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High-performance liquid chromatography: a comprehensive review

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ABSTRACT

High-Performance Liquid Chromatography (HPLC) is a type of column chromatography that is generally used in biochemistry and analysis to separate, identify, and quantify active components. It is the most versatile, safest, reliable, and fastest chromatographic technique for the quality control of medicine factors. 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 article was prepared with an aim to review different aspects of HPLC, such as principle, types, instrumentation and application. Despite its widespread use, challenges related to method development, optimization of separation conditions, detector selection, and matrix interference remain significant. This review consolidates recent advancements and methodological strategies to address these challenges, thereby contributing to a clearer understanding of HPLC applications across pharmaceutical, environmental, food, and agricultural sciences.

Keywords: *High-performance liquid chromatography, instrumentation, elution, applications, mobile phase, validation, quantify, discovery.*

INTRODUCTION

High-performance liquid chromatography (HPLC) is an advanced form of a liquid chromatography technique in which the mobile phase is liquid solvent or mixture of solvents that's they called as liquid chromatography. The technique is sometimes referred to as High-Pressure Liquid Chromatography because the mobile phase is forced through a packed column under high pressure. In this process, the sample is introduced into a column packed with a solid stationary phase, where separation takes place [1][2][3].

HPLC is one of the most powerful, sophisticated, highly accurate, versatile and widely used analytical techniques that enables precise separation, identification, and quantification of the components within a complex mixture. Because of its precision and efficiency, it's a cornerstone of modern analytical chemistry, finding widespread use in various fields, especially the pharmaceutical, environmental, and food industries, clinical diagnostics and the agriculture field [4][5].

PRINCIPLE

HPLC is a type of column chromatography that separates compounds on the principle of differential distribution of sample components between a stationary phase and a mobile phase. The fundamental idea is that different components of a sample will interact with these two phases to varying degrees, such as polarity, molecular size, and charge, leading to their separation [6][7].

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The stationary phase generally consists of porous particles packed in a stainless-steel column, while the mobile phase is a liquid solvent or mixture of solvents delivered at high pressure. The mobile phase is a liquid solvent or mixture of solvents that is pumped through the column at high pressure. The separation occurs as the sample is injected into the mobile phase and carried through the column. Components of the sample interact differently with the stationary phase based on their chemical properties, such as polarity, size, or charge. Compounds with a stronger affinity for the stationary phase are retained longer and move through the column more slowly, while those with a stronger affinity for the mobile phase move faster. This difference in migration speed causes the components to separate, eluting from the column at different times, known as their retention time [8][9][10][11].

INSTRUMENTATION

A typical HPLC system consists of several key components that work together to achieve separation and detection:

1. Solvent Reservoir: Mobile phase contents are contained in a glass reservoir. The mobile phase, or solvent, in HPLC, is usually a mixture of polar and non-polar liquid components whose respective concentrations are varied depending on the composition of the sample. The type and composition of the mobile phase affect the separation of the components. For HPLC we use high-grade solvent. Different solvents are used for different types of HPLC. For normal-phase HPLC, the solvent is usually non-polar, and, in reverse-phase HPLC, the solvent is normally a mixture of water and a polar organic solvent. The purity of solvents and inorganic salts used to make the mobile phase is paramount [12].

2. Pump: The pump is arguably the most critical component, responsible for delivering the mobile phase through the column at a constant flow rate and high pressure (up to 6,000 psi or more).

This high pressure is necessary to overcome the resistance of the tightly packed stationary phase particles.
Isocratic System: Delivers a mobile phase of constant composition throughout the separation.
Gradient System: Delivers a mobile phase whose composition changes over time. This is achieved by mixing two or more solvents in varying proportions, which can significantly improve separation efficiency for complex mixtures [13].

3. Injector: The injector is designed to introduce a small, reproducible volume of the sample into the high-pressure mobile phase stream without interrupting the flow or pressure.
Manual Injectors: Utilize a syringe and a six-port valve to load the sample.
Autosamplers: Computer-controlled devices that automatically inject multiple samples, improving throughput and reproducibility, crucial in busy laboratories [14][15].

4. Column: It is the heart of the system where the actual separation takes place. It's typically a stainless-steel tube packed with very small (1.7 to 5 µm), porous particles that serve as the stationary phase [16].

- **Column Chemistry**: The most common stationary phase material is silica gel modified with various functional groups. For instance, C18 (octadecylsilane) is the most popular for reversed-phase HPLC, providing a non-polar surface.
- **Column Dimensions:** Lengths typically range from 30 mm to 250 mm, and internal diameters from 2.1 mm to 4.6 mm. Shorter columns with smaller particles are used in UHPLC (Ultra-High-Performance Liquid Chromatography) for faster separations and higher resolution.
- **Column Oven:** Most HPLC systems include a column oven to maintain a stable temperature, as temperature can significantly affect retention times and separation efficiency.

