CHAPTER TITLE

**HIGH PERFORMANCE LIQUID CHROMATOGRAPHY**

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**Abstract:**

High Performance Liquid Chromatography (HPLC) is a kind of column chromatography that is generally used in biochemistry and analysis to separate, identify, and quantify active chemicals. HPLC is the commonly used separation technology for detecting, separating, and quantifying the drug. High Performance Liquid Chromatography technique development and validation perform crucial roles in novel drug discovery, development, and manufacturing, as well as a diversity of other human and animal studies. This chapter discusses the many processes involved in developing and validating an HPLC technique. The creation of an High Performance Liquid Chromatography technique is impacted by the chemical structure of the molecules, the synthetic pathway, solubility, polarity, pH and pKa values, and the activity of functional groups, among other factors. Accuracy, Specificity, Linearity, Range, limit of detection and limit of quantification, robustness, and system suitability are comprised in the validation of a High Performance Liquid Chromatography technique according to ICH Guidelines.

Keywords: Pressure Liquid Chromatography, Chromatography, Method validation and development.

**Introduction:**

### 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.
* Liquid chromatography is a separation technique based on a different distribution rate of sample constituents between a stationary and a liquid mobile phase.
* The concentration of each separated constituent in the column effluent which is monitored by online detector and generates chromatogram.
* Most of the drugs are in the multi component dosage forms are analyzed by HPLC method because of the several benefits like alacrity, specificity, accuracy, precision and ease of automation in this method.

**Benefits of High performance liquid chromatography (HPLC) [2,3]**

* Speed (analysis can be achieved in 20 minutes or less)
* High sensitivity
* Enhanced resolution (Various stationary phase)
* Reviable columns
* Absolute for the substances of low volatility
* Easy sample recovery, handling and maintenance
* Instrumentation tends itself to automation and quantitation (fewer time and few labour)
* Precise and Reproducible
* Calculations are done by integrator itself
* Acceptable for preparative liquid chromatography on a much larger scale

#### Principles of High Performance Liquid Chromatography [3]

* The separation process, which is based on the interaction between solute and stationary phase.
* Dilution of the solute in the mobile phase because of diffusion processes; this effect restrain separation.

#### Classification of High Performance Liquid Chromatography [4]

1. On the basis of mode of separation
2. Normal phase chromatography

In this Normal phase chromatography technique, separation is based on polarity. The stationary phase is polar i.e Silica gel type, cyanotype, amino type, and the mobile phase is non-polar i.e hexane, benzene and other organic solvents.

1. Reverse phase chromatography

It is reverse of the normal phase chromatography where the stationary phase is non-polar and the mobile phase is polar. Examples of the mobile phases are organic solvents i.e methanol, acetonitrile, buffer (phosphate buffer).

1. Based on the principle of separation
2. Absorption chromatography

In the absorption chromatography solute molecule straightly bound to the surface of the stationary phase. The elements which has more affinity towards mobile phase elutes first and the component which has been less affinity towards stationary phase elute later.

1. Ion-exchange chromatography

It is a process that let the separation of ions and polar molecules based on their charge. It can be used for almost any kind of charged molecules including large proteins, small nucleotides and amino acids. Retention is rely on the attraction between solute ions and charged sites bound to the stationary phase.

1. Ion-pair chromatography

This is a form of chromatography in which ions in solution can be “paired” or neutralized and separated as an ion pair on a reversed-phase column.

1. Gel permeation chromatography

This kind of chromatography lacks an attractive interaction between the stationary phase and solute. The liquid or gaseous phase cross through a porous gel which is separates the molecules depending to its size.

1. Affinity chromatography

This technique is the most selective type of chromatography employed. It utilizes the specific interaction between one type of solute molecule and a econd molecule that is immobilized on a stationary phase.

1. Chiral chromatography

This includes the separation of stereoisomers. In the case of enantiomers, these have no chemical or physical dissimilarities apart from being three- dimensional mirror images. The permeable chiral separations were took place; either the mobile phase or the stationary phase must themselves be made by chiral, giving differing affinities between the analytes.

1. Based on elution technique
2. Isocratic elution

A separation in which the mobile phase construction remains constant throughout the process is termed isocratic elution. Often used in quality control application those supports and are in close proximity to a manufacturing process.

1. Gradient elution

In Gradient elution the mobile phase construction is changed during the separation process is described as a gradient elution. Frequent used in method development for unknown mixtures.

1. Based on scale of preparation
	* 1. Analytical High Performance Liquid Chromatography

 No recovery of individual components of a substance.

* + 1. Preparative High Performance Liquid Chromatography

 Independent elements of substances can be removed.

1. Based on the type of analysis
2. Qualitative analysis

Analysis of a substance in order to ascertain the nature of its chemical components and we can separate individual constituents but cannot determine the quantity in this analysis.

1. Quantitative analysis

Determining the quantities and proportions of its chemical constituents and the amount of the impurity and the independent components can be assessed.

