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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD DEVELOPMENT AND VALIDATION: A COMPREHENSIVE REVIEW

Pranjal Dattu Avhad* and Madhuri Bhausaheb Nagare

Department of Pharmaceutical Quality Assurance, SND College of Pharmacy, Babhulgaon (Yeola), India.

***Corresponding Author: Pranjal Dattu Avhad**

Department of Pharmaceutical Quality Assurance, SND College of Pharmacy, Babhulgaon (Yeola), India.

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ABSTRACT

High-Performance Liquid Chromatography (HPLC) is a highly efficient analytical technique extensively employed in pharmaceutical, environmental, and food industries for the separation, identification, and quantification of chemical compounds. The accuracy and reliability of HPLC methods are crucial for ensuring consistent quality control in drug formulation and other applications. This review explores the fundamental principles of HPLC, the stepwise approach to method development, and the critical aspects of method validation in compliance with International Council for Harmonisation (ICH) guidelines. The method development process involves optimizing various chromatographic parameters, including mobile phase composition, column selection, flow rate, and detection techniques. Additionally, method validation ensures robustness and reproducibility through parameters such as accuracy, precision, specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), robustness, and ruggedness. The significance of HPLC in pharmaceutical analysis, including drug stability testing, impurity profiling, and bioanalytical studies, is also discussed. Advancements in chromatographic techniques continue to improve analytical performance, making HPLC a critical tool for quality assurance and regulatory compliance in pharmaceutical sciences.

KEYWORD:- HPLC, Method Development, Validation, ICH Guidelines, Analytical Techniques.

INTRODUCTION

High-Performance Liquid Chromatography (HPLC) has become one of the most widely used analytical techniques in modern laboratories for the separation, identification, and quantification of complex mixtures. Since its development, HPLC has played a crucial role in various scientific and industrial fields, particularly in pharmaceuticals, food safety, environmental monitoring, and biochemical research. The versatility and high resolution of HPLC make it indispensable for ensuring product quality, regulatory compliance, and drug development.[1]

HPLC operates on the principle of liquid-solid interactions, where a sample mixture is passed through a stationary phase using a liquid mobile phase under high pressure. The separation efficiency depends on factors such as mobile phase composition, column packing material, flow rate, and detection methods. The technique is categorized into different modes, including Reverse-Phase HPLC (RP-HPLC), Normal-Phase HPLC (NP-HPLC), Ion-Exchange HPLC, and Size-Exclusion HPLC, each suited for specific applications. The development of a reliable HPLC method involves systematic optimization of parameters to achieve accurate, precise, and reproducible results. Key considerations include selecting suitable solvents, column type, pH conditions, and detection wavelengths to ensure proper analyte resolution. Moreover, method validation, as per ICH guidelines, is essential to confirm the method's accuracy, precision, specificity, linearity, and robustness, ensuring that it meets the regulatory and analytical requirements. HPLC is extensively used in pharmaceutical analysis for drug formulation, stability studies, impurity profiling, and bioanalytical testing.[2,3] It also plays a significant role in food safety monitoring by detecting contaminants and preservatives in food products. Environmental applications include the assessment of pollutants in water, soil, and air, making HPLC a valuable tool in quality control and regulatory enforcement. With the continuous advancements in instrumentation and software, HPLC has evolved to include Ultra-High Performance Liquid Chromatography (UHPLC), which offers faster analysis times and improved resolution. Coupling HPLC with mass spectrometry (HPLC-MS) has further enhanced its capabilities in complex sample analysis, expanding its applications in proteomics, metabolomics, and forensic sciences.[4] This review provides a comprehensive discussion on HPLC principles, method development strategies, and validation parameters. It aims to highlight the significance of HPLC in various scientific fields and

the continuous advancements that enhance its analytical performance. By following a systematic approach to method development and validation, researchers and analysts can ensure the reliability and accuracy of HPLC methods in their respective applications.

Principles of HPLC

In a separation column between a stationary and a mobile phase, the purification happens. A separation column contains a granular substance with incredibly small porous particles as the stationary phase. On the other hand, the mobile phase is a solvent or solvent mixture that is pushed through the separation column under high pressure. The sample is injected into the mobile phase flow from the pump to the separation column via a valve with a connected sample loop, which is a tiny tube or a stainless-steel capillary. As a result of interactions with the stationary phase, the various components of the sample are retained to variable degrees, which cause them to migrate across the column at various rates. After leaving the column the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer. At the end of this operation a chromatogram in the HPLC software on the computer is obtained, which allows the identification and quantification of the different substances. HPLC operates on the principle of differential partitioning of analytes between the stationary and mobile phases. The separation efficiency depends on factors such as column type, mobile phase composition, flow rate, and detection techniques. The primary modes of HPLC include:

