

Chromatography latest techniques

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Abstract- High-Performance Liquid Chromatography (HPLC) is a type of column chromatography that is commonly used in biochemistry and analysis to separate, identify, and quantify active chemicals. HPLC is the most often used separation technology for detecting, separating, and quantifying the drug. HPLC technique development and validation serve critical roles in novel drug discovery, development, and manufacturing, as well as a variety of other human and animal investigations. This review discusses the many processes involved in developing and validating an HPLC technique. The creation of an HPLC technique is influenced by the chemical structure of the molecules, the synthetic pathway, solubility, polarity, pH and pKa values, and the activity of functional groups, among other factors. Accuracy, accuracy, specificity, linearity, range, limit of detection, the limit of quantification, robustness, and system suitability testing are all included in the validation of an HPLC technique according to ICH Guidelines.

Keywords: Pressure Liquid Chromatography, Chromatography, Method validation, Method development.

INTRODUCTION:

At the start of the 20th century, Russian-Italian botanist Mikhail Semyonovich Twit devised chromatography, physicochemical method for dividing composite mixtures. Chromatography is a non-destructive method for separating a multi-component mixture into its component parts, whether they are trace, minor, or substantial. Solids, liquids, and gases can all be modified in various ways. Chromatography is primarily a separating tool, though it can also be used quantitatively.

Thin layer chromatography and ion exchange chromatography were two types of chromatography that were established as separation methods in the 1930s. Partition and paper chromatography were first proposed by Martin and Synge in 1941. In 1952, gas chromatography was first used. The routine use of chromatography as a separation technique spread to many fields of study throughout the following ten years, including chemistry, biology, and medicine. It is becoming a possible technology for the preparation of extremely pure compounds, such as in the pharmaceutical business or the production of pure chemicals, in addition to its usage in analysis. The chromatographic methods of biomolecule separation are totally responsible for the recent amazing advancements in the biosciences.

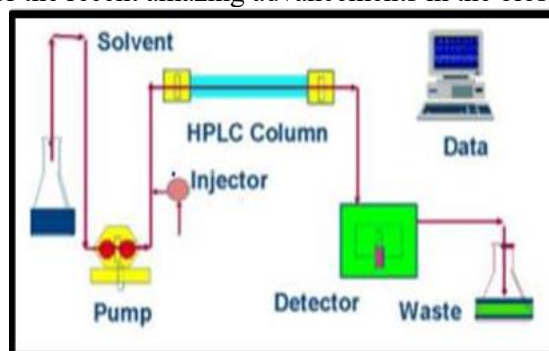


Fig:1 Flow diagram of HPLC

Methods of chromatography HPLC

Method development involves the following steps:

1. Understanding the Physicochemical Properties of the drug molecule,
2. Selection of chromatographic conditions,
3. Developing the approach of analysis,
4. Sample preparations,
5. Method optimization,
6. Method validation.

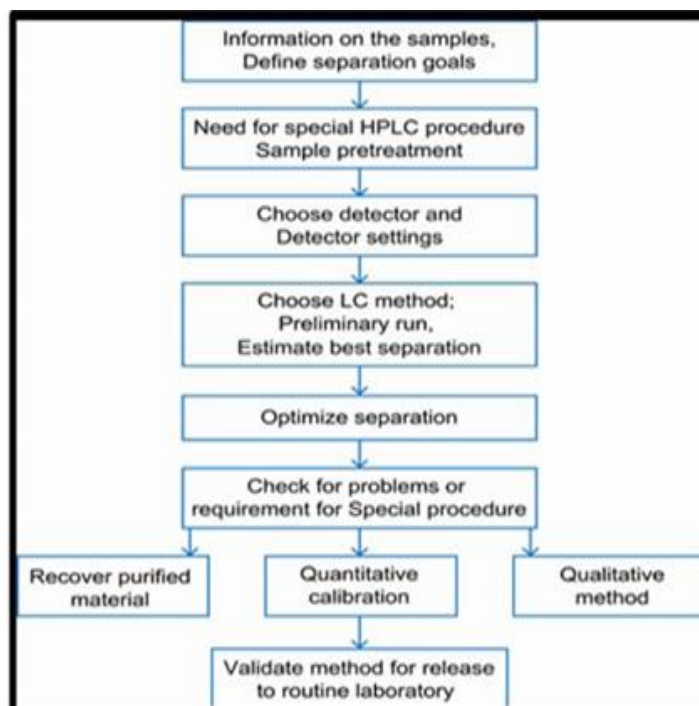


Fig: 2 Steps involved in HPLC Method development.

