



**INTERNATIONAL JOURNAL OF
PHARMACEUTICAL SCIENCES**
[ISSN: 0975-4725; CODEN(USA): IJPS00]
Journal Homepage: <https://www.ijpsjournal.com>



Review Article

An Overview HPLC Method Development and Validation

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ARTICLE INFO

Published: 19 Nov 2025

Keywords:

High performance liquid chromatography (HPLC), Method Development, Method Validation, Instrumentation, Limit of Detection, Limit of Quantification, (LOD, LOQ) Application

DOI:

10.5281/zenodo.17650743

ABSTRACT

High-Performance Liquid Chromatography (HPLC) is presented as a pivotal analytical technique for the qualitative and quantitative assessment of chemical compounds, particularly within pharmaceutical, environmental, clinical, and forensic contexts. The document details HPLC's operational principles, benefits, and limitations, as well as the distinct types—normal phase, reversed phase, size exclusion, and ion exchange chromatography. Key aspects of instrumentation, including mobile and stationary phases, sample injection, and detection systems, are explained. The process of method development covers considerations for chromatographic conditions, sample preparation, buffer selection, and optimization strategies. Comprehensive coverage of method validation is provided, aligning with international guidelines (ICH, USP, FDA), and including specificity, linearity, precision, accuracy, solution stability, detection limits, robustness, and system suitability. This resource serves as a complete guide for practical implementation and validation of HPLC methods in diverse analytical applications.

INTRODUCTION

High-performance liquid chromatography (HPLC) stands as a powerful analytical tool in modern chemistry. It excels at identifying, measuring, and separating components within liquid-dissolved samples. Widely employed in pharmacological product analysis, HPLC is prized for its precision in both quantitative and qualitative assessments, contributing significantly to advancements in analytical chemistry.(1) High performance liquid

chromatography (HPLC), which is widely used for the quantitative and qualitative analysis of drug products as well as for determining drug product stability, is the most precise method of analysis.(2) Compared to gas chromatography, High Performance Liquid Chromatography is more adaptable.(3)

❖ The technique of HPLC has following features.

1. Superior resolution
2. Small diameter, Stainless steel, Glass column

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Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



3. Rapid analysis
4. comparatively higher pressure in the mobile phase
5. controlled mobile phase flow rate.

Principal

The plan is to inject a sample solution into a porous material column and pump a liquid (the stationary phase) through the column at high pressure. The basis for sample separation is the variation in migration rates through the column resulting from the sample's partitioning into the stationary and mobile phases. At, elution takes place. different times based on how various components behave during partitioning.

ADVANTAGES

HPLC has numerous advantages over other chromatographic ways. It has made significant donation to the growth of logical wisdom and its different operation in medicinals, environmental, forensics, foods, polymers and plastics, clinical fields etc.

1. For a wide range of grueling samples, HPLC offers an extremely specific, nicely precise, and fairly quick logical system.
2. Macromolecules can be dealt with using the HPLC system.
3. It's profoundly suitable for utmost ' pharmaceutical medicine substances'.
4. HPLC allows the reliable analysis of a good number of products, including metabolic, biochemical, and non- unpredictable substance, polar composites etc.
5. The sample's medication and donation are simple in HPLC.
6. Both the separation speed and emulsion resolution are high. 8. HPLC software is able of reporting precise and accurate results.

7. The sensors that are used are veritably sensitive.
8. HPLC coupled with mass spectrophotometers and FT- IR system have bettered efficacy.

DISADVANTAGES

1. Complexity of separation of certain antibodies specific to the protein.
2. The cost of developing an HPLC apparatus for assay or method of separation of individual components is tremendous.
3. Due to the speed of the HPLC and its reliance on the different polarities; two compounds with similar structure and polarities can exit the chromatographic apparatus at the same time (coelution). This is difficult in detecting compounds.
4. a lack of sensitivity to some compounds toward column stationary phase is challenging.
5. Some compounds are absorbed or react with the chemicals in the column's packing materials.
6. The column may not be able to separate or withstand pressures that are either too high or too low at certain points.
7. Qualitative analysis may be limited unless HPLC is used mass spectrometry as an interface. (4)

❖ Application

Identification, quantification, and resolution of compound are all examples of the information that can be gleaned from HPLC. The procedure of isolating and purifying compounds is referred to as preparative HPLC. This is in contrast to analytical HPLC, where information about the sample compound is the primary goal.

