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A REVIEW ON HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

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ABSTRACT

High performance liquid chromatography (HPLC) is an important qualitative and quantitative technique, generally used for the estimation of pharmaceutical and biological samples. HPLC is the most often used separation technology for detecting, separating, and quantifying the drug. HPLC technique development and validation serve critical roles in novel drug discovery, development, and manufacturing, as well as a variety of other human and animal investigations. HPLC instrumentation includes a Solvent reservoir, pump, injector, column, detector, and integrator or acquisition and display system. The heart of the system is the column where separation occurs. The information that can be obtained using HPLC includes identification, quantification, and resolution of a compound.

KEYWORDS - *High performance liquid chromatography, instrumentation, elution, applications, mobile phase.*

INTRODUCTION

High Performance Liquid Chromatography (HPLC) is one of the most used analytical techniques. Chromatographic process can be defined as separation technique involving mass-transfer between stationary and mobile phase. HPLC utilizes a liquid mobile phase to separate the components of a mixture. The stationary phase can be a liquid or a solid phase. Common solvents used include any miscible combinations of water or organic liquids the most common are methanol and acetonitrile. This technique is widely used like spectroscopy and is a very powerful tool not only for analytical methods but also for preparative methods. Compounds of high-grade purity can be obtained by this method. This technique is widely used like spectroscopy and is a very powerful tool not only for analytical methods but also for preparative methods. Compounds of high-grade purity can be obtained by this method. Chromatography can be simply defined as It is the technique in which the components of a mixture are separated based upon the rates at which they are carried or moved through a stationary phase (column) by a gaseous or liquid mobile phase.The major applications are in the area of Pharmaceuticals, food, research, manufacturing, forensics, and bio-monitoring of pollutants.

Principle of HPLC

HPLC is a separation technique that involves: The injection of a small volume of liquid sample into a tube packed with tiny particles (3 to 5 micron (µm) in diameter called the stationary phase) where individual components of the sample are moved down the packed tube (column) with a liquid (mobile phase) forced through the column by high pressure delivered by a pump. These components are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles. These separated components are detected at the exit of this tube (column) by a flow-through device (detector) that measures their amount. Output from this detector is called an “HPLC” In principle, LC and HPLC work the same way except for the speed, efficiency, sensitivity, and ease of operation of HPLC are vastly superior. Though HPLC retains major of the credits for the analytical side, the earlier one of simple Liquid Chromatography still finds applications for the preparative Purposes.

Figure 1 HPLC Machine

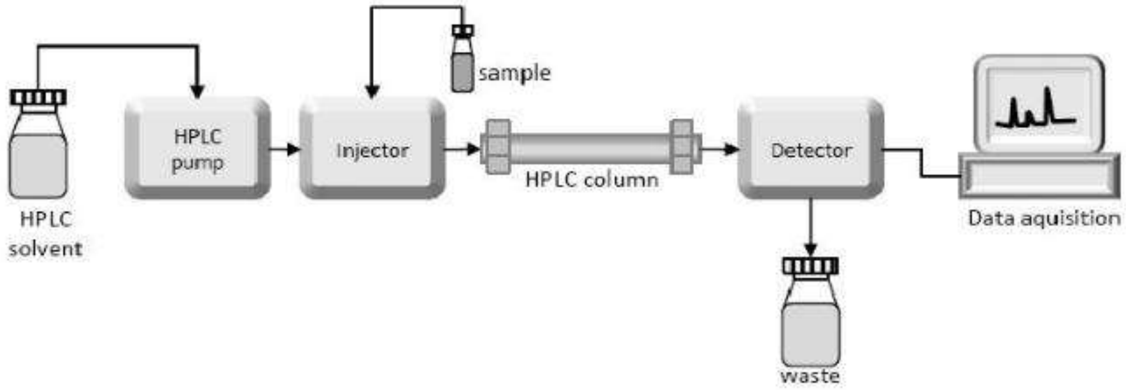


Figure 2 Components Of HPLC

Components of HPLC

1. Pump
2. Injector
3. Column
4. Detector
5. Column Oven/Thermostat:
6. Data Acquisition System:
7. Mobile Phase Reservoirs:
8. Gradient Controller (optional):
9. Waste Collector:

1. **Pump**
   The pump is responsible for delivering the mobile phase at a constant flow rate through the system.
   It maintains pressure to ensure consistent flow and reproducible chromatographic results.
   Types of pumps include binary (two-solvent) or quaternary (four-solvent) pumps.

