

A Concise Review on High-Performance Liquid Chromatography

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Abstract

A review of High-Performance Liquid Chromatography included an introduction, chromatographic terms, different classes, and types of HPLC techniques. Also included a brief introduction to HPLC principle and instrumentation. Columns, pumps, and detectors used in HPLC are also included in detail with their efficiency. Applications of the HPLC and overall new advantage cues are included in this review article.: High performance liquid chromatography (HPLC) is an important qualitative and quantitative technique, generally used for the estimation of pharmaceutical and biological samples.

The chromatography term is derived from the Greek words namely chroma (colour) and graphein (to write). Chromatography is defined as a set of techniques that are used for the separation of constituents in a mixture. This technique involves 2 phases stationary phase and a mobile phase. The separation of constituents is based on the difference between partition coefficients of the two phases. chromatography is a very popular technique and it is mostly used analytically. There are different types of chromatographic techniques namely Paper Chromatography, Thin Layer Chromatography (TLC), Gas Chromatography, Liquid Chromatography, Ion exchange Chromatography, and lastly Liquid Chromatography (HPLC). This review is mainly based on the HPLC technique its principle, types, instrumentation, and applications. The mixture is separated using the basic principle of column chromatography and then identified and quantified by spectroscopy. In the 1960s, the column chromatography LC with its low-pressure suitable glass columns was further developed to the HPLC with its high- pressure adapted metal columns.

Keywords: HPLC, Columns, Pumps, Injections and Detectors etc.

1. General Introduction to Chromatography

Chromatography (from Greek Chroma "colour" and graph in "to write") is the collective term for a set of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase, which separates the analyte to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus changing the separation.

History of chromatography

The history of chromatography begins during the mid-19th century. Chromatography, literally "color writing", was used and named in the first decade of the 20th century, primarily for the separation of plant pigments such as chlorophyll. New types of chromatography developed during the 1930s and 1940s made the technique useful for many types of the separation process. Chromatography became developed substantially as a result of the work of Archer John Porter Martin and

Richard Laurence Millington Synge during the 1940s and 1950s. They established the principles and basic techniques of partition chromatography, and their work encouraged the rapid development of several types of chromatography methods: paper chromatography, gas chromatography, and what would become known as high-performance. Since then, technology has advanced rapidly. Researchers found that the main principles of Teat's chromatography could be applied in many different ways, resulting in different varieties of chromatography. Simultaneously, advances continually improved the technical performance of chromatography, allowing the separation of increasingly similar molecules [01].

2. Introduction to high Performance Liquid Chromatography.

The advanced technique having faster separation time and increased accuracy, precision and sensitivity is High-Performance Liquid Chromatography which is based on adsorption, partition, ion exchange, and gel permeation principle. In this type of chromatography, the separation of compounds is carried out based on their idiosyncratic polarities.

The interaction of these compounds with the stationary phase of the column too is considered. The equipment required for carrying out high-performance liquid chromatography includes a pump (used for moving the mobile phase and analyte through the column), a stationary phase, and a detector. The retention

time for the analyte too is provided by the detector. Depending on the strength of interactions that take place between the analyte and the stationary phase, retention time varies.

