HPLC Method Development and Validation: A Review

Nikita Shelke, Vidya Magar,Sanket Khedkar,Prashant Jadhav,Chetan Jain

Srinath College of Pharmacy, Chh. Sambhajinagar (Aurangabad), Maharashtra, India

Introduction

Analytical chemistry is the branch of chemistry that focuses on the quantitative and qualitative identification of constituents in substances, samples, and mixtures. There are two branches of analytical chemistry: quantitative and qualitative. Analytes of mixtures or samples are identified, and this process is known as qualitative analysis. Quantitative analysis involves counting the components or determining the analyte of a mixture or sample. Along with chemistry, other sciences, including biology, zoology, the arts (including painting and sculpture), archeology, space exploration, and medical testing, all require analytical data. Analytical chemistry finds significant applications in quality control in the manufacturing industry, biological and clinical research, pollution monitoring and control, fundamental and applied research, and geological assays.

The basic principle of instrumental methods of analysis is the use of instruments to measure a substance's physical properties. Determine the chemical composition. Compared with conventional methods, instrumental procedures are easy to use, precise, and reproducible. As a result, analytical techniques created using advanced equipment including spectrophotometers, HPLC, GC, and HPTLC have many uses in verifying both the quality and amount of raw materials and finished products.[1]

Chromatography:

A non-destructive method for separating a multi-component mixture of trace, minor, or major ingredients into its component fractions is chromatography. Gases, liquids, and solids can all be dependent on various variations. Chromatography is mainly used as a separation tool, while it can also be used quantitatively. Chromatography is a technique that applies equilibrium distribution between two phases to separate a mixture of components into distinct components. Chromatography is primarily dependent on the variations in the rates at which mixture

components pass through a porous material (also known as the stationary phase) when a solvent or gas (also known as the mobile phase) is involved.

The following steps generally included in the chromatographic method of separation:

1. A substance's adsorption or retention on the stationary phase.
2. The mobile phase separates the absorbed material.
3. The process known as elution involves recovering the separated materials through a continuous flow of mobile phase.
4. Analysis of the eluted compounds, both qualitative and quantitative.

Table 1 various types of chromatographic technique

Sr. no.	Techniques	Stationary phase	Mobile phase
1.	Column chromatography or Adsorption chromatography	Solid	Liquid
2.	Partition chromatography	Liquid	Liquid
3.	Paper chromatography	Liquid	Liquid
4.	Thin layer chromatography (TLC)	Liquid or Solid	Liquid
5.	Gas- liquid chromatography (GLC)	Liquid	Gas
6.	Gas – solid chromatography (GSC)	Solid	Gas
7.	Ion-exchange chromatography	Solid	Liquid

]

Table no. 2 Classification of Chromatographic Methods[2]

Stationary phase	Mobile phase	Name
Solid	Liquid	Plane chromatography
		Paper chromatography (PC)
		Thin layer chromatography (TLC)
		Adsorption column chromatography
		High Performance Liquid Chromatography (HPLC)
Solid (ion exchange resin)	Liquid	Ion exchange Chromatography (IEC)
Solid	Gas	Gas- solid Chromatography(GSC)
Solid matrix	Liquid	Gel Permeation chromatography (Exclusion chromatography) (GPC)
Liquid	Gas	Gas-Liquid chromatography (GLC)
Liquid	Liquid	Liquid - Liquid chromatography

High Performance Liquid Chromatography

One of analytical chemistry's most potent instruments nowadays is high performance liquid chromatography. Compounds present in any material that can dissolve in a liquid can be separated, identified, and quantified using this method. (HPLC) stands for high performance liquid chromatography is the most precise analytical techniques that are frequently applied to both quantitative and qualitative medicinal product analysis. [3] HPLC is an acronym for High performance liquid chromatography or High Pressure Liquid Chromatography. The fundamental principle is to inject a sample solution into a porous material column (the stationary phase), and then pump a liquid (the mobile phase) through the column at high pressure. Based on variations in the rates of migration across the column resulting from distinct sample partitions between the stationary and mobile phases, the sample is separated. Elution occurs at different times based on the partition behaviour of various components.