Chemical Separation : The foundation of it is because different compounds migrate at different rates in different columns and mobile phases, the



choice of stationary phase and mobile phase most often determines the degree of separation.

Purification : The term "purification" refers to the or contaminants, the process of separating the target compound from a mixture of compounds or extracting it showed a distinctive peak for each compound under specific chromatographic conditions. In order for the pure desired compound to be collected or extracted without introducing any other undesirable compound, the migration of the compounds and contaminants through the column must differ sufficiently.

Identification : Typically, HPLC is used to analyze the compounds in the assay. This assay's parameters should be set so that the chromatograph can see a clear peak of the known sample. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels which the assay will be performed.(5)

Other applications of HPLC : Other applications of HPLC includes

Pharmaceutical applications (6-7)

- Study of how pharmaceutical dosage forms break down.
- Estimates of pharmaceutical dosage forms' shelf lives .
- The active components have been identified.
- pharmaceutical quality control.

Environmental application (8-9)

- Phenolic compounds found in water for drinking.
- Sedimented samples contained diphenhydramine.

- Body monitoring of pollutants.

Types of HPLC

The phase system used determines which High-Performance Liquid Chromatography (HPLC) method is used for analysis. Analytes are categorized according to their polarity using normal phase chromatography (NP-HPLC) or normal phase HPLC. An interaction between a non-polar mobile phase and a polar stationary phase retains polar analytes in NPHPLC. This interaction causes an increase in elution time with increasing polarity of the analyte. NP-HPLC provides effective separation, revealing insights into sample composition based on polarity, making it a valuable analytical tool, particularly in characterizing compounds with different polarities in diverse fields such as chemistry, pharmaceuticals, and environmental analysis.(1)

1. Normal Phase chromatography
2. Reversed Phase chromatography
3. Size exclusive chromatography
4. Ion exchange chromatography

Normal Phase chromatography

In normal phase chromatography, the stationary phase is polar, while the mobile phase is non-polar. As a result, the polar analyte remains in the station phase. Due to a longer elution time, the polarity of solute molecules enhances their ability to adsorb. This is due to the stationary phase's lower affinity for non-polar compounds. The mixture's polar compound is moving through the column. These methods can be used to identify and separate people, but they are rarely used in pharmaceutical applications.(10)

Reversed Phase chromatography



Utilizing an aqueous, moderately polar mobile phase and a non-polar stationary phase, reversed phase chromatography (RP-HPLC or RPC) phase of inactivity Hydrophobic interactions, which are brought about by repulsive forces exerted by two or more non-polar stationary phase, a relatively non-polar analyte, and polar eluent. The area of contact surrounding the non- polar section of the analyte molecule in the aqueous eluent after association with the ligand is proportional to the analyte's binds to the phase that is stationary.(11)

Size exclusive chromatography

It separates molecules according to their molecular mass. Largest molecules are eluted first and the smallest molecules last. This method is generally used when a mixture contains compounds with a molecular mass difference of at least 10%. This mode can be further subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solvents).(12)

Ion exchange chromatography

In exchange of ions retention is based on the attraction in IC chromatography. between charged

sites attached to the stationary and solute ions phase. Solute ions with the same charge as the sites that are charged on the column are not bound, but solute ions are. the column's charged sites are of the opposing charge. kept in place on the column. Ions of solute that remain on the By altering the solvent, the column can be eluted from the column. conditions (such as increasing the solvent system's ion effect) by increasing the solution's salt concentration, increasing the solvent's pH, changing the temperature of the column, and so on...) (13)

Instrumentation Of HPLC

In High-Performance Liquid Chromatography (HPLC), analytical separation involves the high pressure flow of liquid through a stationary phase that can be either liquid (L) or solid (LSC/LLC). Injected compounds separate as they travel through the column, and electronic detection takes place as they clute at the column's end. Even though HPLC is less adaptable than Gas Chromatography, it has a wider range of mobile and stationary phases, making it more useful for precise compound analysis in biochemistry and pharmaceuticals.(14)

