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A REVIEW ON OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Nowadays HPLC is widely used instrumentation technique for separation and purification in various areas such as Biotechnology, Pharmaceuticals, Environmental food and polymer Industries.In this Review of High- Performance liquid chromatography contain Introduction, Chromatographic terms, Types and different classes of HPLC . As well as contain brief introduction to HPLC principle and Instrumentation, pumps, Included in detail with their efficiency.

This article is Written with the intension of reviewing several HPLC related topics including instrumentation , principles , manner of separation, characteristics, key parameter and Numerous application in various fields.

KEYWORDS: *HPLC , Instrumentation , pharmaceutical aid , Techniques , Application, Detector, columns , pumps.*

INTRODUCTION

In the late 1960s and early 1970s, liquid chromatography (HPLC) was developed. The amount that a unit remains in the column is determined by its separation between the liquid mobile phase and the stationary phase. In HPLC, this separation is affected by the interface phase interaction between the solute/residual phase and the solute/stimulus. So, unlike GC, changes in mobile phase composition can have a significant impact on your split , since the compounds have different mobilities , they leave the column at different times. In other words, the retention periods are different [1].Additionally taken into account is how these compounds interact with the column's stationary phase. The apparatus needed to execute high performance liquid chromatography consists of a detector, a stationary phase, and a pump that moves the analyte and mobile phase across the column. The detector also provides the retention time for the analyte. Retention period fluctuates according to the strength of interactions between the analyte and the stationary phase.[2,3



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 # DIFFERENT TECHNIQUES OF CHROMATOGRAPHY

1) **Normal phase chromatography :-** This is basic column chromatography. HPLC is relatively simple. The column is filled with silica molecules and the solvent used is non-polar.The polar components of the mixture passing through the column adhere to the polar silica particles more than the non-polar components. Therefore, the non-polar compounds in the column move faster [4,5].
2) **Reverse phase chromatography :-**Reverse phase HPLC is the most commonly used type of HPLC. In this mode silica is reduced by attaching long hydrocarbon chains to the surface. The polar mobile phase is used [6] Therefore, the bond between the polar metal and the polar molecules in the mixture is stronger than the hydrocarbon chains attached to the silica and the polar molecules in the solution [7]. Non-polar compounds attract hydrocarbon groups due to van der Waals dispersion forces. It Is less soluble in metals [8,9].
3) **Size exclusion chromatography:-** also known as gel permeation chromatography. It is also useful for finding the semi- and tertiary structures of proteins and amino acids. This method is used to determine the molecular weight of polysaccharides.
4) **Ion exchange chromatography: -**The attraction between the dissolved ions and the ion sites associated with the stationary phase is maintained in ion exchange chromatography. This type of chromatography is often used in water purification, ligand exchange chromatography, protein ion exchange chromatography, high pH anion exchange chromatography of carbohydrates and oligosaccharides and other applications [10,11].
5) **Bioaffinity:-** Separation is based on specific reversible interaction of proteins with ligands.It protects the proteins bound to the ligand attached to the column. The formation of these complexes involves the participation of common molecular forces such as van der Waals interaction, electrostatic interaction, dipole-dipole interaction, hydrophobic interaction and hydrogen bonding.

6) Separation Mode:- There are two types of separation in HPLC, based on the composition of the eluent:

I) Isocratic: In the isocratic separation mode there is a constant composition of eluent. This means that the equilibrium conditions in the column and the velocity of the compounds moving through are the same.

II) Gradient: The gradient separation mode contains different combinations of detergents. This method greatly increases the resolution power of the system due to the higher intensity. The width of the peak depends on the speed of mixing of the detergent.

