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Review article on high-performance liquid chromatography

Shaik Sameer*, Khadar Basha Showkath Basha, Shaik Mubina Begum, Shaik Shabreen Begum
Department of Pharmacy, Nimra College of Pharmacy, Nimra Nagar Jupudi, Ibrahimpatnam, Vijayawada, Andhra Pradesh, India

Abstract
High performance liquid chromatography is an important qualitative and quantitative technique, generally used for estimation of pharmaceutical and biological samples. Its properties mainly depends on affinity,polarity and adsorption.High performance liquid chromatography employs a liquid mobile phase that passes through a column packed with a stationary phase,facilitating the separation of analytes based on their interactions with the stationary phase and mobile phase.It is the most versatile,safest,dependable and fastest chromatographic technique for the quality control of drug components.High performance liquid chromatography provides a highly specific,reasonable prices and fairly rapid analytical method for a complicated samples.HPLC method in development and validation plays important role in new discovery. It gives various validations like accuracy, precision, specificity, linearity, range and limit of defective, limit of quantification and system suitability testing. It also used in various industries like pharmaceutical analysis, environmental sciences, quality control, quality research, clinical trails, pharmaceutical chemistry. The technique is capable of analyzing a broad spectrum of substances, ranging from small organic molecules to large biological micro molecules, by utilizing different column types, mobile phase compositions and detection methods.

Keywords: Chromatography, separation, validation, stationary phase, mobile phase

Introduction
High-Performance Liquid Chromatography (HPLC) is a sophisticated analytical technique used for the separation, identification, and quantification of components within a mixture. It leverages high pressure to push a liquid mobile phase through a column packed with a stationary phase, enabling the differentiation of compounds based on their interactions with these phases [1].

Definition of HPLC
High-Performance Liquid Chromatography (HPLC) is an analytical method for separating, identifying, and quantifying the components of a liquid sample. It operates by forcing the mobile phase through a column packed with a stationary phase under high pressure, which allows the separation of compounds based on their varying interactions with the stationary and mobile phases.

Principle of HPLC
1. Chromatographic Column
- **Description:** The core component of HPLC is the column, filled with a stationary phase. This phase can be a solid or a liquid coated onto a solid support, chosen based on the separation needs.

2. Mobile Phase
- **Description:** The mobile phase is a liquid (or sometimes a supercritical fluid) that transports the sample through the column. Its composition can be adjusted to optimize the separation of different compounds.

3. Sample Injection
- **Description:** A precise volume of the sample is injected into the mobile phase stream at the start of the analysis.

4. Separation Mechanism
- **Polarity:** In reversed-phase chromatography (RPC), non-polar compounds interact more strongly with the non-polar stationary phase and are retained longer, while polar compounds elute more quickly. In normal-phase chromatography (NPC), polar compounds interact more strongly with the polar stationary phase.
- **Charge:** In ion-exchange chromatography (IEC), separation is based on electrostatic interactions, with charged molecules interacting with oppositely charged stationary phases.
- **Size:** Size-exclusion chromatography (SEC) separates molecules based on size. Larger molecules elute first as they do not enter the pores of the stationary phase, while smaller molecules penetrate the pores and elute later.
- **Affinity:** In affinity chromatography, the separation is based on specific interactions between target molecules and a ligand on the stationary phase.

5. Detection
- **Description:** Separated components are detected as they exit the column. Common detectors include UV/Vis spectrophotometers, fluorescence detectors, and mass spectrometers. The detector measures the amount of each component, providing data on their concentration and identity.

6. Chromatogram
- **Description:** The detector’s output is recorded as a chromatogram, showing detector response (y-axis) versus time (x-axis). Each peak represents a different component, with the position and area of the peaks indicating their identity and concentration [2].

Types of HPLC Techniques

1. **Normal-Phase HPLC (NP-HPLC)**
- **Stationary Phase:** Polar (e.g., silica gel, alumina)
- **Mobile Phase:** Non-polar or less polar (e.g., hexane, chloroform)
- **Application:** Effective for separating non-polar to moderately polar compounds, such as lipids and natural products.