2. **Injector**
   The injector introduces the sample into the mobile phase stream, typically via an injection loop.
   It must provide accurate and precise sample volumes to ensure reproducible analysis.

3. **Column**
   The column is the heart of the chromatographic system, where separation of analytes occurs.
   It consists of a packed bed of stationary phase material inside a stainless steel or glass tube.
   The choice of column (e.g., reversed-phase, normal-phase, size-exclusion) depends on the properties of the analytes and the separation requirements.

4. **Detector**
   The detector monitors the eluent leaving the column and detects the separated analytes.
   Common types of detectors include UV-Vis absorbance, fluorescence, refractive index, and mass spectrometry detectors.
   Detectors must provide high sensitivity, selectivity, and a wide linear range for accurate quantification.

5. **Column Oven/Thermostat**
   The column oven or thermostat maintains a constant temperature of the column to ensure reproducible chromatographic conditions.
   Temperature control is crucial for optimizing separation efficiency and resolving power.

6. **Data Acquisition System**
   The data acquisition system collects, processes, and analyzes the detector signals to generate chromatograms.
   It typically includes software for instrument control, data visualization, and peak integration.

7. **Mobile Phase Reservoirs**
   The mobile phase reservoirs hold the solvents used to prepare the mobile phase.
   They may include degassing units to remove dissolved gases that can interfere with chromatographic performance.

8. **Gradient Controller**
   In gradient elution chromatography, a gradient controller is used to vary the composition of the mobile phase over time.
   It allows for more complex separations and improved resolution by adjusting solvent composition during the chromatographic run.

9. **Waste Collector**
   The waste collector collects the eluent after it passes through the detector, preventing contamination and ensuring safe disposal of waste solvents.
   Each of these components plays a vital role in the performance and functionality of an HPLC system, contributing to the accuracy, precision, and reliability of the analytical results.

Types of HPLC

1. **Reversed-Phase Chromatography (RPC):**
   In reversed-phase chromatography, the stationary phase is non-polar, while the mobile phase is polar.
   Analytes are separated based on their hydrophobicity, with more hydrophobic compounds eluting later.
   RPC is widely used for the analysis of organic compounds, drugs, and biomolecules like proteins and peptides.

2. **Normal-Phase Chromatography (NPC):**
   In normal-phase chromatography, the stationary phase is polar, and the mobile phase is non-polar.
   Separation occurs based on the polarity of the analytes, with more polar compounds eluting earlier.
   NPC is commonly used for the analysis of non-polar and moderately polar compounds, such as natural products and lipids.

3. **Ion-Exchange Chromatography (IEC):**
   In ion-exchange chromatography, the stationary phase contains charged functional groups that interact with analyte ions.
   Separation is based on differences in ionic charge and affinity for the stationary phase.
   IEC is useful for separating charged molecules such as amino acids, peptides, and proteins.

4. **Size-Exclusion Chromatography (SEC) or Gel Filtration Chromatography**
   In size-exclusion chromatography, the stationary phase consists of porous beads with a range of pore sizes.
   Analytes are separated based on their size and molecular weight, with larger molecules eluting earlier.
   SEC is commonly used for the analysis of polymers, proteins, and biomolecules.

5. **Affinity Chromatography:**
   Affinity chromatography utilizes specific interactions between a ligand immobilized on the stationary phase and the analyte of interest.
   Separation is based on the affinity of the analyte for the ligand, allowing for highly selective purification.
   Affinity chromatography is widely used for the isolation and purification of biomolecules such as proteins, enzymes, and antibodies.