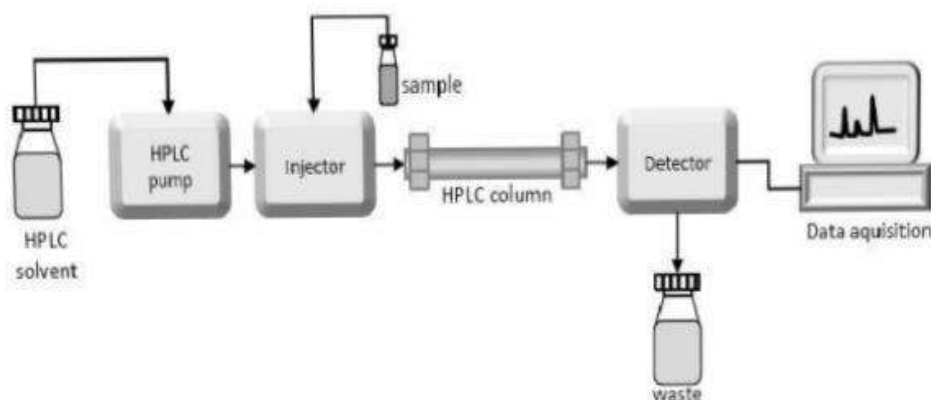


Figure No.1 Flow Diagram of HPLC Instrumentation

The parts of the HPLC system are:

1. Mixing system, solvent reservoir, and degassing system
2. Pump with high pressure
3. Sample injector
4. Column
5. Detector
6. Data Recording System

❖ Mixing system, solvent reservoir, and degassing system

The solvent is kept in the solvent reservoir, also known as the mobile phase. These are stainless steel or glass containers that do not change color. Glass bottles are the most prevalent type of solvent reservoir. Aside from delivering mobile phase, the pump must also combine solvents with extreme accuracy and precision. Mixing at low pressure and high pressure are the two distinct varieties of mixing unit. Air bubbles that have become stuck in the solution are removed by the degassing mechanism. Ultrasonication and filtration are two methods of degassing.(11)

❖ Pump with high pressure

A high-pressure pump also known as a solvent delivery system manager is used to generate and meter a specified flow rate of the mobile phase, usually in milliliters per minute. The pump forces and suctions the mobile phase into the solvent reservoir; it goes to the column and then the detector. The procedure pressure depends on column dimensions, particle size, flow rate and mobile phase's composition. Normal flow rates in HPLC range from 1 to 2 ml/min. Standard pumps can reach pressures between 6000 and 9000 psi (400 and 600 bar).(15)

❖ Sample Injection

There are septum injectors; which sample solution should they be used with? is administered. An injector, also known as an autosampler or sample manager, capable of injecting the sample continuously into the mobile phase stream that transports the sample into the Column for HPLC. A brand-new, cutting-edge rotary valve and loop. With the injector, you can get results that are repeatable. Typical The sizes of the sample are 5 to 20 microliters (l).(15)

❖ Column

HPLC columns are packed with very fine particles (usually a few microns in diameter). To achieve, the extremely fine particles are required. the low dispersion that enables modern plate counts to be high HPLC. It is possible to count more than 25,000 plates per column. with modern columns, however, these very high efficiencies are very rare in actual samples because of the dispersion that comes with data acquisition systems, injection valves, and the dispersion because real samples have a higher molecular weight than the common samples for testing. Introducing these minute particles to the column is a challenging technical issue, even with good packing, a great amount of care needs to be taken of the inlet and the fittings at the column ends. and an outlet connection to minimize dispersion.(16)

❖ Detector

This is a tool for continuously registering particular chemical or physical properties of the column discharge. The most widely used detector in pharmaceutical analysis is UV (ultraviolet), which allows observing and continuously recording the UV absorbance at a particular wavelength or across a range of wavelengths (the detection by diode arrays). The manner in which



The The analyte in the detector flow cell is what alters the absorbance. If the analyte absorbs greater than the a positive signal is obtained from the background (mobile phase).(17)

❖ Data Recording System

The outcomes are depicted as a series of high points, and the computer that is connected to the display calculates the space under each high point automatically.(5)

Method Of Development

Analytical method development and validation play important roles. in the discovery development and manufacture of pharmaceuticals. These methods used to ensure the identity, purity, potency, & performance of drug products. There are many factors to consider when developing methods. The initially collect the information about the analyte's physiochemical properties (pKa, log P. solubility) and determining which mode of detection would be suitable for analysis in case of UV detection.(18-19) The majority of the analytical Validating a stability requires

development effort. describing the HPLC method. The purpose of the HPLC method is to quantify the primary active drug separately, any impurities from reactions, as well as any synthetic intermediate and any degradants.