2. **Reversed-Phase HPLC (RP-HPLC)**
- **Stationary Phase:** Non-polar (e.g., C18, C8 bonded phases)
- **Mobile Phase:** Polar (e.g., water, acetonitrile, methanol)
- **Application:** Widely used for a broad range of compounds, including pharmaceuticals, peptides, and proteins.

3. **Ion Exchange HPLC (IEC)**
- **Stationary Phase:** Charged (e.g., resin with acidic or basic groups)
- **Mobile Phase:** Aqueous buffer with varying ionic strength and pH
- **Application:** Separates ions and polar molecules based on charge, used in protein purification and water analysis.

4. **Size Exclusion HPLC (SEC)**
- **Stationary Phase:** Porous material (e.g., cross-linked polymers or silica beads)
- **Mobile Phase:** Aqueous or organic solvents
- **Application:** Separates molecules based on size, useful for determining molecular weight and purifying proteins.

5. **Affinity HPLC**
- **Stationary Phase:** Contains specific ligands or antibodies that bind selectively to target molecules.
- **Mobile Phase:** Typically aqueous, adjusted to favor or disrupt binding.
- **Application:** Purifies biomolecules based on specific interactions.

6. **Hydrophilic Interaction Chromatography (HILIC):**
- **Stationary Phase:** Polar (e.g., silica with hydrophilic groups).
- **Mobile Phase:** Mixture of organic solvents and water.
- **Application:** Separates highly polar and hydrophilic compounds, such as polar metabolites and peptides.

7. **Chiral HPLC**
- **Stationary Phase:** Chiral (e.g., enantiomeric or chiral selectors)
- **Mobile Phase:** Varies based on the chiral stationary phase
- **Application:** Separates enantiomers or chiral compounds, important in pharmaceutical analysis.

8. **Supercritical Fluid Chromatography (SFC)**
- **Stationary Phase:** Similar to normal or reversed-phase but optimized for supercritical fluids
- **Mobile Phase:** Supercritical fluids (e.g., supercritical carbon dioxide)
- **Application:** Separates non-polar and moderately polar compounds, useful for thermally labile compounds.

9. **Two-Dimensional HPLC (2D-HPLC)**
- **Stationary Phases:** Different phases used in the two dimensions
- **Mobile Phases:** Vary between dimensions
- **Application:** Enhances separation power by combining different HPLC techniques to resolve complex mixtures.

10. **Gradient Elution HPLC**
- **Stationary Phase:** Typically used with reversed-phase or normal-phase columns
- **Mobile Phase:** Gradually changing composition
- **Application:** Improves separation of compounds with a wide range of polarities by adjusting the mobile phase composition over time [3].

Instrumental Requirements for HPLC

1. **Solvent Delivery System (Pump)**
- **Purpose:** Delivers a continuous, precise flow of the mobile phase.
- **Requirements:** High pressure capability, precision, and minimal pulsation.

2. **Injector**
- **Purpose:** Introduces the sample into the mobile phase stream.
- **Types:** Manual injectors, automatic sample loaders, autosamplers.
- **Requirements:** Precision and accuracy in sample volume injection.

3. **Column**
- **Purpose:** Contains the stationary phase where separation occurs.
- **Types:** Packed columns (e.g., with silica) and capillary columns.
- **Requirements:** Proper dimensions, packing material, and compatibility with the mobile phase.

4. **Detector**
- **Purpose:** Detects and measures separated components as they elute from the column.
- **Types:** UV-Vis absorbance, fluorescence, refractive index, conductivity, and mass spectrometry detectors.
- **Requirements:** Sensitivity, selectivity, and appropriate detection range.

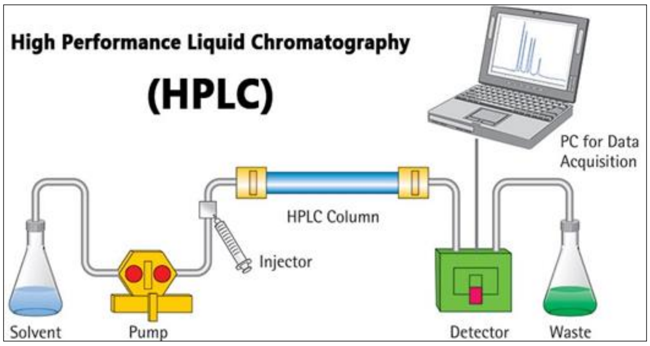
5. **Data Acquisition System**
- **Purpose:** Records and processes detector signals to produce chromatograms.
- **Requirements:** Software for data analysis, integration, and interpretation.