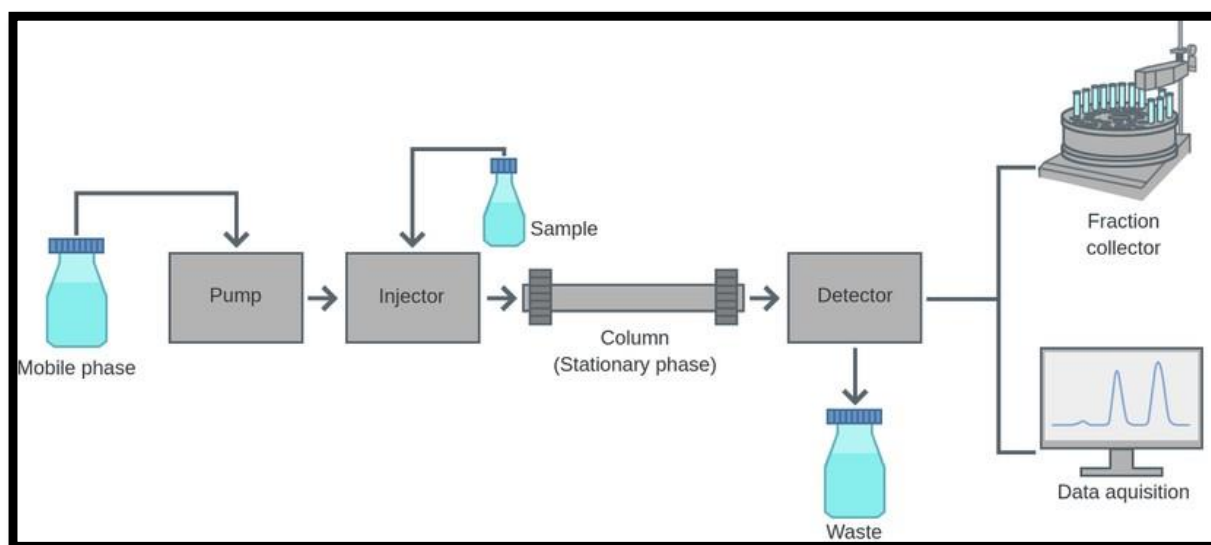


Figure 1: Flow Diagram of High-Performance Liquid Chromatography System

3. Classification

a) Based on Modes of Separation HPLC is classified into the following two types

1. Normal-phase chromatography, or NP: is liquid chromatography's classic form using polar stationary and non-polar mobile phases. The analyte is retained by the interaction of its polar functional groups with the polar groups on the surface of the packing. Analytes elute from the column starting with the least polar compound followed by other compounds in order of their increasing polarity. Normal-phase chromatography is useful in the separation of Analytes with low to intermediate polarity and high solubility in low-polarity solvents. Water-soluble Analytes are usually not good candidates for normal-phase chromatography.

2. Reversed-phase chromatography, or RP: has become the most common mode of liquid chromatographic separation. In RP the stationary phase is non-polar, and the mobile phase is polar. The Analytes are attracted to the surface by their non-polar functional groups. The most polar analyte elutes from the RP column first followed by other Analytes in order of decreasing polarity. RP chromatography is useful for the separation of compounds having high to intermediate polarity [02, 03].

b) Based on the separation mechanism and type of stationary phase used

- Adsorption chromatography
- Partition chromatography
- Ion exchange chromatography
- Pore penetration (permeation) chromatography
- Bio affinity chromatography
- Chiral phase chromatography

This classification is based on different types of stationary phases used while the liquid mobile phase is common for all the above types depending upon the polarity of the solvent and compound which is to be separated.

In Adsorption Chromatography: the chemical mixture is needed to be passed over an adsorbent bed. The different compounds present in this mixture get adsorbed on the bed at different rates. The process is mostly carried out for analytical separation.

In Partition Chromatography: this type of separation technique, a mixture is separated by making use of the partition of a solute between two solvents. In this process, one of the solvents is immobilized with the help of a substance present in the filter paper or column.

In Ion-exchange Chromatography: as the name suggests, the ion-exchange mechanism is used to separate the analytes in this method of chromatography. It can be performed in 2 different modes, i.e., the planar and column. The separation of charged compounds like peptides, amino acids, proteins, etc. is carried out using a charged stationary phase.

In Pore Penetration Chromatography: molecules of the mixture are separated based on their size. This technique of chromatography is also known as gel permeation or size exclusion chromatography. Technically speaking, the separation is carried out based on the hydrodynamic diameter of the molecules. Larger molecules present in the mixture are unable to enter the pores of the media; therefore, these molecules are washed out quickly. The smaller molecules on the other hand

take more time to elute, as they can enter the pores of media.

In Affinity Chromatography non-covalent interaction takes place between the analyte in question and certain molecules. The purification of proteins that are bound to tags is carried out with the help of affinity chromatography.

Chiral Phase Chromatography is a variant of column chromatography in which the stationary phase contains a single enantiomer of a chiral compound rather than being achiral. The two enantiomers of the same analyte compound differ in affinity to the single-enantiomer stationary phase and therefore they exit the column at different times. The chiral stationary phase can be prepared by attaching a suitable chiral compound to the surface of achiral support such as silica gel, which creates a Chiral Stationary Phase (CSP). Many common chiral stationary phases are based on oligosaccharides such as cellulose or cyclodextrin (in particular with β -cyclodextrin, a seven-sugar molecule). Chiral Stationary Phases are much more expensive than comparable achiral stationary phases such as C18[04, 05].

4. Principle of High-Performance of Liquid Chromatography

High-performance liquid chromatography (HPLC) is a separation technique utilizing differences in the distribution of compounds to two phases, called stationary phase and mobile phase. The stationary phase designates a thin layer created on the surface of fine particles and the mobile phase designates the liquid flowing over the particles. Under a certain dynamic condition, each component in a sample has a different distribution equilibrium depending on solubility in the phases and/or molecular size. As a result, the components move at different speeds over the

stationary phase and are thereby separated from each other. This is the principle behind HPLC [06].