The method involves steps. development are:

1. calibrated and qualified instrument
2. outlined procedures
3. Reliable standards of reference
4. specialized analysts
5. Integrity and selection of the samples
6. The analysis ought to take as little time as possible and should be economical.
7. The analyst's accuracy must accept the guidelines of pharmacopoeia.
8. The chosen method should be precise and elective.
9. Configure the HPLC conditions.
10. Preparation of the method's sample solution development.
10. Method optimisation.
11. Development and validation of method (20)

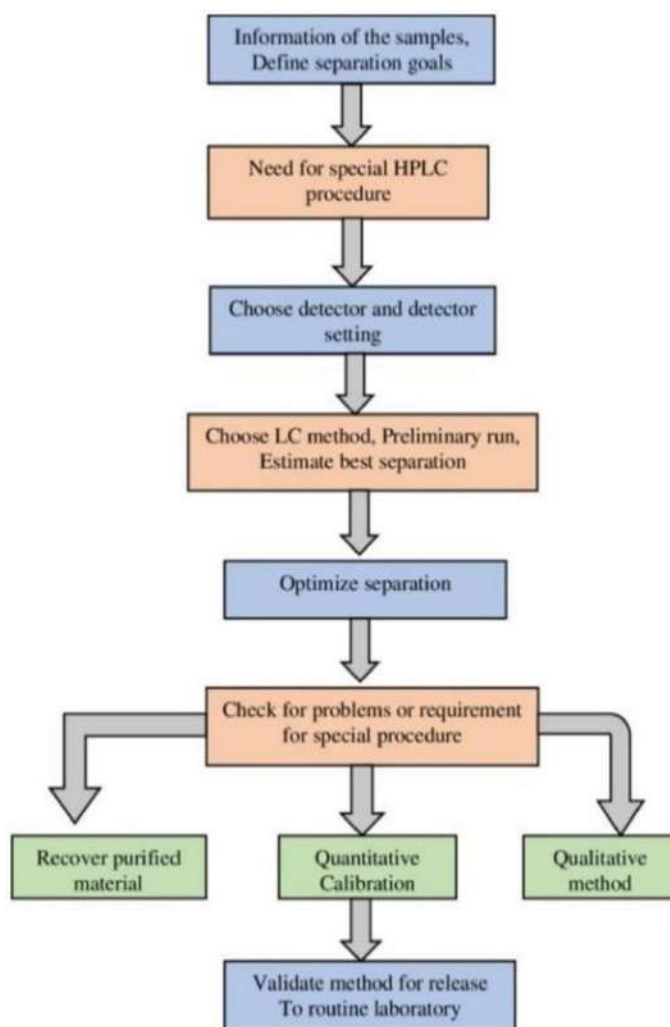


Figure 2. Steps involved in HPLC Method Development Steps involved in Method Development are (21)

- Knowing the physical and chemical properties of the drug molecule
- Set up of chromatographic condition.
- Developing the approach of analysis.
- Sample preparation
- Method Optimization
- Validation

Knowing the physical and chemical properties of the drug molecule

Physiochemical properties of a drug molecule play an important role in method development. For the method development one has to study the physical properties like solubility, polarity, pKa and pH of the drug molecule. Polarity is a physical property of a compound. It helps an analyst, to decide the solvent and composition of the mobile phase. The solubility of molecules can be explained on the basis of the polarity of molecules. Polar, such as water, and nonpolar, e.g. benzene, solvents do not mix. In general, materials of the same polarity dissolve one another, so soluble in each other. The solubility of the analyte is taken into consideration when selecting a mobile phase or diluent. The chemical must not react with any of its constituents and must be soluble in diluents. pH and pKa play

an important role in Method development for HPLC. The negative of the logarithm to base 10 of the pH value is the the amount of the hydrogen ion. $[H_3O^+] = -\log_{10} pH$. When an appropriate pH is chosen for ionizable analytes, HPLC frequently exhibits symmetrical and sharp peaks. Sharp, In quantitative analysis, symmetrical peaks are required to attain low relative and low detection limits. reproducible retention times and standard deviations between injections (22-23)

Set up of chromatographic conditions

A set of initial conditions (detector, column, mobile) were established during the initial method development. phase) is chosen to produce the initial "scouting" chromatograms of the sample. In most instances, On a C18 column equipped with UV detection, reversed phase separations are used. At this point, a It is necessary to choose between developing an isocratic or a gradient method. (24)

• Buffer Selection

The desired pH determines which buffer to use. The typical pH range for reversed phase on silica based packing is pH 2 to 8. Since the buffer must have a pKa that is close to the desired pH, buffer has the highest pKa for pH control. Choosing a buffer with a pKa value less than 2 units of the desired mobile is a rule. pH of a phase.