6. **Mobile Phase System**
- **Purpose:** Carries the sample through the column.
- **Requirements:** High purity, appropriate composition, and degassing to prevent bubble formation.

7. **Column Oven (Optional)**
- **Purpose:** Maintains column temperature for reproducibility and stability.
- **Requirements:** Accurate temperature control and uniform heating.

8. **Degasser**
- **Purpose:** Removes dissolved gases from the mobile phase to prevent interference.
- **Requirements:** Efficient gas removal to ensure consistent flow and detector performance [4].

Parameters Used in HPLC



1. **Mobile Phase**
- **Composition:** The solvents used (e.g., water, methanol, acetonitrile) and their ratios influence the separation. Different solvent mixtures affect the polarity and interaction of analytes with the stationary phase.
- **pH:** The pH of the mobile phase impacts the ionization of analytes and their retention times. Buffers are commonly used to maintain a stable pH.

2. **Flow Rate**
- **Speed:** The flow rate of the mobile phase, typically measured in mL/min, affects the interaction time between analytes and the stationary phase. Higher flow rates reduce retention times but may compromise resolution.

3. **Column**
- **Column Type:** The choice of column (e.g., reversed-phase, normal-phase, ion-exchange) determines the separation mechanism used.
- **Column Dimensions:** The length, internal diameter, and particle size of the column affect separation efficiency. Longer columns generally provide better resolution but require more time.
- **Stationary Phase:** The material and its surface properties (e.g., C18, C8) influence interactions with analytes and affect separation.

4. **Temperature**
- **Column Temperature:** The temperature of the column can affect the viscosity of the mobile phase and interactions between analytes and the stationary phase. Controlled temperature helps achieve consistent results and better separation.

5. **Detector**
- **Type:** Common detectors include UV/Vis absorbance, fluorescence, refractive index, and mass spectrometry (MS). The choice depends on the analytes and required sensitivity.
- **Wavelength:** For UV/Vis detectors, the selected wavelength impacts sensitivity and selectivity.

6. **Injection Volume**
- **Volume of Sample:** The volume of sample injected affects sensitivity and resolution. Excessive volume can lead to peak distortion and reduced resolution.

7. **System Pressure**
- **Operating Pressure:** HPLC systems operate under high pressure. Maintaining proper pressure is crucial for accurate flow and separation. Pressure levels vary with flow rate and column dimensions.

8. **Sample Preparation**
- **Filtration and Solvent:** Proper sample preparation, including filtration and dissolution in compatible solvents, is essential to prevent blockages and ensure accurate analysis.

Applications of HPLC [5]

1. **Pharmaceutical Industry**
- **Drug Development:** Analyzes and purifies new drug compounds.
- **Quality Control:** Ensures the quality and purity of pharmaceutical products by quantifying active ingredients and detecting impurities.
- **Stability Testing:** Assesses drug product stability over time, checking for degradation products.

2. **Clinical and Medical Diagnostics**
- **Biomarker Analysis:** Measures biomarkers for disease diagnosis and monitoring, such as hormones or metabolites.
- **Therapeutic Drug Monitoring:** Monitors drug levels in patient samples to ensure efficacy and avoid toxicity.
- **Drug Screening:** Detects and quantifies drugs and their metabolites in biological fluids.

3. **Environmental Analysis**
- **Water Quality Testing:** Analyzes pollutants like pesticides, heavy metals, and pharmaceuticals in water samples.
- **Soil and Sediment Analysis:** Detects and quantifies contaminants in soil and sediment.