6. **Hydrophilic Interaction Chromatography (HILIC):**
   Hydrophilic interaction chromatography utilizes a polar stationary phase and a less polar mobile phase.
   Separation occurs based on differences in polarity and hydrophilicity of analytes.
   HILIC is suitable for the analysis of polar and hydrophilic compounds, including carbohydrates, amino acids, and small organic acids.
   Each type of HPLC offers unique advantages and is selected based on the physicochemical properties of the analytes and the desired separation mechanism. Understanding these different types of HPLC is crucial for selecting the most appropriate method for specific analytical tasks in research or industrial settings.

Application Of HPLC in Pharmaceutical Analysis

High-Performance Liquid Chromatography (HPLC) is a powerful analytical technique extensively used in pharmaceutical analysis. It plays a crucial role in ensuring the quality, safety, and efficacy of pharmaceutical products. Here are some key applications of HPLC in pharmaceutical analysis.

1. **Drug Purity and Impurity Profiling**
   HPLC is used to assess the purity of drug substances and products by identifying and quantifying impurities and degradation products. It helps ensure that pharmaceutical products meet stringent regulatory standards.

2. **Quantitative Analysis**
   HPLC is used for the quantitative determination of active pharmaceutical ingredients (APIs) in bulk drugs and formulations. This ensures that the dosage of the active ingredient in tablets, capsules, and other forms is accurate and consistent.

3. **Stability Testing**
   HPLC is employed in stability studies to monitor the stability of pharmaceutical products under various environmental conditions (e.g., temperature, humidity, light). It helps in understanding the shelf life and determining expiration dates.

4. **Bioavailability and Bioequivalence Studies**
   HPLC is used in pharmacokinetic studies to measure the concentration of drugs and their metabolites in biological fluids (e.g., blood, plasma, urine). This data is crucial for bioavailability and bioequivalence studies, which compare the drug release profiles of different formulations.

5. **Method Development and Validation**
   Pharmaceutical companies use HPLC to develop and validate analytical methods for new drugs. This includes determining parameters like precision, accuracy, linearity, and robustness, ensuring the method is reliable and reproducible.

6. **Dissolution Testing**
   HPLC is used to analyze samples from dissolution tests, which measure the rate and extent of drug release from solid dosage forms like tablets and capsules. This is essential for ensuring consistent drug release and absorption in the body.

7. **Identification of Compounds**
   HPLC can identify unknown compounds in a mixture by comparing their retention times with those of known standards. This is particularly useful in the identification of impurities and degradation products.

8. **Quality Control**
   HPLC is a routine tool in quality control laboratories for the analysis of raw materials, intermediates, and finished products. It ensures that all components meet predefined specifications and standards.

9. **Chiral Separation**
   Many drugs exist as enantiomers, which can have different therapeutic effects or side effects. HPLC with chiral stationary phases can separate and quantify these enantiomers, ensuring the correct form is used in the drug.

10. **Regulatory Compliance**
    Pharmaceutical companies must comply with various regulatory guidelines (e.g., USP, EP, ICH) that often mandate the use of HPLC for specific analyses. Adhering to these guidelines is crucial for market approval and product registration.

    Example: HPLC in Action
    A practical example could involve the use of HPLC in the analysis of a common drug like paracetamol (acetaminophen). The HPLC method would be developed and validated to quantify paracetamol in tablet form, ensuring it meets the USP standards for content uniformity and dissolution rate.

Method Development In HPLC

1. **Understanding the Purpose**
   Determine the specific goal of the HPLC analysis:
   Identification: To identify the presence of specific compounds
   Quantification: To measure the amount of specific compounds.
   Purity Assessment: To determine the purity of a sample and identify impurities.
   Stability Studies: To evaluate the stability of compounds under various conditions.