The precision of HPLC results from nuanced component behaviours during partitioning, providing a reliable technique for examining many different kinds of samples in industries such as analytical chemistry and pharmaceuticals. Longer distances are covered and faster travel by chemical compounds with a lowered affinity

for the stationary phase, whereas a compound with Higher affinity moves slower and covers a shorter distance . Effective separation is made possible by this differential migration, and examination of the components of the sample. Pharmaceutical analysis benefits greatly from HPLC's ability to effectively isolate and quantify important drugs and reactions, degradation agents, synthesis intermediates, and it's contaminants. HPLC is a leading analytical instrument that is excellent in detecting, quantifying, and isolating the various liquid-soluble components of the sample. Its accuracy is important for both qualitative and quantitative research, drug product analysis, which is essential for figuring out how stable a medication product is. By providing a systematic approach to HPLC is a crucial method for describing pharmaceutical samples and guaranteeing the safety and quality of analytical chemistry and related pharmaceutical formulations.[4] The most common solvents used in HPLC are N-hexane, methylene chloride, chloroform, methyl-t-butyl ether, tetrahydrofuran (THF), isopropanol (IPA), acetonitrile (MeCN or CAN), methanol (MeOH), and water.[5]

HPLC Principle:

A column made of stainless steel that contains stationary phase particles with a diameter of 3–10 μm is pumped with a liquid mobile phase under pressure. The analyte is loaded onto the column head through a loop valve, and the mixture is separated based on how long each component spent in the stationary phase. It should be mentioned that every component in a mixture must spend roughly the same amount of time in the mobile phase before exiting the column. There are several detectors that can be used to monitor the column effluent.

Types of HPLC:

HPLC is classified as follows

1. **Based on a scale of operation**

    1. Preparative HPLC
    2. Analytical HPLC

2. **Based on the principle of separation**

1. Affinity chromatography
2. Adsorption chromatography
3. Size exclusion chromatography
4. Ion- exchange chromatography
5. Chiral phase chromatography

3. **Based on the elution technique**
    1. Gradient separation
    2. Isocratic separation

4. **Based on modes of operation**

1. Normal phase chromatography
2. Reverse-phase chromatography

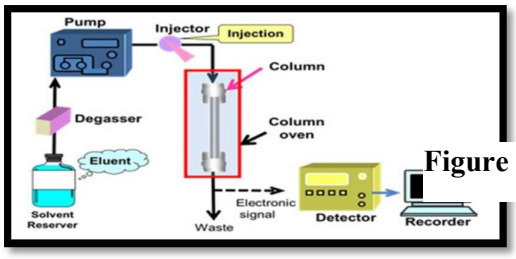

Figure 1 Instrumentation of HPLC

Normal Phase Chromatography

The mobile phase in normal phase chromatography is nonpolar, while the stationary phase is polar. As a result, the polar analyte will remain in the stationary phase. The solute molecules' increased polarity, which increases adsorption capacity, results in a longer elution time. This chromatography uses chemically modified silica (cyanopropyl, aminopropyl, and diol) as a stationary phase. For example, Typically, a column has an inner diameter of roughly 4.6 mm and a length of 150 to 250 mm. The polar chemicals will stick to the polar silica longer than the non-polar ones when the mixture moves through the column. Consequently, the non-polar ones will move quickly through the column.

Reverse-phase chromatography

The stationary phase in RP-HPLC is non-polar, while the mobile phase is either polar or somewhat polar. The concepts of hydrophobic interaction is the core concept of RP-HPLC. Over an extended period of time, analytes in the nonpolar stationary phase will be significantly less polar than those that are significantly more polar in a mixture of components. Thus, the component that is more polar elutes first.[6]

INSTRUMENTATION

Components of the HPLC system:

1. Solvent reservoir, mixing system, and degassing system
2. High pressure pump
3. Sample injector
4. Column
5. Detector
6. Data Recording System

Solvent reservoir, mixing system, and degassing system

The solvent reservoir (mobile phase) is where the solvent is stored. These are colorless containers composed of stainless steel or glass. The most common kind of solvent reservoir is a glass container. The pump's functions include supplying the mobile phase and precisely combining solvents. Both high-pressure and low-pressure mixing are mixing units of the two varieties. Air bubbles trapped in the solution are removed by the degassing mechanism. Ultrasonication and filtration methods are used to achieve degassing.[7]

Figure 2 Solvent Reservoir

High pressure pump

Every HPLC system has at least one pump to push the mobile phase through, and these

are reasonably compactly packed. Depending on the flow rate applied to the mobile phase, its viscosity, and the size of the stationary phase particles, this causes a pressure increase at the injector that can reach 20,000 kPa (200 bars). Even when the mobile phase's composition changes, pumps are made to maintain a steady flow rate without pulsing. In order to prevent flow rate interruptions, these flow rate-metered pumps often have two pistons in series that operate in opposition to one another.