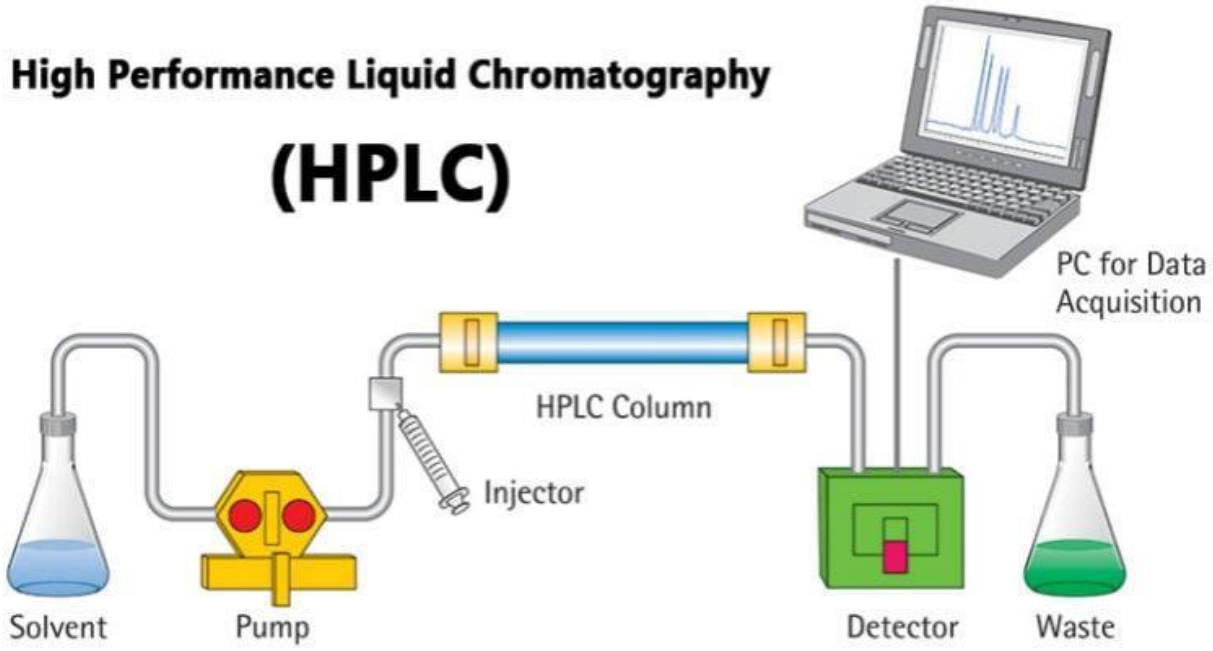
PRINCIPLE OF HPLC

The principle of HPLC is that the sample solution is injected into a column of porous materials(mobile phase) and the liquid phase (mobile phase) is pumped into higher pressure. The principle of phase separation is the introduction of solutes into the stationary phase based on its affinity for the stationary phase [12].

INSTRUMENTATION:

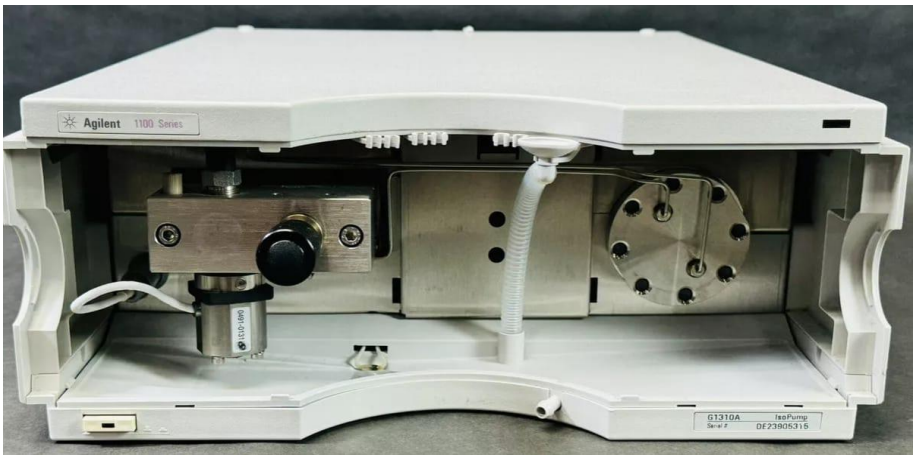
1. Solvent reservoir
2. Pump
3. Sample injector
4. Column
5. Detector
6. Data collection device or integrator

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1)Solvent Reservoir:- The solvent is stored in the solvent reservoir (mobile phase). Here are containers crafted from glass or stainless steel that stay free from discoloration. Glass bottles are considered the most common type of solvent reservoir. In addition to handling the mobile phase, the pump must meticulously and precisely blend solvents together. Low pressure mixing and high pressure mixing are the two types of mixing units. The degassing mechanism eliminates any air bubbles that may have been trapped in the solution. [13].

