as:
   - Phosphate buffer (e.g., potassium dihydrogen phosphate, disodium hydrogen phosphate)
   - Acetate buffer (e.g., acetic acid, sodium acetate)

4. pH adjusters-Chemicals for adjusting the pH of the mobile phase, such as:
   - Orthophosphoric acid
   - Sodium hydroxide

5. Sample preparation reagents: Reagents for preparing samples, such as:
   - Solvents (e.g., methanol, acetonitrile)
   - Dilution buffers (e.g., phosphate buffer)


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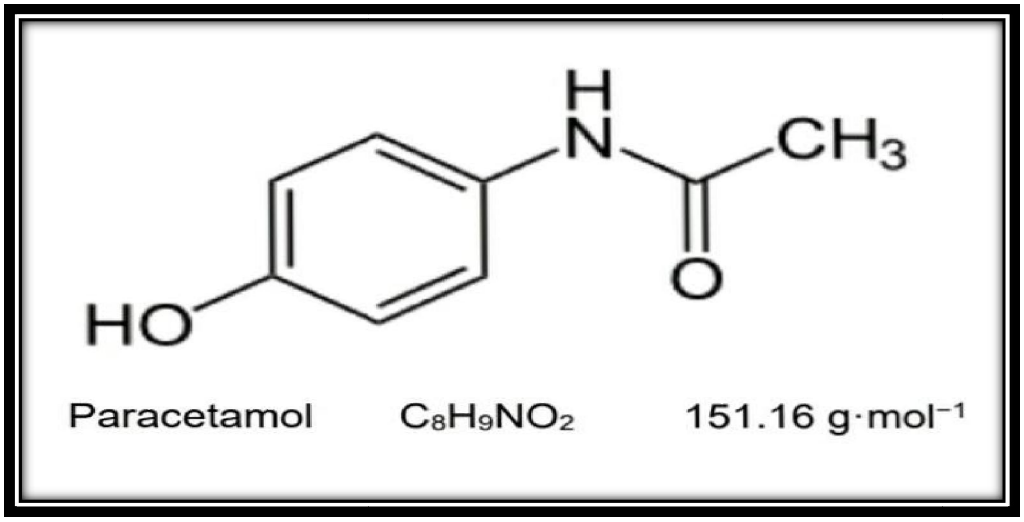
**Method for Paracetamol Analysis**

A method for quantification of paracetamol in a tablet formulation by high pressure liquid chromatography (HPLC) using a reversed phase column and an external standard is described. Method comprises tablet extraction, standard and sample preparation, dilutions and analysis. Pharmaceutical analysis requires accurate results testing the students standard and sample preparation thoroughly.Introduction and Structures

A method for quantification of paracetamol in a tablet formulation by high pressure liquid chromatography (HPLC) using a reversed phase column and an external standard is described. A Tablet extract containing approximately 0.02 % w/v of paracetamol is prepared and about 2 µg of paracetamol is injected into the column in 10 µl of extract solution. The paracetamol is detected using UV detection at a wavelength of 243 nm and quantification is achieved against a five-point calibration curve.




**Reagents and SafetyParacetamol** - Harmful if ingested in quantity; irritant; reproductive effects

**Acetic acid** - Corrosive; harmful to skin and eyes; flammable; vesicant

**Acetonitrile** - Highly flammable; toxic by ingestion, inhalation and skin contact; may be mutagen / teratogen.

Avoid skin and eye contact with reagents by wearing a lab coat, gloves and safety glasses. Do not expose acetonitrile to a source of ignition. Avoid inhalation of acetonitrile vapour.Provided the recommended precautions are adopted, the risk to operators during this procedure is minimal.

**Mobile Phase Preparation**

**MOBILE PHASE A** – Water + 0.1% Acetic Acid• 1 mL Acetic Acid to 1 L of HPLC Grade water

**MOBILE PHASE B** - Acetonitrile• 1L of HPLC or Gradient Grade Acetonitrile

**Standard Preparation**

Stock Solution (0.5 mg/mL)

• Weigh 125 mg (± 10 mg) of paracetamol – analytical grade into a 250 mL volumetric

• Fill to the mark with Mobile Phase A

**Calibration Standards**

• Prepare standards at the following nominal concentration:5, 10, 15, 20 and 25 µg/mL in Mobile Phase A

• Example of preparation of 20 mg/mL solution:1 mL of stock solution made to mark in a 25 mL volumetric with Mobile Phase A

**Sample Preparation**

• Take 1 tablet containing 500 mg of paracetamol

• Grind the tablet into a find powder

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• Dissolve (in duplicate) 125 ± 10 mg of the ground paracetamol tablet into 250 mL volumetric flask
• Add approximately 150 mL of mobile phase A
• Shake or sonicate until dissolved
• Fill volumetric to mark (if sonication is used make sure the solution has cooled first)
• Prepare a further dilution of each solution within the calibration curve

**Analytical Conditions**

• Column : Shimadzu Shimpak GIST C18-AQ 150 x 4.6 mm 5µm
• Temperature : 20°C
• Injected volume : 10 µL
• Mobile phases : A : 0.1% Acetic Acid in Water:
B : Acetonitrile:
C: Water
• Isocratic :90% A : 10% B :
• Flow rate : 1.0 mL/min
• Wavelength : 243 nm (cell at 40 °C)
• Runtime : 10 minutes
• Column Wash : 50:50 Acetonitrile: Water