A liquid (known as the mobile phase) must be pressured through the liquid chromatography system at a precise flow rate, measured in milliliters per minute (mL/min), by the pump. In HPLC, typical flow rates in between 1 and 2 mL/min. Normal pumps are able to reach pressures between 6000 and 9000 psi (400 and 600 bar). Variations in the mobile phase flow rates impact the elution time of sample components and lead to errors during the chromatographic experiment. A pump can deliver a constant mobile phase composition (isocratic) or an increasing mobile phase composition (gradient). Under constant pressure, pumps supply the column with a steady flow of mobile phase.

There are three type of pumps used commonly

1. Syringe type pumps,
2. Constant pressure pumps,
3. Reciprocating piston pumps.

1) **Syringe type pumps**

Small bore columns can use it. A motorized screw arrangement supplies the column with a steady flow rate. By changing the motor's voltage, the solvent delivery rate is adjusted. The main drawbacks of these pumps are their limited solvent capacity and their restriction on gradient operation, but they provide pulse-less flow regardless of column back pressure and viscosity variations.

2) Constant pressure pumps

This uses pressure from a gas cylinder to provide a consistent, continuous flow rate through the column. The solvent chamber can be quickly refilled similar to the valve arrangement. To produce high liquid pressures, a low-pressure gas source is required.

3) Reciprocating piston pumps

These use a piston in a hydraulic chamber that reciprocates to deliver the solvent or solvents. The solvent is derived in on the backstroke and sent to the column on the forward stroke. By varying the piston displacement in each stroke, flow rates can be adjusted. The identical piston chamber units that make up dual and triple head pistons function at 1800 or 1200 phase differences. Reciprocal pump systems have smooth solvent delivery because one pump is in the delivery cycle and the other is in the filling cycle. Both gradient operation and high-pressure output are achievable at a steady flow rate. However, in order to further eliminate pressure pulses, pulse dampening is necessary.

Sample Injector

An automated injection system or a single injection can serve as the injector. An HPLC system's injector should be able to inject a liquid sample at high pressure (up to 4000 psi) and with high reproducibility within the volume range of 0.1-100 mL. The high pressures in the liquid system must also be tolerated by the injector. When manual injection is impractical or the user has a large number of samples to analyze, an autosampler is an automatic version. Samples are continuously injected into the mobile phase stream using injectors. Maintaining a high degree of accuracy requires injection inertness and reproducibility.

The type of injector

1. Manual injection (Rheodyne/Valco injectors)
2. Automatic injection

Column

Columns typically measure between 50 and 300 mm in length, have an internal diameter of 2 to 5 mm, and are composed of polished stainless steel. Usually, a stationary phase with particles ranging in size from 3 to 10 μm filled them. Microbore columns are frequently defined as columns having internal diameters of less than 2 mm.

Types of columns

1. Guard columns
2. Analytical columns

Figure 3 Guard Column

Guard Column

By eliminating contaminants and particulate matter from the solvents as well as sample components that bind irreversibly to the stationary phase, a guard column is placed before the analytical column to extend its lifespan. To reduce solvent losses from the analytical column, the guard column saturates the mobile phase with the stationary phase.

Analytical Column

Figure 4 Analytical Column

High-performance liquid chromatography revolves around it. The length of liquid-chromatographic columns is between 10 and 30 cm. Typically, the columns are straight, but when necessary, they can be made longer by joining two or more of each other. The most typical packing particle size is 5 or 10 m, and the inside diameter of liquid columns is typically 4 to 10 mm. At the moment, the most widely used column is 25 cm long, 4.6 mm in diameter, and filled with 5 mm particles. These columns have between 40,000 and 60,000 plates per meter.

Detector

Analytes elute from the chromatographic column and are detected by the HPLC detector, which is at the end of the column. Evaporative light scattering, UV

spectroscopy, fluorescence, mass spectrometry, and electrochemical detectors are frequently employed. The individual molecules that emerge (elute) from the column can be seen (detected) by the detector. The chemist can quantitatively examine the sample components by using a detector to measure the quantity of those molecules. The liquid chromatogram, also known as the graph of the detector response, is produced by the detector's output to a recorder or computer. For the components that the column separates, a detector provides a particular response in addition to the necessary sensitivity. It must remain unaffected by modifications in the composition of the mobile phase.