2) PUMPS



The pump is an important part in liquid chromatography. The mobile phase is drawn from the metal tank to the column using a pump at high pressure. The pump pressure range is 4000 - 6000 psi. Mobile phase composition, particle size, mobile phase flow and column size are factors that depend on the proper operation of the pumps. The best features of pumps are metal [14,15].

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3) Sample Injector :- Septum machines are available for injection of sample fluid. The ability to introduce [objective] the sample into a continuous flow mobile phase stream which transports the sample to the HPLC column is delivered by an injector (sampler or autosampler). The combination of the ring injector and the new rotary valve can produce repeatable results. Sample volumes range from 5 to 20 microliters (liters).Septum injections are available to inject sample fluid. Sample injection can be done when the mobile phase is running or stopped. The combination of the ring injector and the new rotary valve can produce repeatable results [16, 17].



4) Columns:- Columns are typically manufactured from clean stainless steel, measuring approximately 50 to 300 millimeters in length, with an inner diameter ranging from 2 to 5 millimeters. They are typically filled with a stationary component having a molecular size ranging from 3 to10 millimeter [18-25].



5) Detector :-Detectors in HPLC are placed at the end of the analytical column. The function of the converter (Detector) is to check the solution that came from column. The electrical signal is proportional to the concentration of the individual units in the filter.

a) UV- visible Detector:- This detector is the most commonly used in HPLC. Most organic compounds absorb light in the UV range (190-400 nm) and the visible range (400-750 nm). This is based on the Beer-Lambert law. Deuterium and high pressure xenon lamps are sources of UV radiation. It has many advantages and disadvantages [26].

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b) Refractive index Detector :- Refractive index is an important property of column eluent. In this detector, the detection of the solute change depends on the total refractive index of the mobile phase. Mass spectrometers have a low sensitivity. Refractive index is very useful for detecting non-ionic compounds, and non-ions do not absorb ultraviolet range and fluorescence [27].

The different types of RI players are as follows [28].
i. Christiansen effect detector
ii. Interferometer detector
iii. Thermal lens detector
iv. Dielectric constant detector
• Advantages:
1.Reacts with all solvent
2. Does not affect flow rate
• Disadvantages:
1. Not as sensitive as most other types of detectors
2. Cannot be used with gradient washes.

c) Photodiode detector (PAD), diode array detector :-Photodiode array (radioactive devices) are used in the detection module. DAD detects absorption in the UV to VIS region. Although there is only one light-receiving unit on the sampling side, DAD has multiple photodiode arrays (1024 for L-2455/2455U) to acquire information over a wide range of wavelengths simultaneously. is one of the benefits of DAD [29].

d) Fluorescence Detector:- This detector stands as the most sensitive and specific among all the existing High-Performance Liquid Chromatography (HPLC) detectors. It is capable of identifying the presence of a single analyte molecule within the flow cell. The sensitivity of this detector is measured to be 10 - 1000 times greater than that of a UV detector [30].
• There are several types of fluorescence detectors available,
including:
1. Single Wavelength Excitation Fluorescence Detector
2. Multi-Wavelength Fluorescence Detector
3. Laser-Induced Fluorescence Detector

e) Electrical Conductivity Detector: - This device offers a universal, reproducible, and highly sensitive method for the detection of various charged species, including anions, cations, metals, and organic acids. It measures the conductivity of the total mobile phase, thereby classifying it as a bulk density detector. The electrodes of this detector are typically composed of platinum, stainless steel, or other noble metals [31].