- Reverse-phase HPLC (RP-HPLC)
- Normal-phase HPLC (NP-HPLC)
- Ion-exchange HPLC
- Size-exclusion HPLC.[5,6,7]

History

Prior to HPLC, researchers used conventional liquid chromatographic techniques. Because the flow rate of solvents depends on gravity, liquid chromatographic techniques are inefficient. Separations take many hours, and maybe even days, to complete. Despite the fact that liquid chromatography (LC) at the time was more efficient, it was assumed that gas stage partition and research of extremely polar high atomic weight biopolymers were both impractical. GC was ineffective for some organic chemists because the solutes were thermally unstable. As a result, it was predicted that alternative methods would soon lead to the advancement of HPLC.

Cal Giddings, Josef Huber, and others predicted in the 1960s that LC could be operated in the high-efficiency mode by reducing the pressing molecule measurement significantly below the standard LC and GC level of 150 µm and using pressure to increase the versatile stage velocity, building on the original work of Martin and Synge from 1941. Throughout the 1960s and into the 1970s, these expectations underwent extensive research and improvement. Early research on improving LC particles began, and the development of Zipax, an externally permeable molecule, was encouraging for HPLC technology. Many improvements in machinery and instrumentation were made throughout the 1970s. Injectors and pumps were first used by experts to construct a straightforward HPLC system. Gas amplifier pumps were perfect since they worked at consistent pressure and did not require release free seals or check valves for steady flow and great quantitation. Although improvements in apparatus had a significant role, the history of HPLC is mostly the narrative of the evolution of molecular technology. There has been a consistent trend towards smaller molecules since the introduction of permeable layer particles to increase effectiveness. But when molecule sizes shrank, other problems emerged. The drawback from the unneeded pressure drop is anticipated to be the difficulty of setting up a uniform pressing of extremely fine materials as well as the difficulty of driving versatile liquid through the segment. To handle the pressure, another cycle of instrument advancement should typically take place every time the molecule size is completely reduced.[8-13]

Operation

The sample mixture that will be separated and combined is added to the stream of mobile section that permeates the column in a unique very small volume (typically microliters). The sample is divided into segments that move through the phase at various speeds that are a result of particular physical interactions with the adsorbent (likewise known as stationary stage). Each element's speed is determined by its compound structure and mobile section composition. Retention time refers to the time at which a chosen analytical elutes (rises up out of the column). A characteristic that is customary for a certain analysis may be the retention time measured under particular circumstances.[14–18] There are numerous types of columns available that are filled with adsorbents with different molecular sizes and surface characteristics ("surface science"). The use of packing materials with tiny molecular sizes necessitates the use of increased operational pressure ("backpressure"), which frequently improves action resolution (i.e. the degree of division between sequent analyses rising up out of the column). Stuff particles may have polar or hydrophobic properties. Basic mobile phases combine water with a variety of natural solvents, including any mixable mixture of water (the most widely recognised area unit acetonitrile and methanol). Certain HPLC systems use mobile phases that are not water-soluble. The aqueous phase of the mobile section might contain acids, (for example, formic, element or trifluoroacetic corrosive) or salts to assist with the separation of the sample parts.

During the chromatographic analysis, the composition of the mobile section may remain unchanged ("isocratic extraction mode") or change ("inclination extraction mode"). When it comes to pattern elements that are no longer wholly exceptional in their predisposition for the stationary stage, isocratic extraction typically succeeds.

In gradient extraction, the cellular area's employer frequently varies from low to excessively high eluting tremendous. Analytical maintenance times show the eluting quality of the mobile section, with high eluting quality delivering quick extraction. The stationary stage's stationary stage and the strength of connections between completely separate example pieces ("analyses") determine the structure of the mobile section (also known as eluent) (e.g. hydrophobic connections in turned around stage HPLC). Based on their preference for the stationary and mobile stages, analyses divide between the two during the detachment operation in the sample. This process is similar to what occurs during a liquid-liquid extraction, except that it is continuous rather than stepwise. When the mobile stage becomes more densely packed with acetonitrile in this situation (during a variable amount of upper eluting quality), extra hydrophobic elements may wash (fall off the column) late.[19-27]

Types of HPLC

Varieties of HPLC often depend on the process's utilisation of a phase system [3, 4], the following HPLC types are frequently used in analysis.