The column is a stainless steel (or resin) tube that is packed with spherical solid particles. The mobile phase is constantly fed into the column inlet at a constant rate by a liquid pump. A sample is injected from a sample injector near the column inlet. The injected sample enters the column with the mobile phase and the components in the sample migrate through it, passing between the stationary and mobile phases. Compounds move in the column only when they are in the mobile phase. Compounds that tend to be distributed in the mobile phase, therefore, migrate faster through the column while compounds that tend to be distributed in the stationary phase migrate slower. In this way, each component is separated on the column and sequentially elutes from the outlet. Each compound eluting from the column is detected by a detector connected to the outlet of the column.

When the separation process is monitored by the recorder starting at the time the sample is injected, a graph is obtained. This graph is called a chromatogram. The time required for a compound to elute (called retention time) and the relationship between compound concentration (amount) and peak area depend on the characteristics of the compound. Retention time is therefore used as an index for qualitative determination and peak surface area (or height) as an index for quantitative determination. The retention time of the target compounds and the concentration for each unit of peak area is based on data obtained in advance by analyzing a sample with known quantities of the reference standards. Normally, the reference standards are highly purified target compounds [07].

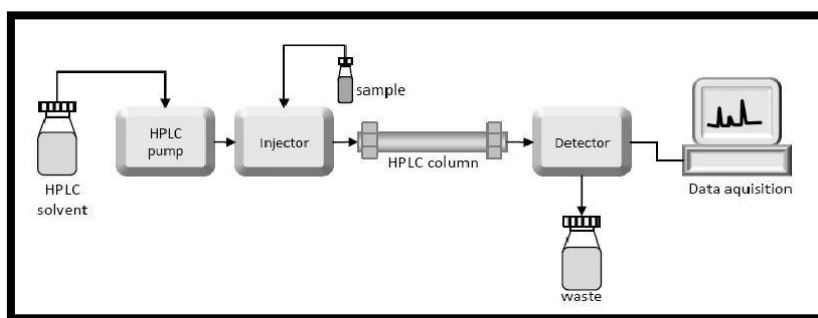


Figure 2: Components of High-Performance Liquid Chromatography

5. Instrumentation of HPLC

- Solvent Reservoir
- Pump of High pressure - 1000 to 5000 psi
- Injector of Low pressure - stop the flow of High-pressure value
- Column of Normal Phase - organic (water-free) mobile phase
 - Silica gel - non-aqueous
- Adsorption of Reverse phase (C8, C18) - aqueous mobile phase
- Partitioning of Ion-exchange - aqueous mobile phase
- Molecular sieve - aqueous mobile phase

- Size
- Detector of Specific
 - Absorbance
 - Fluorescence
 - Electrochemical of Non-specific
 - Refractive index
 - Radioactivity
 - Conductivity [8,9].

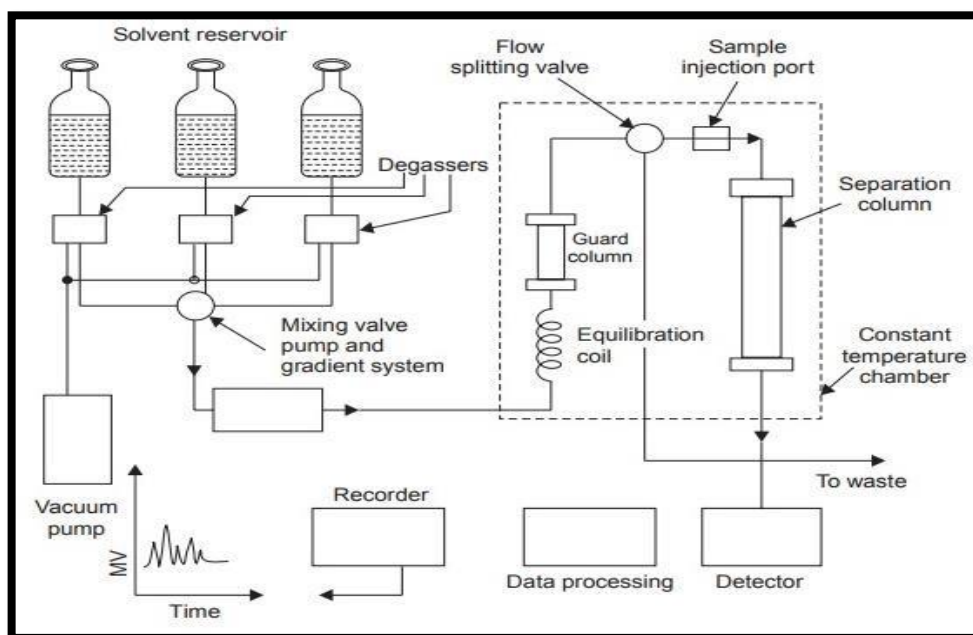


Figure 3: General instrumentation of High-Performance Liquid

a. Solvent Reservoir

Glass or stainless-steel containers capable of holding up to 1-liter mobile phase [pure organic solvents or aqueous solution of salts and buffers]. Inert to a variety of aqueous and non-aqueous mobile phases. Stainless steel should be avoided for use with solvents containing halides ions. Solvent flow through an HPLC system begins in the solvent reservoirs, which contain the solvents used