4. **Food and Beverage Industry**
- **Nutritional Analysis:** Determines the content of vitamins, amino acids, and other nutrients in food products.
- **Flavor and Fragrance:** Identifies and quantifies flavor and fragrance compounds in food and beverages.
- **Contaminant Detection:** Screens for contaminants such as pesticides, toxins, and additives.

5. **Cosmetics Industry**
- **Product Development:** Analyzes cosmetic ingredients to ensure they meet formulation specifications.
- **Quality Assurance:** Ensures the consistency and safety of cosmetic products by checking for impurities and active ingredients.

6. **Chemical Industry**
- **Process Monitoring:** Monitors chemical reactions and processes to ensure they proceed as expected and control product quality.
- **Purity Analysis:** Checks the purity of chemical compounds and intermediates.

7. **Forensic Science**
- **Toxicology:** Analyzes substances in biological samples to identify drugs or poisons in forensic investigations.
- **Drug Identification:** Identifies unknown substances in criminal investigations.

8. **Research and Development:**
- **Metabolomics:** Studies metabolic profiles to understand disease mechanisms or the effects of interventions.
- **Proteomics:** Analyzes proteins and peptides, including their structure, function, and interactions.

9. **Academic Research**
- **Chemical Analysis:** Provides detailed analysis of chemical compounds in various research projects, from organic chemistry to environmental science.

10. **Industrial Manufacturing**
- **Process Optimization:** Used to optimize and control manufacturing processes by analyzing intermediate and final products.

Advantages of HPLC

1. **High Resolution and Sensitivity**
- **Resolution:** Provides excellent separation of complex mixtures, allowing for the resolution of closely related compounds.
- **Sensitivity:** Detects low concentrations of analytes, often in the nanogram or picogram range.

2. **Versatility**
- **Wide Range of Applications:** Analyzes diverse substances including small molecules, proteins, peptides, nucleic acids, and polymers.
- **Adaptability:** Compatible with various detectors (e.g., UV/Vis, fluorescence, MS) for different analytical needs.

3. **Quantitative Analysis**
- **Accuracy:** Provides precise and accurate quantification, essential for pharmaceutical quality control and environmental monitoring.

4. **Speed**
- **Rapid Analysis:** Modern systems offer fast separations, with analysis times ranging from minutes to an hour depending on sample complexity.

5. **Reproducibility and Precision:**
- **Consistency:** Delivers high reproducibility and precision, crucial for reliable results in quality control, research, and regulatory compliance.

6. **Automated Operation**
- **Automation:** Equipped with autosamplers and data processing software for high-throughput analysis and reduced manual intervention.

7. **Minimization of Sample Preparation**
- **Direct Analysis:** Allows direct sample injection with minimal preparation, simplifying workflow and reducing the potential for sample loss or contamination.

8. **Scalabilit**
- **Scalable:** Can be scaled from analytical to preparative applications, enabling both analysis and purification of compounds.

Disadvantages of HPLC

1. **Cost**
- **Initial Investment:** High cost for purchase and installation, which can be prohibitive for smaller labs.
- **Maintenance:** Ongoing costs for maintenance and calibration.

2. **Complexity**
- **Operational Complexity:** Requires skilled operators for setup, calibration, and data interpretation.
- **Data Interpretation:** Analysis of complex samples can be challenging.

3. **Sample Preparation**
- **Preparation Needs:** Some samples require extensive preparation to avoid issues like column clogging or contamination.

4. **Limited to Liquid Samples**
- **Sample State:** Generally restricted to liquid samples; solid samples must be dissolved or extracted first.

5. **Column Lifespan**
- **Column Degradation:** Columns degrade over time, especially with aggressive solvents or high sample loads, leading to higher costs and potential downtime.

6. **Potential for System Issues**
- **Clogging and Fouling:** Issues such as column clogging can occur, particularly with samples containing particulate matter or high protein levels.

7. **Solvent Usage**
- **Environmental Concerns:** High solvent usage can be costly and environmentally impactful, requiring proper disposal.

8. **Limited Detection for Some Compounds**
- **Sensitivity Limitations:** Some detectors may not be suitable for compounds that do not absorb UV light or are not inherently fluorescent.