• General considerations for selecting a buffer:

1. Phosphate is more soluble in methanol/water than in acetonitrile/water or THF/water.
2. Some salt buffers are hygroscopic, which may alter the chromatography and result in more tailing. of basic compounds and possibly differences in selectivity.
3. Ammonium salts are generally more soluble in organic/water mobile phases.

4. With time, trifluoroacetic acid can degrade. It is volatile and absorbs at low UV wavelengths.
5. In buffered mobile phases with little or no organic modifier, microbial growth can occur quickly. The growth accumulates on column inlets and can damage chromatographic performance.
6. Phosphate buffer accelerates the dissolution of silica at a pH higher than 7, significantly reducing its lifetime. of HPLC columns based on silica. Organic buffers with a pH greater than 7 should be used whenever possible.
7. Ammonium bicarbonate buffers usually are prone to pH changes and are usually stable for only 24-48 hours. The pH of this mobile phase tends to become more basic due to to the release of carbon dioxide.
8. A 0.2 micrometer filter should be used to filter the buffers after they have been prepared.
9. Degassing the mobile phase is required.(25)

Buffer Concentration

Generally, a buffer concentration of 10-50 mM is suitable for use with small molecules. Generally, no morethan 50% organic should be used with a buffer. This will be influenced by the particular buffer and its concentration. Phosphoric acid and its sodium or potassium salts are the most common buffer systems for HPLC in the reversed phase. Buffers sulfonate can replace phosphonate buffers when analyzing organophosphate derivatives.(26)

Buffering Capacity

Buffering Capacity is the ability of the buffer to resist changes in pH :



- Buffering Capacity increases as the molar concentration (molarity) of the buffer salt/acid solution increases.
- The closer the buffered pH is to the pka, the greater the Buffering Capacity.

Buffering Capacity is expressed as the molarity of Sodium Hydroxide required to increase pH by 1.0. Consideration of the affect of pH on analyte retention, type of buffer to use, and its concentration, solubility in the organic modifier and its affect on detection are important in reversed-phase chromatography (RPC) method development of ionic analytes. An improper choice of buffer, in terms of buffering species, ionic strength and pH, can result in poor or irreproducible retention and tailing in reverse-phase separation of polar and ionizable compounds (27- 28)

Isocratic and Gradient Separation

Isocratic separation refers to the column's equilibrium conditions and the actual speed at which compounds move through the column are constant. The peak capacity is low and the longer the component is retained on the column the wider is the consequent peak. Gradient mode of separation includes increases in the separation power of a system due to an increase of the apparent efficiency (decrease of the peak width). The peak of width depends on the rate of the eluent composition variation. Isocratic's calculated ratio of 0.25 is sufficient. A gradient would be sufficient when the ratio is greater than 0.25. (21)

Detector Selection

Detector is a very critical component of the HPLC method selection of the detector depends required, the availability and/or the expense of the detector. UV-Visible detector is a flexible. on the chemical

quality of the analytes, the possible disturbance, the detection limit is dualwavelength HPLC absorbance detector. This detector provides the high sensitivity needed for routine UV-based applications for low-level impurity detection and quantitative analysts The Detector provides superior optical detection for water analytical HPLC, Preparative HPLC, or LC/MS system solutions. High chromatographic and spectral sensitivity are provided by its integrated software and optics innovations. This detector is ideal for evaluating components with or without limited UV penetration due to its stability, reproducibility, chromatographic and spectral accuracy, and refractive indexes. The Multiwavelength Fluorescence Detector measures low target chemical concentrations using fluorescence detection with great sensitivity and selectivity. (29 -30)

Column Selection

The column is at the basis of the HPLC technique. Changing the column would have the greatest impact on the resolution of analytes during the production of the process. A successful chromatographic separation and accurate and reliable analysis can be achieved with the right column. Sometimes, an incorrectly used column can cause uncertainty, difficulties, and weak separations that can lead to results that are invalid or difficult to interpret Generally, modern reverse-phase HPLC columns are made by packing column housing with spherical silica gel beads that are coated with a stationary.(31)