Features of Detectors used in HPLC :-
1) It must generate a reliable and reproducible signal
2) It should be non destructive
3) The peaks should not be expanded
4) It must to be unaffected by the gradient or eluent composition
5) The response must not be impacted by temperature changes [32].

6) Data Collection Device:-The data collection device gracefully captures signals from the detector, which are then recorded on graph recorders or electronic integrators. These devices vary in their multifaceted capabilities, including processing, storing, and reprocessing chromatographic information. The interconnected PC monitors the health of the components, coordinating their responses and transferring data to a readable chromatograph. The output is typically captured as a sequence of peaks, with each peak corresponding to a specific compound within the mixture as it flows through the detector and absorbs UV light.

• **Components of Method Validation:** The following are typical analytical
performance characteristics which may be tested during methods validation:
1. Accuracy
2. Precision

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3. Linearity
4. Detection limit
5. Quantitation limit
6. Specificity
7. Range
8. Robustness

1. Accuracy:- Accuracy is defined as the degree to which a measured value approaches the true or accepted value. Accuracy refers to the difference between the observed mean value and the actual value. To determine lt , apply the procedure to samples with known amounts of analyte added. Analyze these against standard and blank solutions to guarantee no interference. The accuracy of the test results is calculated as a percentage of the analyte recovered by the assay. Recovery can be represented as the assay of known levels of analyte [33].

2.Precision :- The degree of agreement (scatter) between measurements acquired from multiple samplings of a homogenous sample under specified conditions. Precision measures the reproducibility of the entire analytical technique [34].It has two components: repeatability and intermediate precision.

Repeatability refers to the variation encountered by a single analyst on the same instrument. The method does not distinguish between variations caused by the instrument or system and those caused by sample processing. Validation involves testing numerous replicates of an assay composite sample using a specific analytical procedure. The recovery value is calculated. Intermediate precision refers to variations within a laboratory, including between days, instruments, and analysts [35,36].

3. Linearity :- Linearity refers to an analytical process producing a result proportional to the analyte concentration in the sample. If the procedure is linear, the test findings are proportional to the concentration of analyte in samples within a specific range. Linearity is typically defined as the confidence limit around the slope of a regression line [37].

4.Limits of detection and quantitation: - The limit of detection (LOD) is the smallest concentration of an analyte in a sample that can be detected but not quantified. LOD is defined as a concentration at a specific signal-to-noise ratio, typically 3:1. The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy using the method's stated operational parameters [38,39].

5.Specificity:- Specificity refers to the capacity to accurately assess an analyte in the presence of predicted components. Impurities, degradants, matrix, and other components are typical examples. Analytical procedures with limited specificity can be supplemented by additional procedures. This term carries the following implications: Identification ensures the identity of an analyte. Purity tests verify the accuracy of an analyte's impurities, including related compounds, heavy metals, residual solvents, and more. An assay measures the content or potency of an analyte in a sample, providing precise results [40].

6.Range:- The method's range refers to the top and lower levels of an analyte determined with acceptable precision, accuracy, and linearity. It is typically expressed in the same units as the test findings and can be based on a linear or nonlinear response curve [41].

7.Robustness :-The robustness of an analytical procedure refers to its ability to withstand tiny but deliberate alterations in method parameters, indicating its reliability throughout routine usage [42].

 # Future Trends

Emerging Technologies

Further advancements in HPLC technology:

- **Nano-HPLC :** offers improved sensitivity with reduced sample consumption.
- **Microfabricated Columns:** Miniaturization leads to faster and more efficient separation.
- **Advanced Detection Methods:** Improved sensitivity and selectivity through detector advancements. Integration with Other Techniques. HPLC and other analytical techniques are increasingly being combined for better results.
- **HPLC-MS :** combines HPLC and mass spectrometry to provide molecular information
- **HPLC-NMR:** Combines high-performance liquid chromatography with nuclear magnetic resonance for structural elucidation.
- **HPLC-FTIR:** Combines HPLC with Fourier-transform infrared spectroscopy for comprehensive compound identification.