to carry the sample through the system. The solvents should be filtered through an inlet solvent filter to remove any particles that could potentially damage the system's sensitive components. Reservoirs used for storing high-performance, low-pressure liquid chromatography mobile phase. Complete reservoir system includes a plastic-coated graduated bottle, a standard cap assembly, and solvent and sparging filters.



Figure 4: An Overview of The High-Performance Liquid Chromatography



Figure 5: High Performance Liquid Chromatography Pump

High Performance Liquid Chromatography Pump

- The pump is one of the most important components of HPLC since its performance directly affects retention time, reproducibility, and detector sensitivity.
- The two basic classifications of the pump are the constant-pressure and the constant-flow pump.
- The constant-pressure pump is used only for column packing.
- The constant-flow pump is the most widely used in all common

HPLC applications.

- Three main types of pumps are used in HPLC to propel the liquid mobile phase through the system.

1. Pneumatic or constant pressure pump: They are pulse free; suffer from limited capacity as well as a dependence of flow rate on solvent viscosity and column back pressure. They are limited to pressure less than 2000 psi.

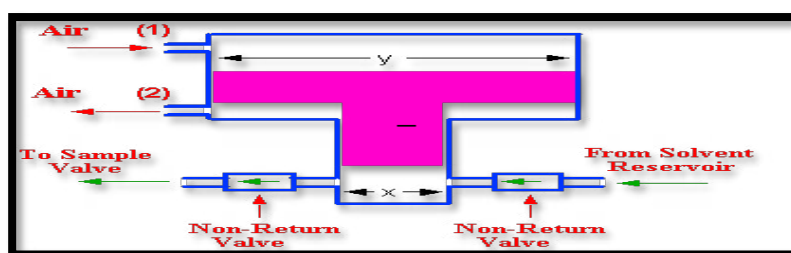


Figure 6: Pneumatic Pump for High Performance Liquid Chromatography

1. Displacement pump (Syringe pump): It produces a flow that tends to be independent of viscosity and back pressure and also the output is pulse free. But it possesses a limited capacity (250 ml). For micro-HPLC applications, a syringe pump allows

for the maintenance of a constant flow at the microliter per minute flow rate range. The syringe pump has to be refilled after it displaces the whole syringe volume.

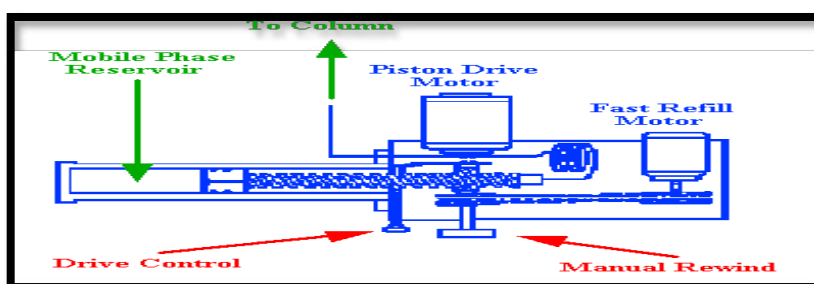


Figure 7: Syringe Pump for High Performance Liquid Chromatography

1. Reciprocating pump: It has a small internal volume (35 to 400 μl), a high output pressure (up to 10,000 psi), and constant flow rates. But it produces a pulsed flow [10-12].

