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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Abstract

One type of column chromatography that is commonly used in biochemistry and analysis for the purpose of separating and quantify active compounds is called High Performance Liquid Chromatography (HPLC). HPLC is a frequently employed separation technique that is utilized to determine, separate, and measure the medication. In addition to a variety of various human and animal study, the development and validation of high-performance liquid chromatography techniques play critical roles in innovative drug identification, development, and production. The several steps needed in creating and verifying an HPLC procedure are covered in this chapter. Among other things, the chemical structure of the molecules, their synthesis pathway, the solubility of polarity, the pH and pKa values, and the activity of functional groups influence the development of an HPLC technology. The validation of a High Performance Liquid Chromatography method includes the consideration of multiple variables, including precision, sensitivity, linearity, range, robustness, system adaptability, limit of detection and limit of quantification, and ICH Guidelines.

Keywords: Chromatography, Pressure Liquid Chromatography, Method Development and Validation.

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I. INTRODUCTION

1. High Performance Liquid Chromatography (HPLC) [1- 3]

- High performance liquid chromatography is one of several chromatographic methods for the separation and analysis of chemical mixtures.
- A separation method known as liquid chromatography relies on the sample ingredients' varying rates of distribution between a stationary phase and a mobile phase of liquid.
- Each separated constituent's concentration in the column effluent, which is recorded online by a detector and gives a chromatogram.
- The HPLC method is used to analyze the majority of pharmaceuticals in multi-component dosage forms due to its numerous benefits, such as speed, automation ease, accuracy, precision, and specificity.

2. Benefits Of High Performance Liquid Chromatography (Hplc) [2,3]

- Quickness (analysis can be completed in less than 20 minutes)
- Extremely sensitive
- Enhanced resolution (Various stationary phase)
- Reviable columns
- Absolute for low-volatility compounds
- Simple sample handling, recovery, and maintaining
- Accurate and Repeatable
- Instrumentation lends itself to mechanization and quantification (less labor and time)
- Capable of significantly larger-scale preparative liquid chromatography
- calculations; performed by integrator itself

3. Principles Of High Performance Liquid Chromatography [3]

- The interaction between the solute and stationary phase, which forms the basis of the separation process.
- Diffusion mechanisms induce the solute to dilution in the mobile phase, which inhibits separation.

4. Classification Of High Performance Liquid Chromatography [4]

a. On the Basis of Mode of Separation

i. Normal phase chromatography
ii. The basis for separation in this Normal Phase Chromatography method is polarity. Hexane, benzene, and other chemical solvents are examples of the non-polar mobile phase, while the polar stationary phase includes types of silica gel, cyanotype, and amino gel.
iii. Reverse phase chromatography
iv. It is the opposite of conventional phase chromatography, in which the mobile phase is polar and the stationary one is non-polar. Organic solvents like methanol, acetonitrile, and buffer (phosphate buffer) are examples of mobile phases.

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b. Based on the Principle of Separation

i. **Absorption Chromatography:** The solute molecule in absorption chromatography adhered directly to the stationary phase's surface. The components with higher affinity for the mobile phase elute first, followed by those with lower affinity for the stationary phase.

ii. **Ion-Exchange Chromatography:** This procedure enables the charge-based separation of polar molecules and ions. Nearly all charged molecules, including big proteins, tiny nucleotides, and amino acids, can be treated with it. The pulling force between ions of the solute and charged sites bonded to the phase that is stationary is what drives retention.

iii. **Ion-Pair Chromatography:** Ions in solution can be "paired," or neutralized, and then segregated as an ion pair on a reversed-phase membrane using this method of chromatography.

iv. **Gel Permeation Chromatography:** There is no attractive relationship between the stationary phase and the solute in this type of chromatography. The gaseous or liquid phase goes through a porous gel that divides the molecules into different sizes.

v. **Affinity Chromatography:** The most selective kind of chromatography that is used is this method. It makes use of a particular form of interaction between an immobilized second molecule on a phase that is stationary and a solute molecule of one kind.

vi. **Chiral Chromatography:** Stereoisomer separation is a part of this. Enantiomers are just mirror reflections of one another in three dimensions; they differ neither chemically nor physically. The analytes had different affinities as a result of the permeability chiral separations, which required that either the stationary phase or the mobile phase be created by chirality.