1. Understanding the Physicochemical Properties of the drug molecule:

The physicochemical qualities of a therapeutic molecule are critical in method development. To develop a method, one must first evaluate the physical properties of the drug molecule, such as solubility, polarity, pKa, and pH. A compound's polarity is a physical property.¹⁶ It supports an analyst in evaluating the solvent and mobile phase composition. Choosing the correct pH for ionizable analytes frequently results in symmetrical and crisp peaks in HPLC. The pH value is defined as the negative of the logarithm to base 10 of the hydrogen ion concentration.

$$\text{pH} = -\log_{10}[\text{H}_3\text{O}^+].$$

Selecting an appropriate pH for ionizable analytes frequently results in symmetrical and sharp peaks in HPLC. In quantitative analysis, sharp, symmetrical peaks are required to achieve low detection limits, low relative standard deviations between injections, and predictable retention durations.

2. Selection of chromatographic condition:

During the early stages of method development, a set of beginning conditions (detector, column, and mobile phase) is chosen to generate the sample's first "scouting" chromatograms. These are typically based on reversed phase separations on a C18 column with UV detection. At this point, a choice should be taken between establishing an isocratic or a gradient method.

3. Developing the approach of analysis:

The initial stage in developing an analytical method for RP HPLC is to select various chromatographic parameters such as mobile phase, column, mobile phase flow rate, and mobile phase pH. All of these characteristics are chosen based on trials, and they are then compared to the system suitability parameters. Typical system suitability parameters include, for example, a retention time of more than 5 minutes, a theoretical plate count of more than 2000, a tailing factor of less than 2, a resolution of more than 5, and a percent R.S.D. of the area of analyte peaks in standard chromatograms of no more than 2.0 %. In the case of simultaneous estimation of two components, the detection wavelength is usually an isobestic point.

4. Sample preparation:

Sample preparation is a vital step in method development that the analyst must study. For example, if there are insoluble components in the sample, the analyst should explore whether centrifugation (determining the ideal rpm and time), shaking, and/or filtration are required. The goal is to show that sample filtering does not affect the analytical result due to adsorption and/or extraction of leachable. The ability of syringe filters to remove contaminants/insoluble components without leaching unwanted artifacts (i.e., extractable) into the filtrate determines their effectiveness. The sample preparation procedure should be fully specified in the appropriate analytical method used on an actual in-process sample or dosage form for later HPLC analysis.

5. Method optimization:

Identify the method's "weaknesses" and optimize the method using experimental design. Understand how the approach performs under varied settings, with different instrument setups, and with different samples. The majority of HPLC technique development optimization has been focused on the optimization of HPLC conditions.³⁴ The compositions of

the mobile phase and stationary phase must be considered. Optimization of mobile phase parameters is always prioritized above optimization of stationary phase parameters since it is considerably easier and more comfortable. After adequate selectivity has been attained, this is utilized to discover the optimal balance between resolution and analysis time. Column dimensions, column-packing particle size, and flow rate are among the parameters at play. These parameters are changeable without impacting the capacity factor or selectivity.

6. Method Validation:

Validation is the evaluation and provision of objective evidence that the specified requirements for a given intended application are met. A way of assessing method performance and demonstrating that it fits a specific condition. In other words, it understands what your method is capable of producing, especially at low concentrations.³⁸ Analytical methods need to be validated or revalidated. Before their introduction into routine use; Whenever the conditions change for which, the method has been validated Whenever the method is changed.

Classification of chromatography:

A. Based on modes of chromatography:

1. Normal-Phase chromatography:

Normal-phase chromatography was one of the kinds of HPLC that chemists developed. Also known as normal-phase HPLC (NP HPLC) this method separates analytes based on their affinity for a polar stationary surface. NP-HPLC uses a non-polar, nonaqueous mobile phase (e.g. Chloroform, Octane), and works effectively for separating analytes readily soluble in non-polar solvents. The analyte associates with and is retained by the polar stationary phase.

2. Hydrophilic interaction chromatography:

Hydrophilic interaction chromatography (HILIC) can be described as a reversed-phase chromatography performed using a polar stationary phase (for example, unmodified silica, amino, or diol bonded phases). The mobile phase employed is highly organic in nature (> 70% solvent, typically acetonitrile) containing also a small percentage of aqueous solvent/buffer or another polar solvent. The water/polar solvent form.

3. Reversed-Phase Chromatography (RPC):

Reversed phase HPLC (RP-HPLC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been surface-modified with RMe₂ Si, where R is a straight-chain alkyl group such as C₁₈ H₃₇ or C₈ H₁₇. With such stationary phases, retention time is longer for molecules that are less polar, while polar molecules elute more readily (early in the analysis). An investigator can increase retention times by adding more water to the mobile phase; thereby making the initially hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase. Similarly, an investigator can decrease retention time by adding a more organic solvent to the eluent.