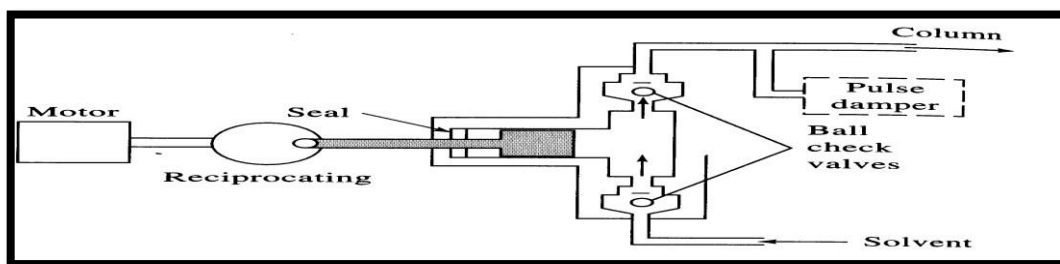


Figure 8: Reciprocating pump for High Performance Liquid Chromatography

Modern Pumps Have the Following Parameters:

- Flow range: 0.01 to 10 ml/min
- Flow rate stability: not more than 1%
- For SEC flow rate stability should be less than 0.2%
- Maximum pressure: up to 5000 psi
- Pressure pulsations: less than 1% for normal and reversed phase mode

c. HPLC injection system



Figure 9: Redone injectors for High Performance Liquid Chromatography

Injection system

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

- **Loop injection:** In which, a fixed amount of volume is introduced by making use of a fixed volume loop injector.
- **Valve injection:** In which, a variable volume is introduced by making use of an injection valve.
- **On-column injection:** In which, a variable volume is introduced using a syringe through a septum.

The commonly used type is the anodyne injector (loop injector) fixed near the pump. It has a range to inject from 20 μ l to 100 μ l volume. In the load, the position sample is injected by a syringe into the injector. In injection, the mode sample is injected from the loop into the pump. When an injection is started, an air actuator rotates the valve: solvent goes directly to the column, and the injector needle is connected to the syringe. The air pressure lifts the needle and the vial is moved into position beneath the needle. Then, the needle is lowered to the vial [13].

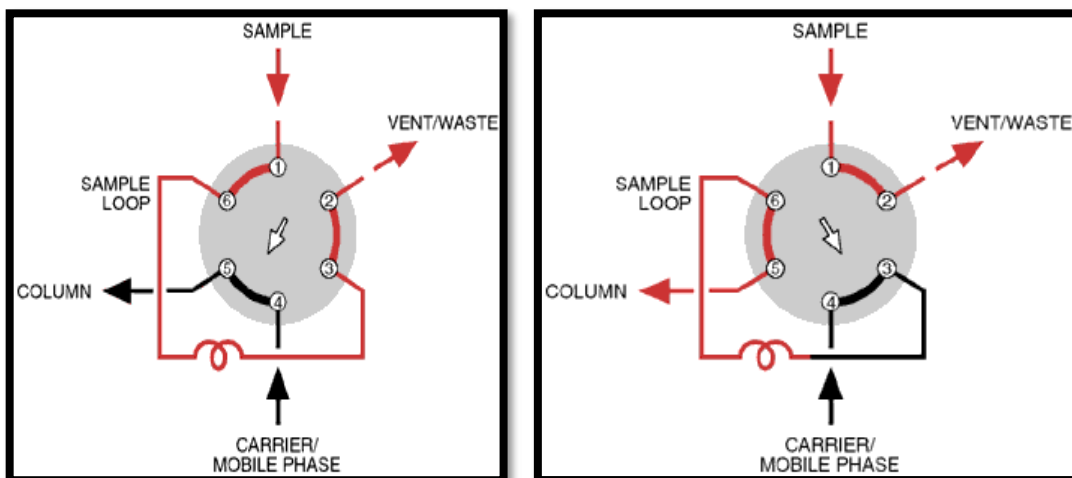


Figure 10, 11: Inject (move the sample loop into the mobile phase flow)