2. **Sample Considerations**
   Understand the nature and composition of the sample:
   Matrix: Is it a biological sample, environmental sample, or a pharmaceutical formulation?
   Physical State: Is it a liquid, solid, or gas?
   Chemical Properties: Consider the polarity, solubility, and stability of the analytes.

3. **Literature Review**
   Research existing methods for similar compounds or matrices to gain insights and starting points.

4. **Selection of HPLC Type**
   Choose the type of HPLC based on the chemical properties of the analytes:
   Reversed-Phase HPLC (RP-HPLC): Suitable for non-polar to moderately polar compounds.
   Normal-Phase HPLC (NP-HPLC): Suitable for polar compounds.
   Ion-Exchange HPLC: Best for ionic compounds.
   Size-Exclusion HPLC: For large molecules like proteins and polymers.

5. **Column Selection**
   Select an appropriate column:
   Column Material: Commonly used columns include C18 (octadecyl), C8, phenyl, etc.
   Column Dimensions: Typically, columns are 150-250 mm in length and 4.6 mm in diameter with particle sizes of 3-5 µm.

6. **Mobile Phase Selection**
   Develop the mobile phase:

- **Solvent System**: Water, methanol, acetonitrile, and buffers are common solvents.
- **pH and Buffer:** Adjust pH using buffers like phosphate or acetate to optimize peak shape and resolution.
  Gradient vs. Isocratic Elution: Gradient elution is useful for complex mixtures, while isocratic elution is simpler for less complex samples.

7. **Detector Selection**
   Choose a detector based on analyte properties:
   UV/Vis Detector: For compounds with chromophores.
   Fluorescence Detector: For fluorescent compounds.
   Mass Spectrometry (MS) Detector: For high sensitivity and structural information.

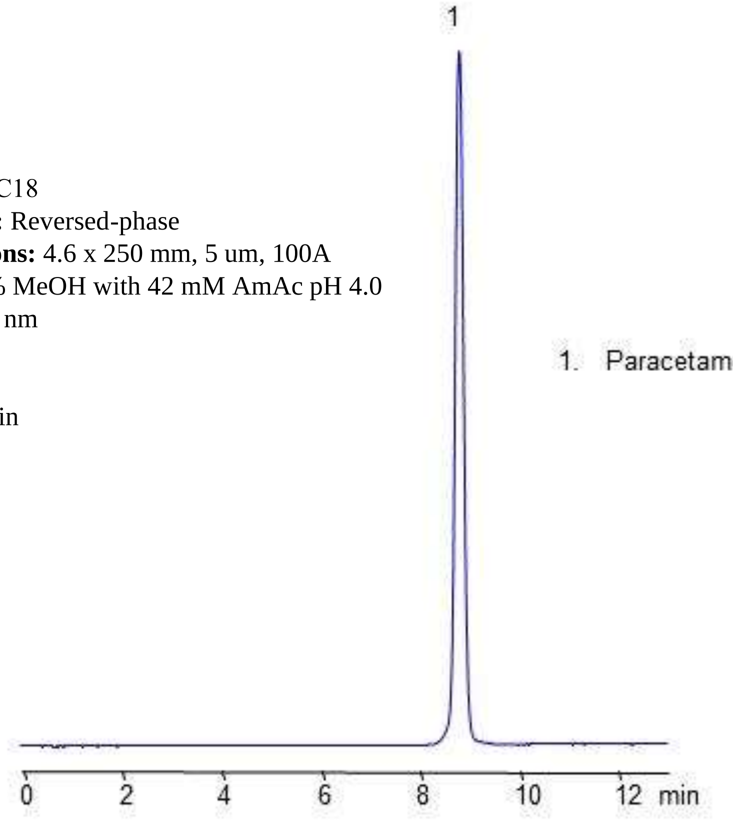
8. **Optimization of Conditions**
   Optimize method parameters:
   Flow Rate: Typically 0.5 to 2.0 mL/min.
   Injection Volume: Usually between 5 to 20 µL.
   Temperature: Often set between 25°C to 40°C to improve reproducibility and peak shape.