A. Based on the principle of separation Ion exchange chromatography:

1. In ion-exchange chromatography:

Retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Solute ions of the same charge as the charged sites on the column are excluded from binding, while solute ions of the opposite charge of the charged sites of the column are retained on the column. Solute ions that are retained on the column can be eluted from the column by changing the solvent conditions (e.g. increasing the ionic strength of the solvent system by increasing the salt concentration of the solution, increasing the column temperature, changing the pH of the solvent, etc...).

2. Size exclusion chromatography:

It is also useful for determining the tertiary structure and quaternary structure of purified proteins. SEC is used primarily for the analysis of large molecules such as proteins or polymers. SEC works by trapping these smaller molecules in the pores of a particle. The larger molecules simply pass by the pores as they are too large to enter the pores. Larger molecules, therefore, flow through the column quicker than smaller molecules, that is, the smaller the molecule, the longer the retention time.

pore opening does not diffuse into the particles, while molecules smaller than the pore opening enter the particle and are separated. Large molecules elute earlier, smaller molecules elute later.

It is classified into two categories based on the nature of the columns and their packing:

Gel Filtration Chromatography:

- I. Which uses hydrophilic packing to separate polar species and uses mostly aqueous mobile phases. This technique is mostly used to identify the molecular weights of large sized proteins & bio-molecules.
- II. Gel Permeation Chromatography: Which uses hydrophobic packing to separate non-polar species and uses non-polar organic solvents. This technique is used to identify the molecular weights of polymers.

Affinity chromatography

Affinity chromatography involves covalently bonding a reagent called an affinity ligand, to a solid support. Typical affinity ligands are antibodies, enzyme inhibitors, cofactor/coenzyme, or other molecules that reversibly and selectively bind to analyte molecules in the sample. The principle is that the stationary phase consists of a support medium (e.g. cellulose beads) on which the substrate (or sometimes a coenzyme) has been bound covalently, in such a way that the reactive groups that are essential for enzyme binding are exposed.

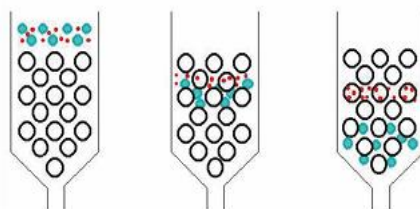


Fig:3 SEC separation

Parameter used in HPLC:

For the accurate analysis of a compound, there are some parameters that 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, and pump pressure. For different compounds, the parameters can be changed according to their nature and chemical properties.

1. Retention time:

Retention time is the difference in time between the point of injection and the appearance of peak maxima. It is also the time required for 50% of a component to be eluted from a column. It is measured in minutes and seconds.

2. Retention volume:

Retention volume is the volume of carrier gas required to elute 50% of the component from the column. It is the product of retention time and low-rate High-Performance Liquid Chromatography (HPLC): A review Retention volume = Retention time \times low rat

3. Separation factor:

Separation factor is the ratio of partition coefficient of the 2 components to be separated.

$$S = K_a / K_b = (t_b - t_0) / (t_a - t_0) \text{ Where } t_0 = \text{Retention time of unretained substance.}$$

$$K_b = \text{Partition coefficient of a, } t_a,$$

$$t_b = \text{Retention time of substance a,}$$

b If there is a more difference in partition coefficient between 2 compounds, the peaks are far apart and the separation factor is more. If the partition coefficient the 2 compounds are similar, then the peaks are closer and the separation factor is less

4. Resolution:

Resolution is the measure of the extent of separation 2 components and the baseline separation achieved.

$$R_s = 2 (R_{t1} - R_{t2}) / w_1 + w_2 \cdot 5$$

Height Equivalent to a Theoretical Plate (HETP):

A theoretical plate is an imaginary or hypothetical unit of a column where the distribution of a solute between the stationary phase and mobile phase has attained equilibrium. It can also be called a functional unit of the column. A theoretical plate can be of any height, which describes the efficiency of separation. If HETP is less, the column is more efficient.

Efficiency:

The efficiency of a column is expressed by the theoretical plates.

$$n = 16 R_{t2} / w_2 \text{ Where } n = \text{no of theoretical plates } R_t = \text{retention time } w = \text{peak width at base.}$$

Asymmetry factor:

A chromatographic peak should be symmetrical about its centre. But in practice due to some factors, the peak is not symmetrical and shows tailing or fronting. Fronting is due to saturation of stationary phase and can be avoided by using less quantity of sample. Tailing is due to more active adsorption sites and can be eliminated by support pretreatment. Asymmetry factor (0.95 to 1.05) can be calculated by $AF = b/a$ (b, a calculated by 5% or 10% of the peak height). Broad peaks occur due to the more conc. of the sample, large injection volume, and column deterioration. Advantage and disadvantages of HPLC.