Types of detector

1. Evaporative Light Scattering (ELSDs)
2. Refractive index Detector
3. U.V detector
4. Flourescence Detector
5. Electrochemical Detector
6. Mass Spectroscopy Detector
7. IR Detector

Data Recording System

Chart recorders and electronic integrators, which differ in complexity and capacity to process, store, and reprocess chromatographic data, can be used to gather signals from the detector. The detector's response to each component is combined by the computer and displayed in an easily readable and interpretable chromatograph. The computer, often referred to as the data system, not only manages every module of the HPLC instrument but also uses the signal from the detector to calculate the quantity of sample (quantitative analysis) and the time of elution (retention time) of the sample components (qualitative analysis).[8]

Method Development

Development and validation of analytical methods are important to drug discovery, development, and manufacturing. The identity, potency, purity, and performance of pharmaceutical products are assured by these techniques. There are a number of factors to consider about when developed method. They initially collect data

regarding the physiochemical characteristics of the analyte (pKa, log P, and solubility) and determine which detection method would be desirable for evaluation in the event of UV detection. A stability-indicating HPLC-method validation occupies most of the analytical development effort. The HPLC method involves separating and quantify the main active chemical, any reaction impurities, and all synthetic inter-mediators and any degradation agents.

Steps involve in method Development

- Understanding the Drug Molecule's Physicochemical Properties
- Chromatographic condition selection
- Developing the analytical methodology
- Sample preparation
- Optimization of the method[9]

Understanding the Drug Molecule's Physicochemical Properties

For method development it is very important to study the physical properties of drug molecules such as pH, pka , polarity and solubility

Polarity

The polarity of compound helps to decide the solvent composition of mobile phase.

Solubility

Solubility helps explain polarity in molecules. Non-polar solvent such as benzene do not interact with polar solvents like water. Generally, like dissolves like. Materials with similar polarity are soluble in one another. The diluents are chosen base on their solubility of analytes.analytes must be soluble in the diluents.must not have a tendency to react with other components.

pH and PKA:

When developing an HPLC technique, the values of pH and PKa are crucial. Generally, a substance’s pH value determines how basic or acidic it is. For ionisable analytes, an appropriate pH setting frequently results in symmetrical and sharp peaks since these features are required. In quantitative analysis to achieve a low threshold for detection, Minimal relative standard deviations among injections, as well as repeatable periods of retention. The pKa is a property of a specific compound that indicates how easily the compound donates a proton.

Chromatographic condition selection

To produce the sample's initial "scouting" chromatograms, a combination of beginning conditions (detector, column, and mobile phase) are selected early in the technique development process. These are usually built on C18 columns that have reversed phase separations and UV sensors. The choice between using an isocratic approach or a gradient method must now be made.

Column selection

The initial and most important step when developing a technique is selecting the stationary phase or column. Establishing an accurate and repeatable approach is impossible without a solid, high-performing column. Columns must be stable and repeatable to avoid problems caused by irreproducible sample retention during technique development. A C8 or C18 column developed specifically for the separation of basic compounds is the best selection for a large number of samples. The silica used to make these columns is extremely pure and less acidic.The most important ones are bonded stationary phase properties, silica substrate properties, and column diameters. Due to its various physical features, silica-based packing is prefferred in the majority of HPLC columns manufactured today. The three primary components of an HPLC column are the matrix, stationary phase, and hardware.

Chromatographic mode selection

The chromatographic modes are dictated by the molecular weight and polarity of the analyte. All case studies will center on reversed-phase chromatography (RPC), as it is the most popular technique for tiny organic compounds. To keep the analytes from ionizing, ion-pairing reagents or buffered mobile phases are frequently used in RPC to separate ionizable compounds (bases and acids).

Selecting a Buffer

Acetate, potassium phosphate, sodium phosphate, and other buffers were examined for overall chromatographic performance and system compatibility factors.

Mobile Phase Selection

The mobile phase affects efficiency, resolution, and selectivity. The strength of the solvent, or the composition of the mobile phase, plays an important role in RP-HPLC separation.

In a normal phase system, hexane and other nonpolar solvents or iso-octane While polar solvents like methanol, acetonitrile, or water are utilised in reverse phase chromatography

Selecting the detectors

The core part of the HPLC system is the detector. Which detector should be used depends on a number of factors, including the chemical composition of the study, potential interference, the required detection limit, and the availability and/or cost of the detector. Liquid chromatography (LC) uses commercial detectors such as mass spectrometry (MS), UV, fluorescence, electrochemical, and refractive index (RI). The detector used depends on the sample and the goal of the analysis.

Developing the analytical methodology

Selecting different chromatographic parameters, such as the mobile phase, column, flow rate, and pH of the mobile phase, is the first step in developing the analytical method on RP-HPLC. The system suitability parameters are taken into consideration after all of these parameters have been chosen based on trials.