5. Detector: It is located at the end of the column and monitor the mobile phase as it elutes from the column. It measures the components as they elute by providing a signal that is proportional to the concentration of the separated analytes [17]. Common detectors include:

- **UV-Vis Detector**: The most common type. It measures the absorbance of UV or visible light by the analytes. It's highly sensitive for compounds that absorb in this region.
- **Diode Array Detector (DAD)**: A more advanced UV-Vis detector that can acquire full UV-Vis spectra across a range of wavelengths simultaneously, allowing for peak purity assessment and identification [18].
- **Refractive Index (RI) Detector**: Measures changes in the refractive index of the mobile phase as analytes elute. It's a universal detector (responds to almost all compounds) but is less sensitive and cannot be used with gradient elution.
- **Fluorescence Detector**: Highly sensitive and selective for compounds that naturally fluoresce or can be derivatized to fluoresce.
- **Evaporative Light Scattering Detector (ELSD)**: Useful for detecting non-volatile and semi-volatile compounds that lack chromophores (don't absorb UV light).
- **Mass Spectrometry (MS) Detector (LC-MS)**: Provides highly specific detection and structural information. It ionizes the separated components and measures their mass-to-charge ratio, offering unparalleled identification capabilities. LC-MS is increasingly common in Indian pharmaceutical R & D.

6. Data System:
A computer equipped with specialized software controls all aspects of the HPLC system, acquires data from the detector, processes the raw data into chromatograms, which show the separated peaks and their retention times, and performs calculations for quantification. This system is crucial for method development, data analysis, and regulatory compliance [19][20][21].

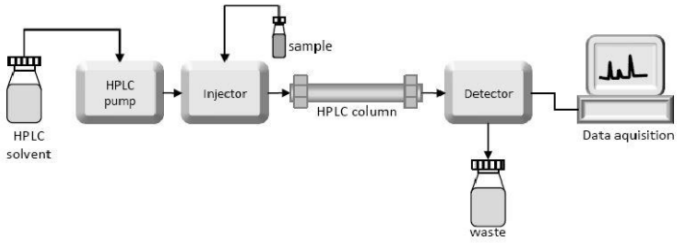

Figure No. 1: HPLC Instrumentation Flow chart

MODES OF SEPARATION
The mode of separation in HPLC depends on the nature of the stationary and mobile phases [22].

1. Normal-Phase HPLC (NP-HPLC):
Stationary Phase: Polar (e.g., bare silica, amino groups).
Mobile Phase: Non-polar (e.g., hexane, chloroform, ethyl acetate).
Mechanism: Compounds are separated based on their polarity. Non-polar compounds elute first, while more polar compounds are retained longer due to stronger interactions with the polar stationary phase. It's less common than RP-HPLC but useful for separating very polar compounds [23][24][25][26][27].

2. Reversed-Phase HPLC (RP-HPLC):
Stationary Phase: Non-polar (e.g., silica bonded with C18).
Mobile Phase: Polar (e.g., water, methanol, acetonitrile,).
Mechanism: Compounds are separated based on their hydrophobicity. More polar compounds elute first (less retention), while more hydrophobic compounds are retained longer. This is the most widely used mode, particularly for pharmaceutical analysis [28][29][30][31].

3. Ion-Exchange HPLC (IE-HPLC)
Stationary Phase: Contains charged functional groups (e.g., sulfonic acid for cation exchange, quaternary ammonium for anion exchange).
Mobile Phase: Aqueous buffers with varying pH and salt concentrations.
Mechanism: Separates ions based on their charge. Analytes with the opposite charge to the stationary phase are retained through electrostatic interactions. Increasing the salt concentration or changing the pH can elute the bound ions. This is vital for proteins, nucleic acids, and small ionic molecules [32][33][34].

4. Size-Exclusion HPLC (SE-HPLC) / Gel Permeation Chromatography (GPC)
Stationary Phase: Porous particles with a defined pore size range.
Mobile Phase: A solvent in which the analytes are soluble.
Mechanism: Separates molecules based on their hydrodynamic volume (size in solution). Larger molecules cannot enter the pores and therefore elute faster, while smaller molecules penetrate the pores and are retained longer. Used for polymers, proteins, and other macromolecules [35][36].

5. Affinity HPLC

Stationary Phase: consists of a support medium on which the ligand has been bound covalently, in such a way that the reactive groups that are essential for enzyme binding are exposed.