## RESULT AND DISCUSSION

The developed and validated method of paracetamol was aimed to establish chromatographic conditions, capable of qualitative and quantitative determination of paracetamol in pharmaceutical preparations. Paracetamol was completely separated on C18 column by HPLC using the isocratic elution of ACN and water as mobile phase. When the ACN percentage was reduced starting from 80% by a decrement of every 5%, broadening, fronting and tailing of peaks were observed (Fig 1). As a result of higher concentrations of ACN in mobile phase and decrease in the percentage of ACN the peak was sharp pointed and well separated. The unusual peaks could be the result of improper dissolution of paracetamol in higher concentration of ACN, therefore the chromatographic column was not able to resolve the paracetamol properly and even there was decrease in the recovery percentage of paracetamol (Table 1). As ACN concentration gradually decreases the peak broadening, fronting and tailing were remarkably reduced. It is evident that the flow rate of mobile phase in chromatography plays an important role in resolving the paracetamol, as the flow rate increases from 0.75 mL/min to 1.50 mL/min the retention time also decreased with fluctuation in paracetamol recovery (Table 1), eventually proper resolution was achieved at flow rate of 1mL/min and retention time of 3.6 minutes.

**Table no- 2 Calibration of the HPLC method for the estimation of paracetamol**

| Concentration of paracetamol (mg/ml) | Mean ( s.d.) of AUC of drug to internal standard (n=5) | % CV |
|---|---|---|
| 0 | 0 | 0 |
| 0.1 | 0.150 | 2.30 |
| 0.2 | 0.32 | 1.27 |
| 0.4 | 0.68 | 1.7 |
| 0.8 | 1.21 | 1.69 |
| 1 | 1.18 | 2.60 |
| 2 | 3.57 | 1.97 |
| 4 | 5.81 | 1.47 |
| 8 | 11.94 | 3.22 |

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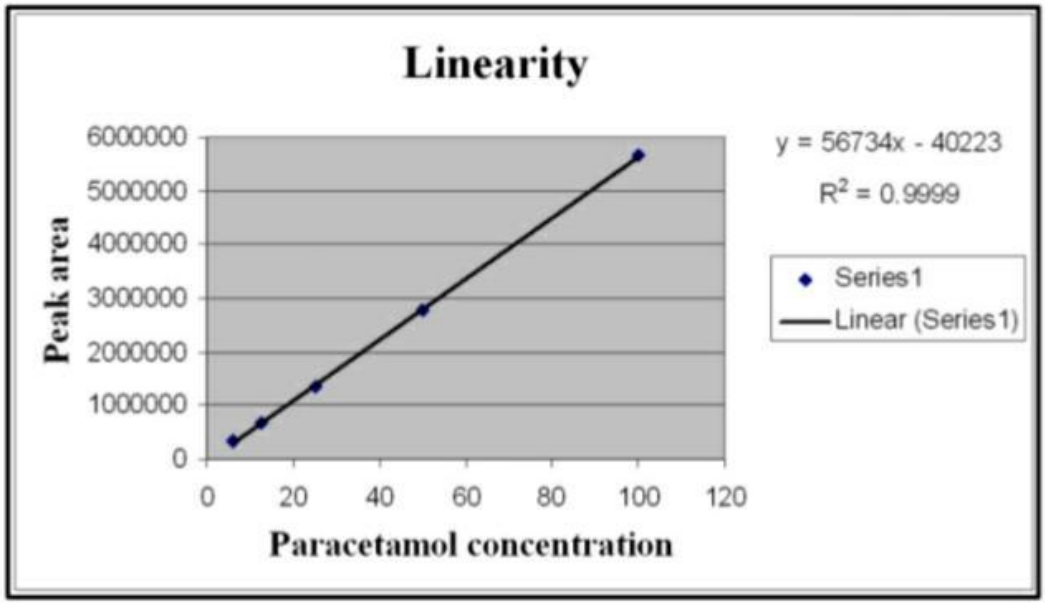
| | | |
|---|---|---|
| 20 | 29.75 | 2.19 |
| 30 | 45.78 | 3.19 |

Linearity

The method gave a linear response to paracetamol drug within the concentration range of 6.25 - 100 µg/mL with $r^2$= 0.999

The paracetamol was recovered in the range of 98.8 to 102.0 % for various concentrations as




shown in the table 2.

Table 2: Recovery percentage of paracetamol in accuracy studies

| Concentration (µg/mL) | Area | Amount Recovered | % Recovered |
|---|---|---|---|
| 10 | 520308 | 09.88 | 98 |
| 35 | 1963621 | 35.32 | 101 |
| 55 | 3143689 | 56.20 | 102 |

## II. CONCLUSION

It can be concluded from the entire review that HPLC is a versatile, reproducible chromatographic technique for the estimation of drug products.

It has wide applications in different fields in term of quantitative and qualitative estimation of active molecules.

Analytical method development can be thought of as a process that begins with planning, which The optimized reverse phase HPLC method for paracetamol is linear, accurate, precise, robust, simple, rapid and selective. It can be adopted ap-parently for routine quality control analysis of raw materials, formulations and testing.

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