#### Instrumentation of HPLC [5-10]

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**Fig. 1 Schematic diagram of High Performance Liquid Chromatography**

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

1. Solvent reservoirs

Storage of sufficient amount of High Performance Liquid Chromatography solvents for constant operation of the system is required. They could be equipped with an online degassing system and special filters to isolate the solvent from the influence of the environment.

1. Solvent degasser

Degassing is utilised to eliminate dissolved gases in the mobile phase.

1. Gradient valve

Restrict liquid movement in and out of the pump head.

1. Mixing vessel for transporting of the mobile phase
2. High pressure pump

This provides the constant and continuous flow of the mobile phase through the system. Latest pumps enable controlled mixing of different solvents from different reservoirs. There are three main types of pumps are utilised in High Performance Liquid Chromatography are displacement pump, reciprocating pump and pneumatic pump.

1. Types of pump
2. Displacement pump
3. Reciprocating pump
4. Pneumatic or constant pressure pump
5. Switching valve in “injection position” and Switching valve in “load position”
6. Sample injection loop

This permetes an introduction (injection) of the analyte mixture into the stream of the mobile phase before it enters the column. Most modern injectors are auto samplers, which allow programmed injections of different volumes of samples that are withdrawn from the vials in the auto sampler tray. Three important ways to introduce samples are loop injection, valve injection, on column injection.

1. Pre-column (guard column)
2. Analytical column

This is the heart of HPLC system. It actually produces a separation of the analyte in the mixture. A column is the site where the mobile phase is in contact with the stationary phase, forming an interface with enormous surface. Most of the chromatography development in recent years went toward the design of many different ways to enhance this interfacial contact.

* Type of column for HPLC
1. Analytical
2. Preparative
3. Capillary
4. Nano
5. Detector

This is a device for continuous registration of specific physical or chemical properties of the column effluent. The most common detector utilised in pharmaceutical analysis is UV detector, which allows monitoring and continuous registration of the UV absorbance at a selected wavelength or over a span of wavelengths (diode array detection). Appearance of the analyte in the detector flow cell causes the change of the absorbance. If the analyte absorbs greater than the background (mobile phase), a positive signal is obtained.

* The detectors are of two types,
1. Bulk property detector
2. Solute property detector.
* Detector used in HPLC
1. Concentration-Sensitive and Mass- Flow-Sensitive Detector
2. UV - VIS Absorption Detector
3. Diode-Array Detector
4. Fluorescence Detector
5. Conducto-metric Detector
6. Potentiometric Detector
7. Refractive index detector
8. Electrochemical detector
9. IR detector
10. Data Acquisition and Control System

Computer based system that controls all parameters of High Performance Liquid Chromatography instrument like eluent composition, temperature, injection sequences and acquires data from the detector and continuous monitoring of the system performance.

1. Waste reservoir

**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 no. 1 Acceptance criteria for chromatographic conditions**

|  |  |
| --- | --- |
| **Parameter** | **Recommendation** |
| Capacity factor (K) | The peaks should be well resolved from other peaks and the void volume, generally K’ > 2 |
| Repeatability | RSD ≤ 1%N ≥ 5 is desirable |
| Relative Retention | Not essential as the resolution stated |
| Resolution | Rs of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipients, degradation product, internal standard, etc.) |
| Tailing Factor | T ≤2 |
| Theoretical Plates | In general should be > 2000 |

#### Application of HPLC [11]

1. It also aids in chemical separation and purification.
2. Pharmaceutical Applications
* To control drug stability
* Tablet dissolution study of pharmaceutical dosage form
* Pharmaceutical quality control
1. Environmental applications
* Detection of phenolic compounds in drinking water
* Bio-monitoring of pollutants
1. Application in Forensics
* Quantification of drugs in biological samples.
* Identification of steroids in blood, urine etc.
* Forensic analysis of textile dyes.
* Determination of cocaine and other drugs of abuse in blood, urine etc.
1. Food and flavor
* Analysis of polycyclic compounds in vegetables.
* Measurement of quality of soft drinks and water.
* Sugar analysis in fruit juices.
* Preservative analysis
1. Application in clinical tests
* Urine analysis, antibiotics analysis in blood.
* Analysis of bilirubin, biliverdin in hepatic disorders.
* Detection of endogenous neuropeptides in extracellular fluid of brain etc.

#### HPLC Method Development [10,11]

#### HPLC method development requires the scientific approach for developing the method for the chemical substances and drug products. Method development will involves the following steps like,

* + - 1. Literature survey
			2. Chemical properties of the molecules
			3. Buffer selection
			4. Column selection
			5. Mobile phase selection
			6. Detector selection
			7. Isocratic and gradient elution
			8. Chromatographic parameters selection
			9. Pre-validation of the method

**Table no.2 commonly used HPLC buffers for R-HPLC**



**Conclusion:**

This chapter gives an overview of the RP-High Performance Liquid Chromatography Technique method development and validation. Method development and validation are constant and interconnected activities that quantify a parameter as intended and determine the measurement's performance limits. The makeup of the buffer and mobile phase (organic and pH) has a remarkable impact on separation selectivity. The advantages of the HPLC technique were its great selectivity, sensitivity, low detection limit, and inexpensive cost.

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