Mobile Phase: Aqueous buffers with varying pH

Mechanism: As the mixture of proteins is passed through the chromatography column, those proteins that have a binding site for the immobilized substrate will bind to the stationary phase, while all other proteins will be eluted from the column. When the sample passes through the column, only the molecules that selectively bind to the affinity ligand are retained. Molecules that don't bind pass through the column with the mobile phase. After the undesired molecules are removed, the retained analytes can be eluted by changing the mobile-phase conditions. Once they bond themselves later, they must be separated from the bonded stationary phase using another solvent that has a good capacity for separation. Mostly it is useful for the separation of biomolecules like protein [37][38].

METHOD DEVELOPMENT AND OPTIMIZATION

A. Choosing the Mobile and Stationary Phases

The proper mobile and stationary phase are very important in developing an effective HPLC method. Considering:

- Analyte nature: polarity, charge, molecular size, and other chemical properties.
- Solubility: The solubility of the analytes in the mobile phase interferes with the efficiency of transport.
- Separation Efficiency: The composition of the mobile phase and the type of stationary phase will be optimized with respect to resolution and peak shape.
- Interaction Mechanism: The interaction mechanism desired, whether hydrophobic, polar, or ionic, dictates the appropriate stationary phase choice.

B. Column Selection

Column selection is a critical step in HPLC. The considerations involve:

- Particle Size: The smaller the particles, the higher the resolution. But the higher the pressures take this.
- Column Dimensions: Column dimensions pertain to separation efficiency and analysis time.
- Stationary Phase Material: C18, silica, ion exchange resins are all materials offering different separation characteristics [39].

METHOD VALIDATION

An HPLC method is validated to prove its reliability and reproducibility for [40]:

- **Precision**:

Precision reflects the closeness of agreement (or the extent of scatter) among a series of measurements obtained from multiple samplings of the same homogeneous sample under prescribed conditions. It is generally expressed as the standard deviation (SD) or relative standard deviation (RSD) of repeated data sets. Precision is further categorized into three levels: repeatability (intra-assay precision), intermediate precision (variability within a laboratory across different days, analysts, or instruments), and reproducibility (inter-laboratory precision). Evaluation is typically carried out by analyzing a sufficient number of aliquots from a homogeneous sample to derive statistically valid estimates of variability [41][42][43][44][45].

- **Accuracy**:

Accuracy represents the degree of closeness between the value obtained and the accepted reference or true value. It is commonly determined using certified reference materials or by applying the analytical procedure to samples spiked with known amounts of analyte. The closeness of a measured value to the true or accepted value is defined as accuracy. In practice, accuracy is assessed as the difference between the mean measured value and the true value. It is calculated by applying the procedure to samples containing known levels of analyte. To confirm that there is no interference, these should be compared to standard and blank solutions. Results are often expressed as percentage recovery of the analyte, calculated by comparing the test outcome against standard or blank solutions to confirm the absence of interference [46][47][48].

- **Specificity**:

Specificity is the ability of an analytical procedure to measure the analyte unequivocally in the presence of other expected components such as impurities, degradation products, or matrix substances. A lack of specificity in one method can sometimes be compensated for by using an alternative or complementary analytical procedure. This concept encompasses identification (confirming the analyte's identity) and purity testing (ensuring accurate characterization of impurities or degradants) [49][50].

- **Linearity**

Linearity describes the capacity of an analytical method to provide results that are directly proportional to the concentration of the analyte within a defined range. A linear response is typically evaluated by preparing multiple concentrations (at least five, as recommended by ICH) from a standard stock solution and analyzing them using the proposed method. The linearity is usually assessed through regression analysis, with results expressed as the correlation coefficient and confidence limits around the regression line's slope [51][52].

- **Limits of detection and quantitation**

The limit of detection (LOD) is the lowest concentration of analyte that can be detected but not necessarily quantified with precision. It is often estimated based on a signal-to-noise ratio of 3:1. The limit of quantitation (LOQ) is the minimum analyte concentration that can be quantified with acceptable accuracy and precision, generally corresponding to a signal-to-noise ratio of 10:1. Alternatively, LOD and LOQ may be calculated using the standard deviation of the response and the slope of the calibration curve, according to the following equations:

$LOD = 3.3 \times (\sigma / S)$

$LOQ = 10 \times (\sigma / S)$

Where, σ is the standard deviation of the response and S is the slope of the calibration curve [53][54].

- **Robustness**:

Robustness refers to the reliability of an analytical method under small, deliberate variations in experimental conditions, such as changes in pH, mobile phase composition, temperature, or instrument parameters. A robust method demonstrates consistent performance under routine operating conditions and indicates the method's suitability for practical use [55].