Column temperature

The use of high temperatures can be advantageous for a number of reasons. First, operating at a temperature higher than ambient lowers the viscosity of the mobile phase and, as a result, the total back pressure of the column. Because



temperature influences selectivity, temperature control of the column is essential for long-term reproducibility. A target temperature of 30-40 °C is typically appropriate for good reproducibility. Lower system pressures allow higher flow speeds and hence faster analysis. Temperature can also affect selectivity patterns because analytes may react appropriately to different temperatures. (32)

Selection of Mobile Phase

The mobile phase effects resolution, selectivity and efficiency. The mobile phase In RPHPLC separation, composition, also known as solvent strength, plays a significant role. Acetonitrile (ACN), methanol (MeOH) and tetrahydrofuran (THF) are commonly used solvents in RP-HPLC having low UV cut-off of 190, 205 and 212nm respectively. These solvents are miscible with water. Mixture of acetonitrile and water is the best option for the mobile phase of method development at the outset. (33)

Mode	Solvent type used	Type of compound used
Reversed Phase	Water/Buffer, CAN, Methanol	Neutral or non-ionized compounds which can be dissolved in water/organic mixtures.
Ion-pair	Water/Buffer, ACN, Methanol	Ionic or Ionizable compounds
Normal phase	Organic solvents	Mixtures of isomers and compounds not soluble in Organic/Water mixtures.
Ion exchange	Water/Buffers	Inorganic ions proteins, nucleic acids, organic acids.
Size exclusion	Water, Tetrahydrofuran Chloroform	High molecular weight compounds

Developing the approach analysis

While developing the analytical method on RP-HPLC the first step is followed is the selections of various chromatographic parameters like selection of mobile phase, selection of column, selection of flow rate of mobile phase, selection of pH of mobile phase. All of these parameters are selected on the basis of trials and followed by considering the system suitability parameters. Typical parameters of system suitability are e.g. retention time should be more than 5min, the theoretical plates should be more than 2000, the tailing factor should be less than 2, resolution between 2 peaks should be more than 5, % R.S.D of the area of analyte peaks in standard chromatograms should not be more than 2.0% like other. Detection wavelength is usually isobestic point in the case of simultaneous estimation of 2 components. After

this the linearity of the drug is studied in order to know the range of concentrations up to which the drug follows the linear pattern. Analysis of the laboratory mixture is also carried out in order to the developed method for simultaneous estimation's applicability. After that analysis of marketed formulation is carried out by diluting the marketed formulation up to concentration range of linearity. (34-35-36-)

Sample preparation

The solvent in which the sample is dissolved plays an important role in terms of peak band broadening and retention time of the solute. If the sample solvent is different than the mobile phase, one or more distinct system peaks, either positive or negative, may be obtained. The best chromatographic result is obtained if sample and

dilutions are prepared in mobile phase. A sample may be difficult to dissolve in the mobile device in some instances due to its lack of solubility. phase. In such cases, a stronger, i.e., less polar solvent may be used to dissolve the sample. Utilization of a less polar solvent can cause peak band broadening or distortion and reduction in retention time. It is important to inject a blank of the solvent to identify system peaks. (37)

Method Optimization

Identify the method's "weaknesses" and optimize the method using experimental design. Understand how the method performs under different conditions, with different instrument setups, and with different samples.(38)

Validation

Validation is the systematic process of assessing and providing objective evidence that specific requirements for a particular intended use are met. It entails assessing a method's effectiveness and demonstrating its capacity to fulfill particular requirements. Essentially, validation provides a thorough understanding of what your technique can reliably produce, particularly when dealing with low doses or challenging conditions in analytical methods like High-Performance Liquid Chromatography (HPLC).(39)

Method Validation

The methods were validated in conjunction with the International Conference on Harmonization (ICH) recommendations for the validation of analytical methods. Validation is necessary for any new or updated medication to ensure that it can produce reproducible, consistent, and effective outcomes. The process by which laboratory experiments have established the method's performance requirements is known as validation

of the analytical method. Most the requirements of the anticipated analytical application. The guidelines of the USP, ICH, FDA, etc. can also provide a basis for validation of pharmaceutical methods.(40-41)

Scope Of Process Validation

Validation is one of the broadest and most complicated areas within manufacturing levels. In various fields, the scope of validity is as follows

- Analytical
- Calibration of the Instruments
- Services for utility processing
- Raw materials
- Packaging materials
- Equipment
- Facilities
- Manufacturing processes
- Product Design
- Cleaning
- Operators