Types of HPLC:

Normal Phase HPLC:

This technique uses polarity to separate materials. Hexane, chloroform, and diethyl ether are employed as the nonpolar stationary phase while silica serves as the primary polar stationary phase. On a column, the polar samples are kept.

Reverse Phase HPLC:

HPLC is used in reverse to normal phase. The stationary phase is hydrophobic or non-polar while the mobile phase is polar. The non-polar character will be kept more the more of it there is.

Size-exclusion HPLC:

The column will be incorporating with precisely controlled substrate molecules. Based on the difference in molecular sizes the separation of constituents will occur.

Ion-exchange HPLC:

The stationary phase has a surface that is electrically charged in the opposite direction of the sample charge. Aqueous buffer is utilized as the mobile phase and will regulate the pH and ionic strength.

Instrumentation:

The various components that are present in HPLC equipment are:

- HPLC Solvent
- Pump
- Injector
- Column
- Detector
- Data handling device and microprocessor control.

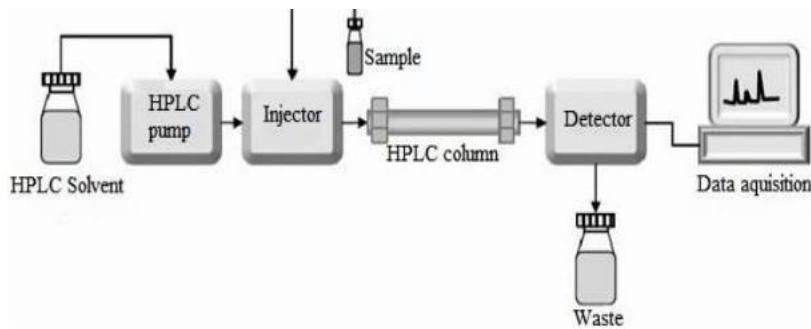


Fig: 4 schematic instrumentations of HPLC

HPLC Solvent:

The solvent reservoir is another name for this component. Here, we keep mobile phase. We use water with a resistivity of 18.2 M cm at 25°C and highly purified solvents such as HPLC grade solvents to prepare the mobile phase.

Pump:

A mobile phase flow rate, commonly measured in millilitres per minute, is generated and metered by a high-pressure pump (solvent delivery system or solvent manager). The mobile phase is drawn from the solvent reservoir by the pump, forced into the column, and then passed on to the detector. The column's dimensions, particle size, flow rate, and mobile phase composition all affect the operating pressure. In HPLC, flow rates typically vary from 1 to 2 ml/min. Normal pumps have a pressure range of 6000 to 9000 psi (400-to 600- bar).

1. Displacement pump: It produces a flow that tends to independent of viscosity and back pressure and also output is pulse free. But it possesses limited capacity (250 ml).
2. Reciprocating pump: Its internal capacity is tiny (35 to 400 l), its output pressure is high (up to 10,000 psi), and its flow rates are constant. However, it results in a pulsed flow.
3. The capacity of pneumatic or constant pressure pumps is constrained, and their flow rate is dependent on the viscosity of the solvent and the column back pressure. They can only be used at pressures under 2000 psi.

Injector:

There are septum injectors available, which are used to inject the sample solution. The ability to introduce the sample into the continuously circulating mobile phase stream that transports the sample onto the HPLC column is provided by an injector (sample manager or autosampler). The combination of a loop injector and a new, sophisticated rotary valve can result in repeatable outcomes. Sample quantities typically range from 5 to 20 microliters (µl).

There are three important ways of introducing the sample into injection port.

1. Loop injection: Using a fixed volume loop injector, a fixed volume of volume is introduced.
2. Valve injection: A variable volume is introduced by using an injection valve in this technique.
3. On column injection: A syringe is used to inject a changeable volume via a septum.