9. **Resolution vs. Speed Trade-off**
- **Balance Required:** Achieving high resolution may require longer analysis times, which might not be ideal for applications needing rapid results.

10. **Temperature Sensitivity**
- **Temperature Control:** Accurate control of column temperature is essential for maintaining performance, as temperature fluctuations can affect separation quality.

High-Performance Liquid Chromatography (HPLC) and Ultra-Performance Liquid Chromatography (UPLC) are both powerful analytical techniques used for separating, identifying, and quantifying compounds in a mixture. While they share fundamental principles, they have distinct differences and advantages. Here's a comparison between HPLC and UPLC [6-14].

1. Principle and Technology

- **HPLC**
- Operates using high pressure to push the mobile phase through a column packed with a stationary phase.
- Traditional HPLC systems operate at pressures up to 4000 psi (pounds per square inch).

- **UPLC**
- Uses ultra-high pressure to drive the mobile phase through a column with a more advanced design.
- UPLC systems operate at pressures up to 15,000 psi or higher.
- Features smaller particle sizes (typically < 2 µm) and more efficient column designs compared to HPLC.

2. Column and Particle Size

- **HPLC**
- Columns typically use particle sizes ranging from 3.5 µm to 5 µm.
- Larger particle sizes can lead to longer analysis times and lower resolution.

- **UPLC**
- Utilizes columns with smaller particle sizes (usually < 2 µm).
- Smaller particles enhance resolution and allow for faster separations.

3. Resolution and Sensitivity

- **HPLC**
- Provides good resolution and sensitivity but can be limited by larger particle sizes and lower pressure capabilities.

- **UPLC**
- Offers higher resolution and sensitivity due to smaller particle sizes and higher operating pressures.
- Better separation efficiency and peak resolution in shorter analysis times.

4. Analysis Speed

- **HPLC**
- Typically requires longer analysis times due to larger particle sizes and lower pressures.
- Analysis times range from several minutes to an hour.

- **UPLC**
- Significantly faster analysis times due to enhanced resolution and efficiency.
- Can complete analyses in a fraction of the time compared to HPLC.

5. Sample Throughput

- **HPLC**
- Suitable for routine analyses with moderate sample throughput.
- Less efficient for high-throughput scenarios compared to UPLC.

- **UPLC**
- Ideal for high-throughput analysis with faster run times and higher sample throughput.
- Allows for more samples to be analyzed in the same amount of time.

6. System Cost

- **HPLC**
- Generally less expensive in terms of initial investment and maintenance compared to UPLC systems.
- Lower cost of consumables such as columns and solvents.

- **UPLC**
- Higher initial cost due to advanced technology and higher pressure requirements.
- Potentially higher costs for consumables and maintenance.

7. Column Lifespan

- **HPLC**
- Columns typically have a longer lifespan but may need more frequent replacement due to lower pressure tolerance and larger particle sizes.

- **UPLC**
- Columns can have a shorter lifespan due to higher pressure and smaller particle sizes, which can lead to faster column degradation.

8. Resolution vs. Speed Trade-off

- **HPLC**
- Often requires a balance between resolution and analysis time, with longer analysis times needed to achieve high resolution.

- **UPLC**
- Achieves higher resolution with shorter analysis times, reducing the need for a trade-off between resolution and speed.

9. Method Development

- **HPLC:**
- Established methods are widely available, and development is generally straightforward.

- **UPLC:**
- Method development may require more optimization due to the newer technology and different column characteristics.

10. Applications

- **HPLC**
- Widely used in various industries including pharmaceuticals, environmental analysis, food and beverage, and clinical diagnostics.

- **UPLC**
- Increasingly used in high-throughput environments such as pharmaceutical research and development, proteomics, and metabolomics.

Conclusion

This article provides an overall review about HPLC method development, validation, analytical techniques and importance of quality control. By the above all review it is possible to produce very pure compounds. HPLC method is usefull for both in laboratory and clinical trails. In this publication the authors tried to conclude that HPLC is reproducible and versatile chromatographic method for analyzing drug product which have wide range applications in both qualitative and quantative estimation of various biological and drug molecule.

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