Importance stages in validation Stage 1

This includes the pre-validation qualification stage covering all exercises that define and improve product studies, pilot batch testing formulation, scale-up analysis, exchange of product studies, Innovation for groups on a market scale, setting the conditions for stability, In process, completed pharmaceutical formulations, and maintenance of Equipment, master, and process limit approval.(42)



Stage 2

This contains the validation step of the procedure. Its function is to guarantee that any installed limit of critical process parameter is not exceeded. is significant and that satisfactory products can be generated even in the most dire circumstances.(42)

Stage 3

It is also known as the validation maintenance stage, and it requires that all archives relating to the procedure, including validation of the review reports, to ensure that The production procedure has not undergone any modifications, failures, or errors, and that all change control procedures in standard operating procedures (SOPs) must be detected. The approval team, which consists of representatives from each major departments, guarantees that no improvements or deviations have occurred that would require revalidation and requalification.(42)

Types of process validation

- Prospective validation
- Concurrent validation
- Retrospective validation
- Re-validation

Prospective validation

It is defined as the established, documented evidence that a system performs as intended under predetermined conditions. protocol. This validation is typically carried out prior to the distribution of a new or previously developed product. changed the way things are made. It was performed on at least three consecutive batches. Prospective Validation employs the validation protocol is executed before the process is put into

commercial distribution of product. During the product during the development phase, the production procedure ought to be broken down into distinct steps. Each step should be evaluated on the basis of experience or theoretical considerations to determine the critical parameters that may affect the quality of the finished product.(43)

Concurrent validation

End-product output current testing and in-process tracking of essential processing steps may provide documented evidence that the manufacturing process is under control. Except for the fact that the operating entity will sell the product during the qualification runs, it will be sold to the general public at a price that is comparable to the market price.(44)

Retrospective validation

Such processes can be validated with historical evidence. The steps involved include creating a specific protocol and publishing the data review's findings, which lead to a conclusion and a recommendation. Retrospective validity is based on the requirement that true in-process requirements for those characteristics be compatible with the drug product's final product requirements.(45)

Batch size/strength/producer/year/period.

File masters for production and packaging.

Current particulars for active ingredients/finished materials. (46)

Re-Validation

It is a validated phase or element that has been reproduced. Changes of the source of the active



raw material producer are among the changes in re validation.(46)

changes made to crude materials.

changes made to the active crude material producer's source.

Alteration of packing material

Modifications inside the plant/facility.(46)

Validation Parameters

The following are typical parameters recommended by the FDA, USP, and ICH.

1. Specificity
2. Linearity & Range
3. Precision
 - I. precision of the method (repeatability)
 - II. Intermediate precision (Reproducibility)
4. Accuracy (Recovery)
5. Solution stability
6. Limit of Detection (LOD)
7. Limit of Quantification (LOQ)
8. Robustness
9. Range
10. Suitability of the System (24)

Specificity

In strategy approval, selectivity and specificity are sometimes used interchangeably to represent the same concept. Specificity is the ability to evaluate the analyte unequivocally in the vicinity of components that might need to be shown. A test

system's specificity is controlled by contrasting test results obtained from an investigation of tests containing contaminations, debasement products, or placebos with substances obtained from an investigation of tests without contaminations, debasement items, or placebo fixings.(47-48)

Linearity and Range

Linearity in an analytical process refers to its ability to produce test results that are directly proportional to the concentration of the analyte in the sample, within a specified range. It is crucial to evaluate this linear relationship, across the spectrum of the analytical technique. The suggested approach involves diluting a normal stock solution containing the constituent parts of the medicinal product to directly demonstrate linearity on the drug substance.

The range of an analytical method is defined as the interval between the higher and lower values that have been demonstrated to be determined with precision, accuracy, and linearity using the method. ICH recommendations recommend a minimum of five concentrations for establishing linearity. The confidence interval around the slope of the regression line is commonly used to establish linearity. Over a predetermined concentration range, this comprehensive evaluation guarantees the analytical method's validity and reliability.(24)

Precision

The degree of consistency between the individual test results obtained by applying the method to repeated sampling of a homogeneous population trail determines an analytical method's precision. The reproducibility of the entire analytical process is measured by precision. It is divided into two sections. Repeatability and intermediate precision.(49)



Accuracy

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose “true value” is known), the measured value is analyzed, and identical to the true value. Typically, accuracy is represented and determined by recovery studies.