Normal phase chromatography: This technique, also known as Normal phase HPLC (NPHPLC), divides analytes according to polarity. Polar stationary phase and non-polar mobile phase are both used in NP-HPLC. The polar stationary phase reacted with the polar analyte and held it. Increased analyte polarity results in stronger adsorption forces, and the interaction of the polar analyte with the polar stationary phase lengthens the elution time.

Reversed phase chromatography: Reversed phases the stationary phase of HPLC (RPHPLC or RPC) is non-polar, and the mobile phase is aqueous and moderately polar. As a result of repulsive forces between a polar eluent, the comparatively non-polar analyte, and the non-polar stationary phase, RPC works on the theory of hydrophobic interactions. Upon association with the ligand in the aqueous eluent, the analyte's affinity for the stationary phase is proportional to the contact surface area around its non-polar segment.

Size exclusion chromatography (SEC): Also known as gel permeation chromatography or gel filtration chromatography, is a type of chromatography that primarily uses size to separate particles. Also, it is helpful for figuring out the quaternary and tertiary structures of proteins and amino acids. This method is frequently used to determine the molecular weight of polysaccharides.

Ion exchange chromatography: The attraction between solute ions and charged sites bound to the stationary phase drives retention in ion-exchange chromatography. Same-charge ions are not included. This type of chromatography is frequently employed in the purification of water, ligand-exchange chromatography, protein ion-exchange chromatography, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides, among other applications.[3,4]

Bio-affinity chromatography: Separation based on a particular, reversible interaction between ligands and proteins. A bio-affinity matrix has ligands covalently bonded to a solid support that holds onto proteins that interact with the ligands connected to the column. A bio affinity column can elute proteins bound to it in one of two ways:

- A specific elution: change in pH, salt, etc. which weakens interaction protein with column-bound substrate.
- Biospecific elution: inclusion of free ligand in elution buffer which competes with column bound ligand.
- Because of specificity of the interaction, bio affinity chromatography can result in very high purification in a single step (10 - 1000-fold).

Instrumentation: Basic instrumentation of HPLC as shown in figure 1.

a. **Solvent reservoir:** Mobile stage substances are contained in a glass reservoir. The versatile stage, or dissolvable, in HPLC is typically a blend of polar and non-polar liquid segments whose particular fixations are changed relying upon the arrangement of the specimen.

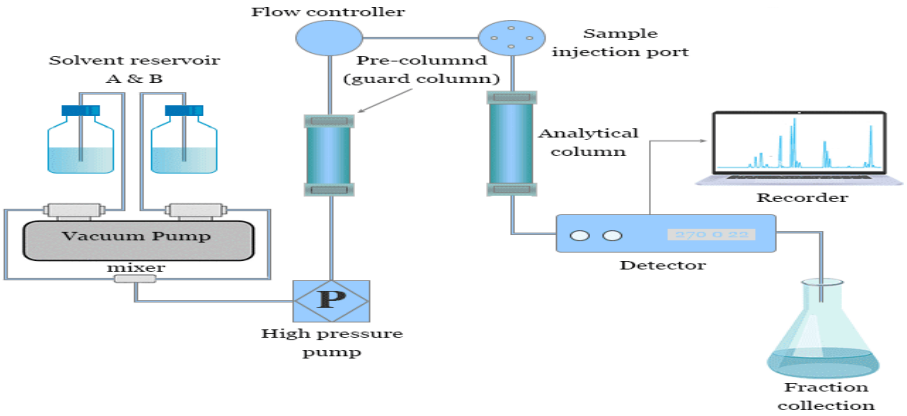

Fig. 1: Instrumentation - High Performance Liquid Chromatography (HPLC).

b. Pump: A pump suctions the versatile stage from the dissolvable reservoir and drives it through the framework's column and detector. Contingent upon various components including column measurements, molecule size of the stationary stage, the stream rate and synthesis of the versatile stage, working weights of up to 42000 kPa (around 6000 psi) can be created.

c. Sample Injector: The injector can be a solitary infusion or a mechanized infusion framework. An injector for a HPLC framework ought to give infusion of the liquid specimen inside the scope of 0.1–100 mL of volume with high reproducibility and under high weight (up to 4000 psi).

d. Columns: Columns are generally made of cleaned stainless steel, are in the vicinity of 50 and 300 mm long and have an inside distance across of in the vicinity of 2 and 5 mm. They are normally loaded with a stationary stage with a molecule size of 3–10 µm. Columns with interior distances across of under 2 mm are regularly alluded to as microbore HPLC columns. In a perfect world the temperature of the portable stage and the column ought to be kept steady amid an examination.

e. Detector: The HPLC indicator, situated toward the finish of the column distinguishes the analytes as they elute from the chromatographic column. Regularly utilized finders are UV spectroscopy, fluorescence, mass-spectrometric and electrochemical indicators.