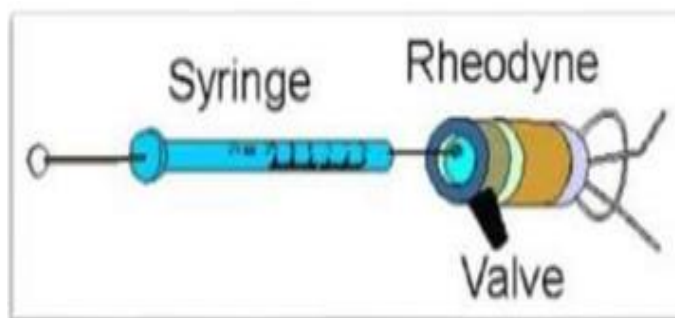


Fig5: Loop of injector

Columns:

It is the location of the real separation. The chromatographic packing material required for the separation is present in the column. Due to the fact that the column hardware keeps this packing material in place, it is known as the stationary phase. It is a stainless-steel tube with an internal diameter of 2.46 cm and a length of 5 to 25 cm. The packing material is either completely porous or only slightly porous.

Modern HPLC uses packing made of tiny, stiff particles with a limited particle size distribution. In HPLC, there are three different forms of column packing. Porous, polymeric beds.

1. Porous layer beds
2. Totally porous silica particle

Detectors:

The detector is capable of identifying the specific molecules that elute from the column. In order for the chemist to quantitatively examine the sample components, a detector measures the quantity of those molecules. A recorder or computer receives an output from the detector, which produces the liquid chromatogram (i.e., the graph of the detector response). When a substance has travelled through the column, it can be determined in a number of different ways. UV spectroscopy is typically used to detect the particular chemicals. Numerous organic substances are UV-absorbing at various wavelengths. The amount of a specific substance that is travelling through the beam at any one time will determine how much light is absorbed.

There are basically two types of detectors:

1. Bulk property detectors:

It contrasts the overall alterations in a mobile phase physical characteristic with and without an eluting solute. such as density, dielectric constant, or refractive index.

2. Solute property detectors:

It reacts to a solute physical characteristic that the pure mobile phase does not display. for instance, diffusion current, fluorescence, or UV absorption.

Data handling device and microprocessor control:

Each peak in the output is a different compound in the mixture that has passed through the detector and absorbed UV light. The area beneath the peak, which is proportionate to the quantity of drug detected, can be determined automatically by the computer connected to the display.

Applications:

Pharmaceutical applications There is a wide variety of applications throughout the process of creating a new drug from drug discovery to the manufacture of formulated products that will be administered to patients.

This Process to create a new drug can be divided into 3 main stages

- 1) Drug discovery
- 2) Drug development
- 3) Drug manufacturing.

LC-MS is the best tool for compound identification and characterization. It may be used as a measurement tool during high throughput screening. Preparative HPLC is also used to isolate and purify hits and lead compounds as required.

E.g.: a combinatorial synthesis.

1. Tablet dissolution study of the pharmaceutical dosage form.
2. To control drug stability, Shelf-life determination.
3. Identification of active ingredients.
4. Pharmaceutical quality control.
5. Tablet dissolution of pharmaceutical dosage forms.

Food and Favor analysis:

1. Rapid screening and analysis of components in non-alcoholic drinks.
2. Measurement of quality of soft drinks and water.

3. Sugar analysis in fruit juices.
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6. Multiresidue analysis of lots of pesticides in food samples by LC triple quadrupole MS.

Environmental applications:

1. Detection of phenol compounds in drinking water.
2. Identification of diphenhydramine in sedimented samples.
3. Bio-monitoring of pollutant.
4. Rapid separation and identification of carbonyl compounds by HPLC.
5. LC/MS/MS solution for pharmaceuticals and personal care products in water, sediment, soil and biosolids by HPLC/MS/MS.
6. Determination of 3-mercaptopropionic acid by HPLC

Forensics applications:

1. Quantification of the drug biological samples.
2. Identification of anabolic steroids in serum, urine, sweat & hair.
3. Forensic analysis of textile dyes.
4. Determination of cocaine and other drugs of abuse in blood, urine, etc.
5. Determination of benzodiazepines in oral fluid using LC/MS/MS.

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CONCLUSION:

One of the most popular analytical techniques is HPLC. It has several benefits over traditional chromatographic methods. HPLC makes precise and quick identification and determination of a variety of natural and synthetic substances possible because of its ease of use and effectiveness. In terms of quantitative and qualitative estimation, it has several applications in a variety of sectors, including pharmaceutical, environmental, forensic, food and flavour, clinical, and many others. It can be used in both laboratory and clinical science. Essentially, it is a greatly enhanced kind

of column chromatography. Solvent is externally driven through a column at high pressures of up to 400ATMs rather than dripping through it while only being affected by gravity. This greatly speeds up the chromatographic procedure. Additionally, it permits the use of column packing material with very fine particle size, providing a lot more surface area for interactions between the stationary phase and the molecules passing through it. As a result, it makes it possible to separate the mixture's components better. The cost of HPLC.

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