- **ADVANTAGES OF HPLC**

HPLC combines speed, sensitivity, precision, and versatility, making it one of the most widely used analytical techniques in pharmaceuticals, biochemistry, environmental science, and the food industries [56][57][58][59][60].

- **High Resolution and Sensitivity**

HPLC provides excellent separation efficiency, even for complex mixtures. It can detect and quantify compounds present in trace amounts (nanogram to picogram levels). Very useful in pharmaceutical, biomedical, and environmental analyses.

- **Wide Range of Applications**

Suitable for analysis of polar, non-polar, ionic, thermally unstable, and high-molecular-weight compounds. Unlike Gas Chromatography (GC), it does not require the analytes to be volatile or thermally stable.

- **Versatility of Detection**

Adaptable to a wide range of compounds, from small molecules to macromolecules.

Multiple types of detectors (UV-Vis, fluorescence, refractive index, conductivity, electrochemical, and mass spectrometry) can be coupled with HPLC. Allows qualitative and quantitative analysis of diverse compounds.

- **Speed and Efficiency**

Modern HPLC systems can achieve separation within minutes to an hour, compared to traditional column chromatography, which takes much longer. Automation and computer integration enable rapid, reproducible results.

- **Accuracy and Precision**

HPLC provides highly reproducible retention times and peak areas. Excellent for quality control in pharmaceuticals, food, and chemical industries.

- **Non-Destructive Analysis**

Some HPLC methods are non-destructive, meaning the separated sample can be recovered and used for further analysis.

- **Applicable to Thermally Labile Compounds**

Unlike GC, HPLC operates at room or mild temperatures, so compounds that decompose on heating can be analyzed safely.

- **Quantitative and Qualitative Information**

Provides both identification (qualitative) and concentration (quantitative) of compounds. Widely used in pharmacokinetics, drug development, and environmental monitoring.

- **Automation and Data Handling**

Equipped with autosamplers, online data acquisition, and software-controlled systems, which improve reproducibility, reduce human error, and allow high-throughput analysis.

- **Flexibility of Mobile and Stationary Phases**

A wide range of mobile phases (aqueous, organic, gradient elution) and stationary phases (normal phase, reverse phase, ion exchange, size exclusion) is available.

Makes HPLC adaptable to almost all kinds of chemical and biological samples.

APPLICATIONS

HPLC's versatility makes it indispensable across numerous industries:

Pharmaceuticals and Biotechnology: HPLC is a cornerstone of the pharmaceutical industry, playing a critical role throughout a drug's lifecycle [61][62][63].

- **Drug Discovery and Development:** HPLC is used to analyse and purify new drug candidates. It helps characterize their chemical properties, assess stability, and monitor reaction progress during synthesis.
- **Quality Control (QC) and Assurance (QA)**: It's the gold standard for ensuring the purity and potency of drug products. HPLC can identify and quantify active pharmaceutical ingredients (APIs), excipients, and any impurities or degradation products that may form over time. This is essential for meeting strict regulatory standards.
- **Bioavailability and Bioequivalence Studies**: In clinical trials, HPLC is used to measure drug concentrations in biological fluids like blood and urine. This helps determine how a drug is absorbed, distributed, metabolized, and excreted in the body, which is crucial for establishing proper dosage and demonstrating equivalence between generic and brand-name drugs.
- **Therapeutic Drug Monitoring (TDM):** HPLC is used to measure the levels of certain drugs in a patient's bloodstream, allowing doctors to adjust dosages to maintain a therapeutic effect while minimizing side effects.
- **Environmental Analysis:** HPLC is vital for monitoring and ensuring the safety of our environment by detecting and quantifying pollutants [64][65][66][67].
- **Water Quality Testing**: It's used to detect and quantify a wide range of contaminants in water sources, including pesticides, herbicides, heavy metals, and organic pollutants. This helps ensure drinking water is safe and that industrial wastewater is treated properly before release.
- **Soil Contamination Analysis**: HPLC can analyse soil samples to identify and quantify pollutants like polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), helping to assess and manage contaminated sites.
- **Air Pollution Monitoring**: HPLC is used to analyse air samples, particularly to detect and measure harmful volatile organic compounds (VOCs) and other particulate matter that can affect human health.
- **Food and Beverage Industry:** HPLC applications in the food industry focus on quality control, safety, and nutritional analysis [68].
- **Food Safety**: It's used to detect and quantify harmful substances like pesticides, mycotoxins, and other contaminants that can pose health risks. HPLC also helps monitor the levels of preservatives and additives to ensure they are within safe limits.
- **Nutritional Analysis**: HPLC can accurately measure the content of essential nutrients, such as vitamins, amino acids, and sugars. This is important for labelling and ensuring products meet nutritional claims.
- **Quality and Authenticity**: The technique is used to analyse flavour compounds, colours, and other components to maintain product consistency and quality. It also helps detect food fraud, such as the adulteration of high-value products like olive oil or honey.