f. Data collection devices: Signals from the indicator might be gathered on outline recorders or electronic integrators that differ in many-sided quality and in their capacity to process, store and reprocess chromatographic information. The PC coordinates the reaction of the identifier to every part and places it into a chromatograph that is anything but difficult to peruse and decipher.[28,29,30]

g. Degasser: The eluent used for LC analysis may contain gases such as oxygen that are nonvisible to our eyes. When gas is present in the eluent, this is detected as a noise and causes unstable baseline. Generally used method includes sparging (bubbling of inert gas), use of aspirator, distillation system, and/or heating and stirring. However, the method is not convenient and also when the solvent is left for a certain time period (e.g., during the long analysis), gas will dissolve back gradually. Degasser uses special polymer membrane tubing to remove gases. The numerous very small pores on the surface of the polymer tube allow the air to go through while preventing any liquid to go through the pore. By placing this tubing under low pressure container, it created pressure differences inside and outside the tubing (higher inside the tubing). This difference let the dissolved gas to move through the pores and remove the gas. Compared to classical batch type degassing, the degasser can be used online, it is more convenient and efficient. Many of new HPLC unit system contain a degasser.[31,32,33]

h. Column heater: The LC separation is often largely influenced by the column temperature. In order to obtain repeatable results, it is important to keep the consistent temperature conditions. Also, for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperature (50 to 80°C). It is also important to keep stable temperature to obtain repeatable results even it is analyzed at around room temperature. There are possibilities that small different of temperature causes different separation results. Thus, columns are generally kept inside the column oven (Column heater).

Applications of HPLC

The identity, quantification, and resolution of a compound are all pieces of information that can be discovered via HPLC. The isolation and purification of chemicals is known as preparative HPLC. This contrasts with analytical HPLC, where learning more about the sample substance is the primary goal. The following are the main applications:

Pharmaceuticals: In order to determine API and related compounds in a single run, high-performance liquid chromatography delivers dependable quantitative precision and accuracy in addition to a high linear dynamic range. The dispersion of samples in water or aqueous solutions that have been modified with acetonitrile or methanol is a practical method for solid dosage form manufacturing. There are numerous ways to separate chiral compounds into their individual enantiomers using HPLC. Precolumn derivatization to create diastereomers is one of them. You might also employ custom columns made with cyclodextrins or unique chiral moieties as stationary phases. To put it briefly, reverse phase HPLC the pharmaceutical industry most widely used option for quantitative analysis is HPLC.

Foods

In the area of food analysis, high-performance liquid chromatography has brought about a number of desired benefits. Food matrices are typically complex, making analyte extraction a difficult operation. The fact that both desired and unwanted components are frequently present in trace quantities further complicates issues, and traditional extraction and analysis do not offer the necessary levels of accuracy and precision. Due to the numerous stationary phase and mobile phase possibilities, HPLC provides workable solutions. Common applications in foods are;

- Residual pesticides such as 2, 4-D, and Monochrotophos.
- Fat-soluble vitamins (A, D, E, and K)

- Antioxidants such as TBHQ, BHA, and BHT.
- Residual antibiotics
- Water-soluble vitamins (B-complex vitamins such as B1, B2, B3, B6, Folic acid, Pantothenic acid, B12, Vitamin C)
- Sugars: Glucose, Fructose, Maltose, and other saccharides
- Mycotoxins such as Alfatoxins B1, B2, G1, G2, M1, M2, and ochratoxin
- Steroids and flavonoids
- Cholesterol and sterols
- Amino acids
- Aspartame and other artificial sweeteners.

Manufacturing

Both in the laboratory and in the field of clinical science, HPLC has several uses. Since it is a reliable method for obtaining and ensuring product purity, it is a common approach employed in pharmaceutical development. Despite the fact that HPLC can create products of incredibly high quality (purity), it isn't always the main technique employed in the manufacturing of bulk medicinal ingredients. Only 15.5% of syntheses, according to the European Pharmacopeia, use HPLC. In contrast, it's involved in 44% of the syntheses in the US Pharmacopeia. Given that HPLC can be an expensive procedure when used on a big scale, this might be the result of different time and financial restrictions. Unfortunately, an increase in cost is correlated with an increase in HPLC's specificity, precision, and accuracy.

Research

Research can use similar techniques to find concentrations of prospective medicinal candidates like antifungal and asthma medications. This method certainly works well for observing several species in samples that have been gathered, but it is necessary to utilise standard solutions when trying to identify the species. Purity is crucial in this kind of research; hence it is employed as a way to verify the outcomes of synthesis reactions.