9. **System Suitability Testing (SST)**
   Perform system suitability tests to ensure the method works properly:
   Resolution (Rs): Ensure sufficient separation between peaks.
   Theoretical Plates (N): Indicator of column efficiency.
   Tailing Factor (T): Check for peak symmetry.
   Retention Time (tR) and Retention Factor (k'): Ensure reproducibility.

10. **Method Validation**
    Validate the developed method according to ICH guidelines:
    Specificity: Ensure the method can uniquely identify the analyte.
    Linearity: Establish a calibration curve to demonstrate the method's response is proportional to analyte concentration.
    Accuracy and Precision: Evaluate by analyzing spiked samples and calculating recovery and relative standard deviation (RSD).
    Limit of Detection (LOD) and Limit of Quantification (LOQ): Determine the smallest amount of analyte that can be reliably detected and quantified.
11. **Documentation**
    Document all steps, observations, and results comprehensively. Include:
    Method Development Report: Detailing the rationale, parameters tested, optimization steps, and final method.
    Validation Report: Summarizing the validation results and demonstrating the method meets all required criteria.

Developing an HPLC Method for Paracetamol

- **Purpose:** Quantification of paracetamol in tablet formulation.
- **Literature Review:** Existing methods suggest RP-HPLC with UV detection.
- **Column Selection:** C18, 250 x 4.6 mm, 5 µm.
- **Mobile Phase**: 15% MeOH with 42 mM AmAc pH 4.0.
- **Detector**: UV at 255 nm.
- **Optimization**: Flow rate of 1 mL/min, injection volume of 10 µL, column temperature at 30°C.
- **SST**: Resolution >2, theoretical plates >2000, tailing factor <1.5.
- **Validation**: Linearity over 5-100 µg/mL, accuracy 98-102%, precision RSD <2%.



- **Column:** Heritage C18
- **Separation Modes:** Reversed-phase
- **Column Dimenstions:** 4.6 x 250 mm, 5 um, 100A
- **Mobile Phase:** 15% MeOH with 42 mM AmAc pH 4.0
- **Detection:** UV 255 nm
- **Sample:** 0.2 mg/ml
- **Injection:** 5 uL
- **Flow rate:** 1 mL/min

Figure 1 Developing an HPLC Method for Paracetamol

Validation Of HPLC Method

1. Accuracy

The closeness of a measured Value to the true or accepted value is defined as accuracy.
To measure the closeness of the test results to the true value.

- **Procedure**
  Prepare samples with known amounts of paracetamol (e.g., 80%, 100%, and 120% of the target concentration).
  Analyze each sample in triplicate.

2. Precision

To demonstrate the reproducibility of the method under normal operating conditions.

- **Procedure**
  Prepare multiple (e.g., six) replicates of a single concentration of paracetamol.
  Analyze all replicates on the same day.

3. Limit of Detection (LOD) and Limit of Quantification (LOQ)

To determine the smallest amount of analyte that can be reliably detected (LOD) and quantified (LOQ) with acceptable precision and accuracy.

- **Procedure**
  Prepare and analyze a series of dilute solutions of paracetamol.
  Determine the signal-to-noise ratio for each solution.

4. System Suitability Testing (SST)

To ensure the system's performance before running analytical samples.

- **Procedure**
  Inject a standard solution of paracetamol.
  Calculate system suitability parameters like retention time, theoretical plates, resolution, and tailing factor.

Validation Of HPLC Method for Paracetamol

- **Specificity**
  No interference at the retention time of paracetamol.
- **Linearity**
  $R^2 > 0.999$ over the concentration range 5-100 µg/mL.
- **Accuracy**
  Mean recovery between 98-102%.
- **Precision**
  RSD < 2% for both repeatability and intermediate precision.
- **LOD and LOQ**
  LOD with S/N ~ 3:1, LOQ with S/N ~ 10:1.
- **Robustness**
  Method performance remains stable under small variations in parameters.
- **SST**
  Resolution > 2, theoretical plates > 2000, tailing factor < 1.5, consistent retention time.