There are three ways to assess accuracy:

1. Comparison to a reference standard.
2. The analyte was recovered and spiked into a blank matrix.
3. The analyte is added as usual.
4. It should be clear how the individual or total impurities are to be determined.(50)

Solution Stability

During normal validation, the stability of standards and samples is established. conditions, typical conditions of storage, and occasionally in the instrument to determine whether Requirements for special storage conditions include light protection or refrigeration. (24)

Limit of Detection (LOD)

The most fundamental measure of an analyte in a sample that can be identified but cannot be accurately quantified is the detection limit of a single analytical method. A fundamental indicator of the method's sensitivity is this limit, which represents the lowest concentration at which the analyte can be reliably detected.(51)

Limit of Quantification (LOQ)

The limit of Quantitation (LOQ) or Quantitation limit of a specific analytical method is the lowest amount of analyte that can be found in a sample quantitatively determined with suitable precision and accuracy.

For analytical procedures such as HPLC that exhibit baseline noise, and the LOQ is typically calculated using most of the time, the LOQ is estimated because of baseline noise. from a determination of S/N ratio (10:1) and is typically verified by injecting standards that provide this ratio of S/N and a respectable percentage of also the standard deviation. (52-53)

Robustness

The robustness of an analytical procedure is a measure of its inability to be influenced by small, but deliberate variations in method parameters and provides an indication of its reliability during regular use. The variable method parameters in HPLC technique may include flow rate, pH, sample temperature, and column temperature mobile phase structure. (54- 55)

Range

The range of an analytical method refers to the interval between the higher and lower values of an analyte that have been demonstrated with sufficient linearity. precision, and accuracy. This range is typically determined based on a linear or nonlinear response curve and is expressed in the same units as the test findings. To accurately evaluate and report results within the method's validated and reliable concentration limits, it is necessary to establish a defined range.(56-57)

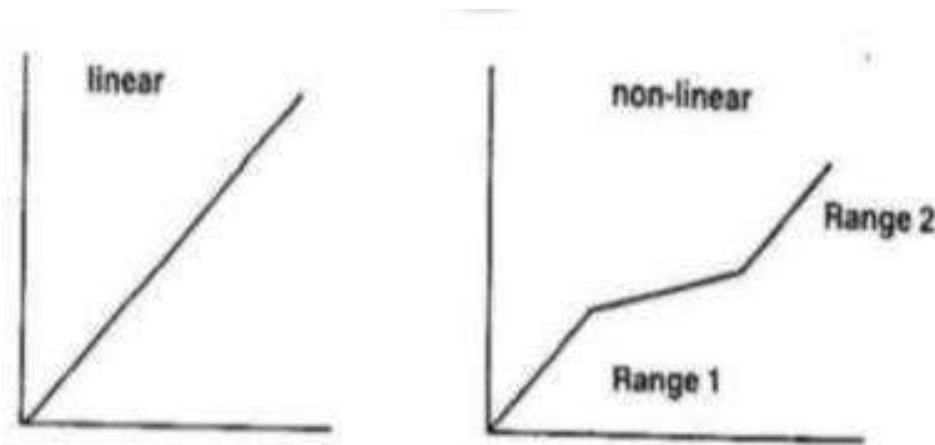


Figure 3. Range determination 10. Suitability of System

Liquid chromatographic methods include system suitability tests as a standard procedure. They are utilized to guarantee that the resolution, detection sensitivity, and for the analysis, and reproducibility are adequate. The tests are predicated on the concept that Analytical procedures, equipment, and samples to be analyzed are all components of a that can be evaluated as a whole as a whole. To determine the suitability of the used method, aspects like peak resolution, theoretical plate count, peak tailing, and capacity were measured. (56)

CONCLUSION -: HPLC is a gold standard analytical technique offering precise, reproducible results for the identification, purification, and quantification of diverse compounds. Its effectiveness, however, depends on rigorous method development, validation, and optimization tailored to the specific context and sample matrix. For best results, continual monitoring of instrument performance and strict adherence to international validation guidelines are essential. The document provides a comprehensive reference for both practical implementation and theoretical understanding of HPLC, highlighting its central role in modern chemical analysis alongside recommendations for mitigating its technical challenges.

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HOW TO CITE: Gayatri Jadhav*, Monali Khatke, Aarti Nanhe, An Overview HPLC Method Development and Validation, *Int. J. of Pharm. Sci.*, 2025, Vol 3, Issue 11, 2945-2962 <https://doi.org/10.5281/zenodo.17650743>