Medical

HPLC can be used for medication analysis in medicine, however nutritional analysis is more closely associated with this use. Blood serum is the sample used for the majority of medical HPLC tests, even though urine is the most used medium for assessing drug concentrations. HPLC has been compared against other approaches, especially immunoassays, for the detection of compounds useful for clinical studies. In one instance, the sensitivity of HPLC and competitive protein binding assays (CPBA) for the detection of vitamin D was evaluated. It was discovered that while this CPBA was helpful for identifying vitamin D deficiency in children, its sensitivity and specificity only reached 40% and 60%, respectively, of HPLC's capacity. HPLC is a costly tool, yet it has almost unmatched accuracy.[34-38]

Method development

Developing an HPLC method involves a series of steps to optimize separation, selectivity, and sensitivity for a given analyte. The key aspects of method development include:

- **Selection of mobile phase:** The choice of solvents, pH, and buffer composition significantly affects analyte retention and peak shape. A suitable mobile phase should ensure efficient separation while maintaining compatibility with the detector.
- **Selection of stationary phase:** The column type, particle size, and stationary phase material play a crucial role in achieving desired selectivity and resolution. Reverse-phase columns are widely used due to their versatility.
- **Optimization of chromatographic conditions:** Parameters such as flow rate, column temperature, and gradient elution must be fine-tuned to maximize efficiency and minimize run time.
- **Detection method:** The selection of an appropriate detector (UV-Vis, fluorescence, or mass spectrometry) depends on the analyte's properties and sensitivity requirements.
- **System suitability testing:** Ensuring reproducibility and efficiency by evaluating parameters such as resolution, tailing factor, theoretical plates, and repeatability before sample analysis.[39,40]

HPLC Method Validation

Method validation is essential to confirm that the analytical procedure is suitable for its intended use. The validation process, based on ICH Q2 (R1) guidelines, involves assessing the following parameters:

- **Accuracy:** Assessed by spiking known analyte concentrations into a matrix and measuring recovery.
- **Precision:** Evaluated at intra-day and inter-day levels through repeatability and reproducibility tests.
- **Specificity:** Ensures that the method can accurately identify the target analyte in the presence of excipients, degradation products, or other interfering substances.
- **Linearity and Range:** Demonstrated by constructing calibration curves over a range of analyte concentrations, confirming a proportional response.
- **Limit of Detection (LOD) and Limit of Quantification (LOQ):** Determine the smallest amount of analyte that can be reliably detected and quantified.
- **Robustness:** The ability of the method to remain unaffected by small variations in analytical parameters, such as changes in mobile phase composition or column temperature.
- **Ruggedness:** Evaluates method reproducibility under different conditions, including changes in analysts, instruments, and laboratories.[41]

Regulatory Aspects and ICH Guidelines

HPLC method validation is conducted in compliance with the International Council for Harmonisation (ICH) guidelines, particularly ICH Q2 (R1). Key aspects include:

- **ICH Q2 (R1) Guidelines:** Define validation parameters and criteria for analytical methods used in pharmaceutical applications.
- **Good Laboratory Practices (GLP) Compliance:** Ensures proper documentation, reproducibility, and reliability of HPLC data.
- **Pharmacopeial Standards:** Regulatory authorities such as the United States Pharmacopeia (USP), European Pharmacopeia (EP), and Indian Pharmacopeia (IP) provide standardized methods and validation criteria for HPLC.
- **Regulatory Submissions:** Proper method validation is required for new drug applications (NDAs), abbreviated new drug applications (ANDAs), and stability studies submitted to regulatory agencies such as the FDA and EMA.[42,43]

CONCLUSION

The primary medication and any reactive contaminants must be separated and quantified using the HPLC technique. The mobile phase in HPLC is a liquid. The most used kind of HPLC is reversed-phase HPLC. Reversed-phase refers to a situation in which the stationary phase is relatively non-polar and the movable phase is substantially polar. Consequently, compared to polar chemicals, non-polar compounds will be kept more and have longer retention durations. The stationary phase is relatively polar in normal phase HPLC, while the mobile phase is generally non-polar. Column packing, which involves various chemical and/or physical interactions between the components' molecules and the packing particles, keeps these parts apart from one another. At the departure of a column, a low-through device (detector) that measures their quantity detects these separated components. Principle-wise, LC and HPLC operate in a similar manner, but HPLC has far better speed, efficiency, sensitivity, and ease of use. The output from this detector is known as a "HPLC." Also, it is the most reliable analytical technique frequently used to assess the stability of drug goods and conduct quantitative and qualitative analyses of drug products.

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