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• Advantages

1) It has made significant contribution to the growth of analytical science and its diverse application in pharmaceuticals, environmental, forensics, foods, polymers and plastics, clinical fields etc.
2) HPLC provides a highly specific, reasonably precise, and fairly rapid analytical method for a plethora of complicated samples.
3) HPLC is capable of tackling macromolecules.
4) It is profoundly suitable for most 'pharmaceutical drug substances'.
5) It offers an efficient means of analysis pertaining to 'labile natural products'.
6) Preparation and introduction of sample is easy and simple in HPLC.
7) Resolution of compounds and speed of separation is high.
8) HPLC software is capable of reporting precise and accurate results.
9) Sensitivity of detectors used is high.

• Disadvantages

1) HPLC is considered one of the most important techniques of the last decade of the 20th century. Despite of the several advantages there are certain limitations also. Limitations include price of columns, solvents and a lack of long term reproducibility due to proprietary nature of column packing. Others include:
2) Complexity of separation of certain antibodies specific to the protein.
3) The cost of developing an HPLC apparatus for assay or method of separation of individual components is tremendous.
4) Low sensitivity of some compounds towards the stationary phase in the columns is difficult.
5) Certain compounds get absorbed or react with the chemicals present in the packing materials of the column.
6) Sometimes the pressure may get too high or low that the column cannot withstand or separation may not takes place.
7) Qualitative analysis may be limited unless HPLC is interfaced with mass spectrometry.
8) Resolution is limited with very complex samples.
9) Newer trends with better efficacy have been established.

APPLICATION

- HPLC is used in the food business as well as in the pharmacy, environmental, clinical, and forensic domains. Resolution, identification, and quantification of a molecule are among the data that HPLC may provide. It also helps with purification of mixtures of substances, molecular weight measurement, and chemical separation.

Other applications includes:-

• Pharmaceutical application:- 1) Research on the dissolution of tablets used to administer medicinal dosages.stability research and shelf-life calculations. 2) Determining the pharmacological active components in dose formulations. 3) Pharmaceutical formulation assay and impurity analysis. 4) Quality assurance. 5) Development and research.

• Environmental application:- include the identification of phenolic chemicals in potable water. Diphenhydramine detection in sedimented samples. Pollutant biomonitoring .

• Forensic:- Drug quantification in biological samples is part of forensics. Anabolic steroid detection in sweat, hair, urine, and serum. identifying whether cocaine and its metabolites are present in the blood. textile industry forensic analysis .

• Clinical:- Quantification of ions in human urine. Antibiotic analysis in blood plasma. Estimation of bilirubin and bilivirdin in blood plasma in the presence of hepatic diseases. Endogenous neuropeptides can be detected in extracellular fluids .

• Food and Flavor:- Maintaining the standard of drinking water and soft drinks. evaluation of alcohol and its byproducts.analysis of fruit liquids for sugar. Polycyclic chemicals in vegetables are analyzed. trace examination of agricultural produce containing military high explosives. checking fruits for pesticides and insecticides .

USES OF HPLC: 1) This method is employed in chemistry and biochemistry research to analyze complex mixtures, purify chemical compounds, create synthesis processes for chemical compounds, isolate natural products, or forecast physical characteristics.
2) It is also employed in quality control to monitor degradation, quantify tests of finished products, manage and enhance process yields, and guarantee the purity of raw materials.
3) It is also employed for the analysis of water and air contaminants.
4) Food and pharmaceuticals goods are surveyed by federal and state regulatory bodies using HPLC.

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CONCLUSION

The HPLC has mostly used an analytical technique. It can be used in both laboratory and clinical science. Reverse phase elution is advantageous over the normal phase. Since isocratic elution decreases the loading capacity, gradient elution is more useful. C8 and C18 columns are generally used. UV detectors are widely used. The typical average pH of reversed-phase on silica-based packing is 5.0. Adequate buffer concentration is 10-50 mM. Different applications are in the field of pharmaceutical analysis,environment, forensic, food, and clinical. Other applications include preparation, chemical separation, purification, and identification. The only disadvantage of HPLC is the high cost.

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