Clinical and Forensic Applications

- **Clinical Diagnostics**: In clinical labs, HPLC is used to analyse biological samples for biomarkers of disease. For example, it can measure glycated haemoglobin (HbA1c) levels to monitor long-term blood glucose control in diabetic patients or analyse amino acids for metabolic disorders [69].

- **Forensic Science**: HPLC helps in forensic investigations by analysing biological samples (blood, urine, hair) to identify and quantify drugs of abuse, poisons, or other substances. It's also used to analyse trace evidence, such as dyes from textiles or ink from documents [70][71].

Plant and Soil Health Monitoring: HPLC can be used to analyse samples from plants and their environment to monitor health and optimize growth [72].

- **Plant Hormones**: It's used to analyse plant tissues for phytohormones (plant hormones) like auxins, gibberellins, and abscisic acid. Changes in the concentration of these hormones can provide early warnings of stress, disease, or nutrient deficiencies, allowing farmers to take corrective action.
- **Soil and Water Analysis**: HPLC is used to analyse soil and water samples for the presence of nutrients and contaminants, such as fertilizers and chemicals. This helps in optimizing soil health and minimizing the environmental impact of farming practices.

Pesticide and Contaminant Analysis: One of the most critical applications of HPLC in agriculture is the detection and quantification of contaminants [73][74][75][76][77][78].

- **Pesticide Residues**: HPLC is a primary tool for screening and quantifying pesticide residues in fruits, vegetables, grains, and water. By using detectors like tandem mass spectrometry (HPLC-MS/MS), scientists can detect and measure a wide range of pesticides at very low concentrations, ensuring they are below maximum residue limits (MRLs) set by regulatory bodies.
- **Mycotoxins**: Mycotoxins, toxic compounds produced by fungi, can contaminate crops like corn, wheat, and nuts. HPLC is used to detect and quantify these harmful substances, such as aflatoxins and ochratoxin A, ensuring food safety and preventing health risks to consumers and livestock.
- **Antibiotics and Other Residues**: In animal agriculture, HPLC can be used to monitor for antibiotic residues in meat and dairy products, which helps prevent the development of antibiotic-resistant bacteria.
- **Quality and Nutritional Analysis:** HPLC is also vital for assessing the quality and nutritional content of agricultural products [79][80][81][82].
- **Vitamins and Amino Acids**: The technique is used to determine the exact concentration of vitamins (e.g., Vitamin C, Vitamin A) and amino acids in food and animal feed. This is crucial for accurate nutritional labelling and for ensuring a balanced diet for livestock.
- **Phytochemicals**: HPLC enables the analysis of various plant-based compounds (phytochemicals) like flavonoids, polyphenols, and carotenoids, which are known for their antioxidant and health-promoting properties. This helps in understanding the medicinal and nutritional value of different crops.
- **Sugars and Organic Acids**: It can be used to profile and quantify sugars (e.g., glucose, fructose) and organic acids in fruits and juices, which are key indicators of ripeness, taste, and overall product quality.

CONCLUSION

HPLC is a highly sophisticated chromatographic technique for separating, identifying, and quantifying compounds in complex mixtures. Among them, Reversed-phase HPLC remains the most widely employed mode due to its versatility. Compared with conventional liquid chromatography, HPLC offers superior efficiency, sensitivity, and reproducibility, making it indispensable in modern research and industry. The chromatographic output, known as a chromatogram, provides valuable qualitative and quantitative information, ensuring accurate analysis across scientific disciplines. With advancements such as LC-MS and UHPLC, its role continues to expand, making it a cornerstone of modern analytical science.

FUTURE SCOPE

Future developments in HPLC are expected to focus on improving sensitivity, speed, and sustainability of analytical methods. The integration of ultra-high-performance liquid chromatography (UHPLC) with advanced detectors such as tandem mass spectrometry (LC-MS/MS) will further enhance selectivity and structural elucidation capabilities. Green chromatography approaches, including the use of eco-friendly solvents and reduced solvent consumption, are gaining importance to minimize environmental impact. Additionally, the application of artificial intelligence and chemometric tools in method development, optimization, and data interpretation is anticipated to improve efficiency and reproducibility. Expanding the use of HPLC in precision agriculture, metabolomics, and biomarker discovery represents a promising direction for future research.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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