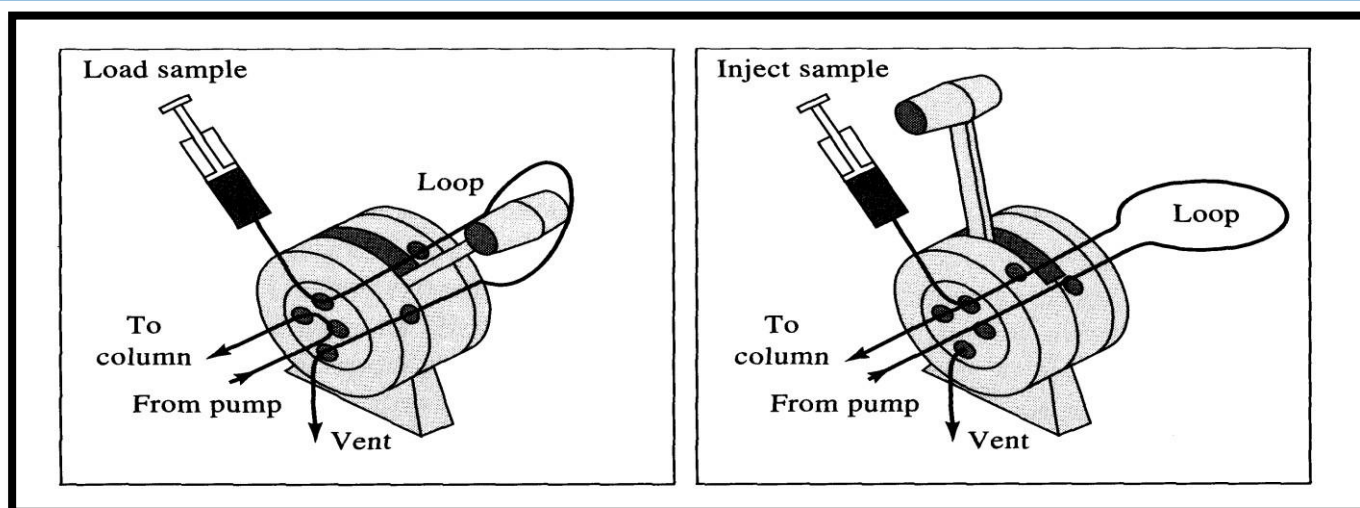


Figure 12: Inject (move the sample loop into the mobile phase flow)

d. Columns

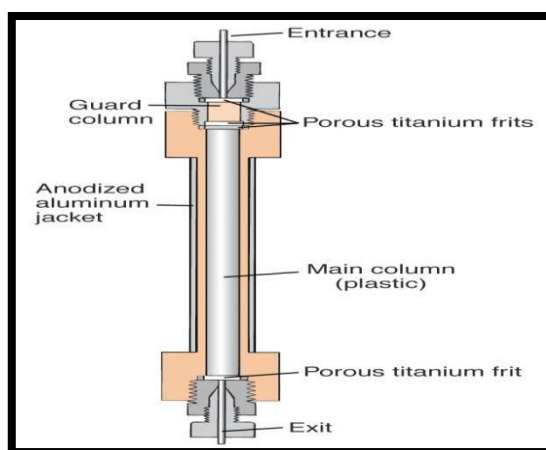


Figure 13: Construction of High-Performance Liquid Chromatography column

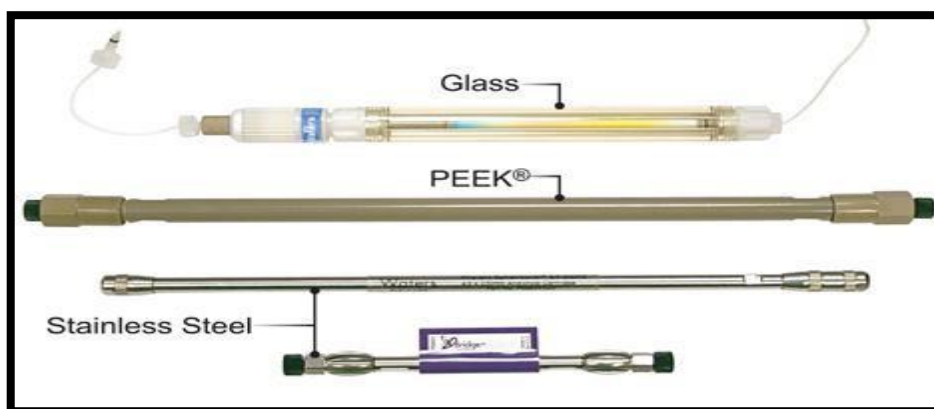


Figure 14; High Performance Liquid Chromatography Column

Conventional liquid chromatography uses plastic or glass columns that can range from a few centimeters to several meters. The most common lengths are 10-100cm, with the longer columns finding a use for preparative-scale separations. High-performance liquid chromatography (HPLC) columns are

stainless steel tubes, typically of 10-30 cm in length and 3-5 mm inner diameter. Short, fast analytical columns, and guard columns, which are placed before an analytical column to trap junk and extend the lifetime of the analytical column, are 3-10 cm long [14,15].



Figure 15: High Performance Liquid Chromatography column for preparative and analytical operation



Figure 16 Picture of High-Performance Liquid Chromatography Column

The column is the most important and sensitive part of HPLC. They are commercially available as microporous, pellicular, and bonded phase types based on the physical and chemical nature of the packed stationary phase in the column. They can withstand a pressure of 8000 psi. They are available in lengths of 20 to 50cm and internal diameters of 1 to 4mm. The particle size range is 5 to 30 microns but mostly lower sizes are preferred for better separation. For reverse phase columns (i.e., the stationary

phase is non-polar) C18 columns are used. While normal phase columns (stationary phase is polar).