c. According to the Elution Method

i. **Isocratic elution:** Isomeric elution is the name given to a separation in which the mobile phase construction does not change during the procedure, frequently employed in applications for quality control that support and are near a manufacturing process.

ii. **Elution gradient:** Gradient elution is the term used to describe a separation procedure in which the mobile phase structure is altered, frequently employed while developing methods for unknown combinations.

d. Based on Scale of Preparation

i. Analytical High Performance Liquid Chromatography
No retrieval of a substance's components.

ii. Preparative High Performance Liquid Chromatography
Independent elements of substances can be taken out.

e. Based on the Type of Analysis

i. **Quantitative Analytical:** We can isolate specific elements in an analysis to discover the type of the substance's chemical components, but we are unable to estimate how many there are in this process..

ii. **Quantitative analysis:** It is possible to evaluate the proportions and amounts of its chemical ingredients as well as the quantity of impurity and independent components.

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5. Instrumentation of HPLC [5-10]

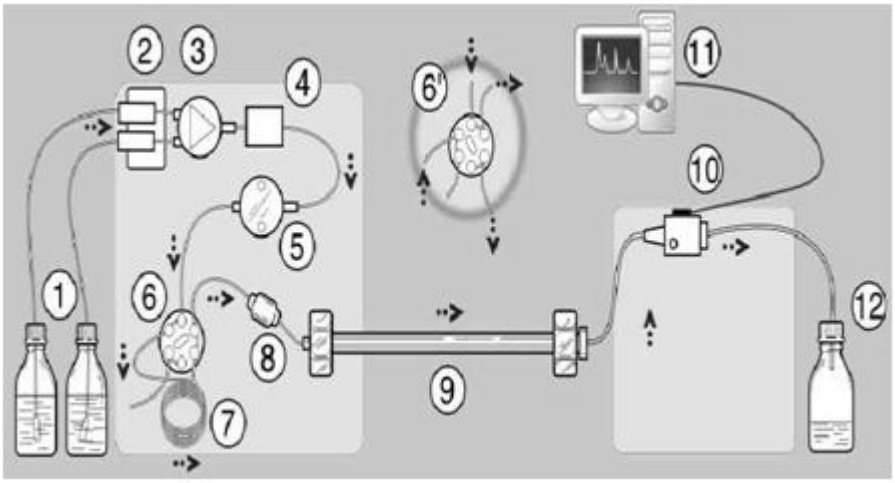

Figure 1: Schematic diagram of HPLC

The components of High Performance Liquid Chromatography are stated below and its schematic diagram is shown in figure 1.

A. Reservoirs for solvents
B. Keeping enough High Performance Liquid Chromatography in storage solvents are necessary for the system to run continuously. They could have unique filters and an online the degassing system installed to keep the solvent safe from outside influences.
C. Solvent degasser
D. The process of degassing is used to remove dissolved gases from the mobile phase.
E. Gradient valve
F. Restrict liquid movement in and out of the pump head.
G. Mixing vessel for mobile phase transportation
H. A pump with high pressure
I. This ensures that the mobile phase flows through the system continuously and continuously. Modern pumps allow various solvents from various reservoirs to be mixed under supervision. In high performance liquid chromatography, three primary pump types are used: displacement, reciprocating, and pneumatic pumps.
J. Types of pump
   - Displacement pump
   - Reciprocating pump
   - Pneumatic or constant pressure pump

a. Switching valve in “injection position” and Switching valve in “load position”

b. Sample Injection Loop: This makes it possible to introduce (inject) the analyte combination into the mobile phase stream prior to it entering the column. The majority of contemporary injector are auto samplers, which enable preprogrammed injections of various sample volumes taken out of the containers in the auto sampler tray. Loop

injections, valve injection, & on-column injection are three crucial methods for introducing samples.

c. Pre-column (guard column)

d. Analytical Column: This is where the HPLC system's heart is. In actuality, it causes the analyte in the mixture to separate. The location where the stationary phase and mobile phase come into touch to produce an enormously surface-aread interface is called a column. The majority of recent advances in chromatography have been focused on creating various strategies to improve this interfacial contact.