Sample preparation

The active component in the drug under analysis needs to be stable in a diluent. The diluent should be maintained in the amber-colored flask during the first phase of method development until The active ingredient is shown to be stable at room temperature and unaffected by typical laboratory conditions. To remove particles, the solution should be filtered through 0.22 or 0.45 μm pore-size filter paper, which is typically recommended. Filtration is an analytical precautionary maintenance technique. [10,11].

Optimization of the method

Determine the "weaknesses" of the approach and use experimental design to improve it. Understand the performance of the method using different samples, instrument variations, and conditions.[12,13]

Method Validation

The FDA (Food and Drug Administration) states that validation is a production and process system of control developed to ensure the identity, strength, quality, and purity of drug products. FDA guidelines in May 1987 state that the validation package must include the information and test procedures that are required to verify that the procedure and system fulfill the criteria that are required.[14]

Parameters of Validation

1. Accuracy
2. Precision
   i) Repeatability
   ii) Intermediate precision
   iii) Reproducibility
3. Linearity
4. Range
5. Limit of detection
6. Limit of Quantitation
7. Specificity/ Selectivity
8. System suitability Studies
9. Robustness
10. Ruggedness

1) Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

2) Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

a) **Repeatability**

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision .

b) Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

c) Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology)

3) Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

4) Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

5) Limit of Detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

6) Limit of Quantitation

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays

for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

7) **Specificity**

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

The implications of this definition are as follows:

Identification: to ensure the identity of an analyte.

Purity Tests: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.

Assay (content or potency): to provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

8) **System Suitability Studies**

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. See Pharmacopoeias for additional information.

9) **Robustness**

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage[15]

10) **Ruggedness**

Ruggedness is a measure of test results' ability to be replicated under settings that differ from one lab to another and between analysts. The degree of reliability of test obtained results by analyzing the same samples under various conditions such as different laboratories, analysts, instruments, reagents, temperature, time, etc. is known as the reproducibility of an analytical process.[16]

References:

1. Azim Md. Sabir, Mitra Moloy, Bhasin Parminder S. HPLC method development and validation: A review. Int. Res. J. Pharm. 2013; 4(4):39-46.
2. 2Gurdeep R. Chatwal, Sham K. Anand Instrumental Method of Chemical Analysis pg. no. 2.566- 2.567.
3. Rao BV, Sowjanya G. N., Ajitha A, Rao Uma MV. A review on stability indicating HPLC method development,World journal of pharmacy and pharmaceutical sciences.2015; 4(8):405-423.
4. Vishal Nilkant Chavan, Dr. Hemant V. Kamble and Prof. Santosh A. Waghmare HPLC Method development and validation : A Review IJARIIE 10 (3) : 5569-5580.
5. Mc Polin . An introduction to HPLC for pharmaceutical analysis. Lulu. 2009.
6. Vaishnavi R. Mankar, Dhirendra B. Sanghai, Arpita R. Yeotkar, Pranita A. Thakre, Hanuman T. Borkar A REVIEW ON HPLC METHOD DEVELOPMENT AND VALIDATION International Research Journal of Modernization in Engineering Technology and Science, 2024 06(04): 11816-11822.
7. Jena AK. HPLC: highly accessible instrument in pharmaceutical industry for effective method development.
8. Pharm Anal Acta 2011; 3.
9. Ali AH. High-Performance Liquid Chromatography (HPLC): A review. Ann Adv Chem. 2022; 6: 010-020.
10. Mayer ML (1996) Selecting filters for chromatographic applications. LC GC 14(10): 902-905.
11. Mayer M (1997) Filtration Preventative maintenance for HPLC. American laboratory Fairfield 29(1): 34-37.
12. Kayode J, Adebayo. Effective HPLC method development. Journal of Health, Medicine and Nursing. 2015; 12: 123-133.

13. R. D. Chakole et al. Ijppr. Human, 2021; Vol. 21 (4): 66-82.
14. Jaha S. M., Validation in Pharmaceutical Industry: Cleaning Validation - A Brief, Vol. 5, Issue 1, January 2017.
15. ICH Harmonized tripartite guidelines, Validation of Analytical Procedures': Text and Methodology, Q2 (R1), current step 4 version; 27 Oct 1994.
16. Lavanya G, Sunil M, Eswarudu MM, Eswaraiah MC, Harisudha K and Spandana BN: Analytical Method Validation: An Updated Review. Int J Pharm Sci Res 2013; 4(4); 1280-1286.