Guard Columns: These are small columns with a similar stationary phase to that of columns. They are used to prevent the passage of any particulate matter to prevent clogging and damage to the column [16].

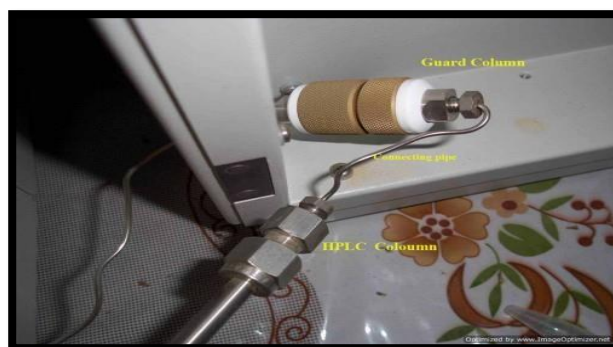


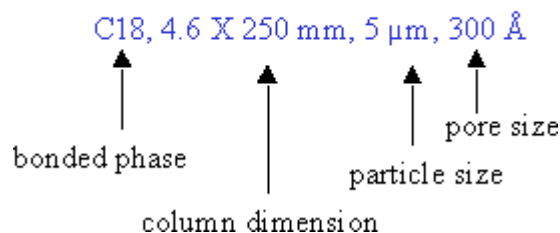
Figure 17: High Performance Liquid Chromatography Guard Column

HPLC Column Components and Specifications



Figure 18: High Performance Liquid Chromatography Column Specifications

Column Specifications



- column dimension (size)
- particle size and pore size
- stationary phase

Column dimension (size): Since columns are tubular, column dimensions usually take the following format, internal diameter X length (4.6mm X 250mm). Columns ranging in internal diameter from 0.050 to 4.6 mm are used or even larger columns for performing large-scale preparative chromatography. This is an important fact that shorter columns are generally cheaper and generate less back pressure. Why is less back pressure important? If a column runs at low pressure, it allows the user more flexibility to adjust the flow rate. Sometimes shorter columns are used to do fast chromatography at higher-than-normal flow rates. In terms of length, we routinely run 100 mm columns; however, 50 mm or 30 mm columns may be adequate for many LC/MS separation needs.

Particle size and pore size: The most common columns are packed with silica particles. The beads or particles are generally characterized by particle and pore size. Particle sizes generally range between 3 and 50 microns, with 5 μ m particles being the most popular for peptides. Larger particles will generate

less system pressure and smaller particles will generate more pressure. The smaller particles generally give higher separation efficiencies. The particle

pore size is measured in angstroms and generally ranges between 100-1000 Å. 300 Å is the most popular pore size for proteins and peptides and 100 Å is the most common for small molecules. Silica is the most common particle material. Since silica dissolves at high pH, it is not recommended to use solvents that exceed pH 7. However, recently some manufacturers have introduced silica-based technology that is more resistant to high pH. In addition, the combination of high temperatures and extremes of pH can be especially damaging to silica [17].

e. Detector

This part of HPLC helps in the detection and identification of compounds in the sample. Based on the types they are available as UV-Visible, Fluorescent, Electrochemical, and Photo diode-array detectors [17].



Figure 19: High Performance Liquid Chromatography Detector

These detectors are designed to have certain properties like...

- Being inert (non-reactive) to the sample injected and the mobile phases passing through
- Preferably non-destructive to the sample
- Produce a quick and quantitative response
- Produce uniform, reliable and reproducible detection and analytic data.
- Compatible with all types of compounds under testing.

HPLC Detector	Commercially Available	Mass LOD (typical)	Linear Range (decades)
Absorbance	Yes	10 pg.	3 – 4
Fluorescence	Yes	10 FG	5
Electrochemical	Yes	100 pg.	4 – 5
Refractive index	Yes	1 ng	3
Conductivity	Yes	100 pg-1 ng	5
Mass spectrometry	Yes	<1 pg.	5
FAIR	Yes	1 µg	3
Light scattering	Yes	1 µg	5
Optical activity	No	1 ng	4
Element selective	No	1 ng	4 – 5
Photoionization	No	<1 pg.	4

Table 1: Performance of HPLC detectors

HPLC Fluorescence detector

In this detector, the fluorescence rays emitted by the sample after absorbing incident light are measured as a function of the quality and quantity of the sample. The equipment comprises accessories in order as a light source, a sample passing through tubing exposed to rays, grating (for light refraction), a photocell, a charged conductor, etc. detector that is suitable for compounds that can produce fluorescence. Some compounds are 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 nanograms quantities.