Type of Column for HPLC

- Analytical
- Preparative
- Capillary
- Nano

e. Detector: This apparatus is used to continuously record particular physical or chemical characteristics of the column discharge. UV detectors, which provide continuous registrations of the UV absorbance at a chosen wavelength or over a range of wavelengths (diode array detection), are the most often used detectors in pharmaceutical investigation. The absorbance changes as a result of the analyte's appearance in the detector flow cell. A positive signal is produced if the analyte absorbs more than the surrounding air (mobile phase).

There are two varieties of detectors:

a. Bulk property detector
b. Solute property detector.

Detector Used in HPLC

- Concentration-Sensitive and Mass- Flow-Sensitive Detector
- UV - VIS Absorption Detector
- Diode-Array Detector
- Fluorescence Detector
- Conducto-metric Detector
- Potentiometric Detector
- Refractive index detector
- Electrochemical detector
- IR detector

f. Data Acquisition and Control System: All of the High Performance Liquid Chromatography instrument's parameters, including eluent composition, temperature, and injection sequences, are controlled by a computer-based system that also collects data from the detector and continuously monitors system performance. Waste reservoir

II. System Suitability of Chromatographic Method [11]

System suitability is the examining of a system performance before or during the analysis. Parameters for system suitability testing of HPLC are given as below:

Table 1: Acceptance criteria for chromatographic conditions

Measurement	Suggestion
Factor of capacity (K)	The peaks should be clearly separated from one another and the void volume; in most cases, K' > 2.
Replicability	RSD ≤ 1% N ≥ 5 is preferred.
Comparative retention	Not necessary, as the resolution indicated
Resolution	Rs of more than two between the closest eluting possible interferent (internal standard, degradation product, contaminant, etc.) and the peak of interest
Tailing Factor	T ≤2
Theoretical Plates	Generally speaking, it should be greater than 2000.

III. APPLICATION OF HPLC [11]

1. Chemical purification and separation are also aided by it.

2. Usage in Pharmaceuticals
   - Pharmaceutical quality control;
   - pharmaceutical dosage form tablet dissolution research; and
   - medication stability control

3. Uses in the environment
   - Phenolic chemical detection in drinking water;
   - Pollution bio-monitoring
   - Use in the field of forensics

4. Quantification of drugs in biological samples.
   - Determining the presence of steroids in urine, blood, etc.
   - Fabric dye forensic analysis.
   - Measuring the presence of cocaine and other drugs of abuse in urine, blood, etc.

5. Food and flavor
   - Analysis of polycyclic compounds in vegetables.
   - Water and soft drink quality measurements.
   - Analysis of fruit juice sugar content.
   - Analysis of preservative
   - Use in clinical investigations

6. Urine analysis, antibiotics analysis in blood.
   - Analysis of problems with the liver involving bilirubin and biliverdin.

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- Finding endogenous neuropeptides in the brain's extracellular fluid, etc.

IV. HPLC METHOD DEVELOPMENT [10,11]

The scientific technique is needed to design the HPLC method for chemical compounds and pharmaceutical items. The following steps will be involved in method development.

1. A survey of literature
2. The molecules' chemical properties
3. Choosing a buffer
4. The choice of columns
5. selection of mobile phase
6. Choosing a detector
7. Gradient and isocratic elution
8. Selection of chromatographic parameters
9. Pre-approval of the technique

Table: Commonly used HPLC buffers for R-HPLC

Buffer	pKa	Buffer Range	UV Cut off (nm)
Phosphate	2.1	1.1-3.1	200
	7.2	6.2-8.2	
	12.3	11.3-13.3	
Formic acid*	3.8	2.8-4.8	210
Acetic acid*	4.8	3.8-5.8	210
Citrate	3.1	2.1-4.1	230
	4.7	3.7-5.7	
	5.4	4.4-6.4	
Tris	8.3	7.3-9.3	205
Triethylamine*	11.0	10.0-12.0	200
Pyrrolidine	11.3	10.3-12.3	200

* Volatile buffers

V. CONCLUSION

This chapter gives an overview of the RP-High Performance Liquid Chromatography Methodology development and validation for techniques. The continuous and linked processes of method development and validation establish the measurement's performance limitations and quantify a parameter as intended. The organic content and pH of the buffer and mobile phase have significant effects on the selectivity of the separation process. The excellent specificity, sensitivity, low detection limit, and low cost of the HPLC technology were major advantages.

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