Recent Advancement in HPLC

High-Performance Liquid Chromatography (HPLC) has seen significant advancements over the years, driven by the need for higher efficiency, sensitivity, and speed in analytical processes. Here are some recent advancements in HPLC technology:

1. **Ultra-High-Performance Liquid Chromatography (UHPLC)**
   UHPLC operates at higher pressures (up to 15,000 psi or more) compared to traditional HPLC, allowing the use of columns packed with smaller particles (sub-2 µm). This results in:
   Improved Resolution: Higher efficiency and better separation of complex mixtures.
   Faster Analysis: Reduced run times without compromising resolution.
   Lower Solvent Consumption: More eco-friendly and cost-effective.

2. **Core-Shell Particles**
   Core-shell particles, also known as superficially porous particles, have a solid core and a porous outer shell. They offer:
   High Efficiency: Similar efficiency to smaller fully porous particles but with lower backpressure.
   Enhanced Performance: Improved resolution and faster separations.

3. **Multidimensional HPLC (2D-HPLC)**
   2D-HPLC involves coupling two different HPLC systems, allowing:
   Complex Sample Analysis: Better separation of complex mixtures by using different separation mechanisms in each dimension.
   Increased Peak Capacity: Significantly higher resolution for complex samples, such as proteomics and metabolomics.

4. **Green HPLC Techniques.**
   There is an increasing focus on environmentally friendly HPLC methods, which include:
   Reduced Solvent Usage: Using water or less harmful solvents instead of traditional organic solvents.
   Miniaturized HPLC Systems: Smaller systems that consume less solvent and produce less waste.
   Supercritical Fluid Chromatography (SFC): Using supercritical CO_2 as the mobile phase, which is non-toxic and reduces environmental impact.

5. **Advances in Detectors**
   Recent developments in detectors have enhanced HPLC performance:
   Charged Aerosol Detectors (CAD): Universal detectors with high sensitivity for non-volatile and semi-volatile compounds.
   Photodiode Array Detectors (PDA): Improved spectral resolution and sensitivity for UV-visible detection.
   Fluorescence Detectors: Enhanced sensitivity for fluorescent compounds.

6. **Automation and Software Enhancements**
   Advancements in automation and software have streamlined HPLC operations:
   Automated Sample Preparation: Reduces manual handling and increases reproducibility.
   Advanced Data Analysis Software: Improved algorithms for peak integration, deconvolution, and identification.
   Remote Monitoring and Control: Internet of Things (IoT) integration for real-time monitoring and control of HPLC systems from remote locations.

CONCLUSION

High-Performance Liquid Chromatography (HPLC) remains a cornerstone analytical technique in pharmaceutical analysis due to its versatility, precision, and sensitivity. Over the years, HPLC has evolved significantly, driven by the need for higher efficiency, speed, and sensitivity in analytical processes.

The primary applications of HPLC in pharmaceutical analysis include the quantification of active pharmaceutical ingredients (APIs), the detection of impurities, and the study of drug stability. HPLC's ability to provide detailed qualitative and quantitative analysis of complex mixtures makes it indispensable in ensuring the safety, efficacy, and quality of pharmaceutical products.

Recent advancements in HPLC, such as Ultra-High-Performance Liquid Chromatography (UHPLC), core-shell particle technology, and multidimensional HPLC (2D-HPLC), have substantially improved the resolution, speed, and robustness of the method. Innovations in detector technologies, particularly mass spectrometry integration, have enhanced the sensitivity and specificity of HPLC, enabling the detection of trace levels of impurities and detailed structural elucidation of analytes.

The development and validation of HPLC methods are critical for ensuring reliable and reproducible results. Validation parameters such as specificity, linearity, accuracy, precision, robustness, and system suitability testing are essential to demonstrate that the method is fit for its intended purpose. Following regulatory guidelines during validation ensures that the method meets industry standards and is acceptable for regulatory submissions.

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