HPLC Electrochemical detector

This detector is especially suitable to estimate oxidizable & reducible compounds. The principle is that when the compound is either oxidized or reduced, the chemical reaction produces electron flow. This flow is measured as the current which is the function of the type and quantity of the compound. The system has electrodes like a working electrode where oxidation or reduction takes place and a reference electrode that acts to subtract conductivity of the mobile phase from that of the

sample. This electrode is suitable for compounds that can't be assayed by a UV detector especially due to their similarities in light absorption properties ex: monoamines. This detector has super sensitivity which ranges till picograms measurement; so, a very minute quantity of compounds present in the samples can be measured. This electrochemical detector produces severe noise or fluctuations in peaks. So, it is tedious to work with when compared to other detectors.

Photo diode-array detector

They are quite versatile Advances in computer technology since ~1985 have led to the development of Diode Array instruments. They are Non-destructive and non-universal. DAD scans a range of wavelengths every second or a few seconds. At each point in the chromatogram, one gets a complete UV-VIS spectrum Detailed spectrum for each peak and each region of each peak can be obtained using a photo diode-array detector. A diode array permits qualitative information to be obtained beyond simple identification by retention time. The best wavelength(s) can be selected for actual analysis. Absorbance rationing at several wavelengths helps to decide whether the peak represents a single compound or, in fact, a composite peak [18].

Sr. No.	Detector	Analytes	Solvent requirement	Comments
1	UV visible detectors	Any with chromospheres	UV-grade non-UV absorbing solvents	Has a degree of selectivity and is useful for many HPLC application
2	Fluorescence detectors	Fluorescent compound	UV-grade non-UV absorbing solvents	Highly selective and sensitive. often used to analyze derivatized compound
3	Refractive index detector	Compounds with a different refractiveindex to the mobile phase	Cannot run mobile phase gradients	Virtually a universal detector but has limited sensitivity
4	Conductivity detector	Charged or polar compound	The mobile phase must be conducting	Excellent for ion exchange methods
5	Electrochemical detector	Readily oxidized or reduced compounds, especially biological samples	The mobile phase must be conducting	Very selective and sensitive
6	Evaporative light scattering detector (ELSD)	Virtually all compounds	Must use volatile solvents and volatile buffers	A universal detector that is highly sensitive. Not selective
7	Mass spectrometer detector	A broad range of compounds	Must use volatile solvents and volatile buffers	Highly sensitive and is a powerful 2- dimensional analytical tool. Many modes are available. Needs trained operator

Table 2: Application of different detectors

6. Applications of High-Performance Liquid Chromatography

In addition, it is used for analysing air and water pollutants, monitoring materials that may jeopardize occupational safety or health, and monitoring pesticide levels in the environment. Federal and state regulatory agencies use HPLC to survey food and drug products, for identifying confiscated narcotics, or to

check for adherence to label claims.

High-Performance Liquid Chromatography (HPLC) is widely used in the pharmaceutical industry, food and beverage industry, research and development, quality control, production process control, environmental analyses, and clinical tests.

Preparative HPLC refers to the process of isolation and purification of compounds. Important is the degree of solute purity and the throughput, which is the amount of compound produced per unit of time. This differs from analytical HPLC, where the focus is to obtain information about the sample compound. The information that can be obtained includes identification, quantification, and resolution of a compound [18, 19].

I. Purification

Purification refers to the process of separating or extracting the target compound from other (possibly structurally related) compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic conditions. Depending on what needs to be separated and how closely related the samples are, the chromatographer may choose the conditions, such as the proper mobile phase, to allow adequate separation to collect or extract the desired compound as it elutes from the stationary phase. The migration of the compounds and contaminants through the column needs to differ enough so that the pure desired compound can be collected or extracted without incurring any other undesired compound.

II. Chemical Separation

Chemical Separations can be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase. Thus, the chromatographer can separate compounds (more on chiral separations) from each other using HPLC; the extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase [20].

7. Conclusion

HPLC is probably the most universal type of analytical procedure; its application areas include quality control, process control, forensic analysis, environmental monitoring, and clinical testing. In addition, HPLC also ranks as one of the most sensitive analytical procedures and is unique in that it easily copes with multi-component mixtures. It has achieved this position as a result of the constant evolution of the equipment used in LC to provide higher and higher efficiencies at faster and faster analysis times with constant incorporation of new highly selective column packing.

UPLC system provides high speed of analyses so a greater number of analyses can be performed in a shorter amount of time thereby increasing sample throughput and better assay sensitivity. Analysis of operation cost and sample throughput found UPLC cost advantageous over HPLC.

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