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## Review Article

# HPLC Development Method and Validation

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### ABSTRACT

Chromatography, while primarily a separation technique, is widely used in chemical analysis. High-performance liquid chromatography (HPLC) is a highly versatile technology that separates analytes by passing them through a column filled with micrometer-sized particles. Today, reversed-phase chromatography is the most extensively utilized separation technique in HPLC. This is due to the reversed-phase method's simplicity, versatility, and scope, which can handle compounds with a wide range of polarity and molecular mass. This article discusses tactics and difficulties for planning HPLC method development and validation. Method development frequently follows well-established processes, such as buffer selection, mobile phase selection, and column selection. The approach developed should be as basic as feasible, and the optimum strategy is theoretical and empirical approach.

### INTRODUCTION

High-Performance Liquid Chromatography (HPLC) has emerged as one of the most powerful and versatile analytical procedures in modern laboratories. HPLC was first introduced in the late 1960s and has since become the gold standard for compound separation, identification, and quantification in a variety of sectors. Its uses include pharmaceutical drug analysis, environmental testing, food safety review, clinical research, and forensic investigations.

HPLC's success stems from its capacity to handle a diverse spectrum of chemical substances,

including those that are non-volatile, thermally unstable, or have large molecular weights. HPLC, as opposed to classical liquid chromatography, delivers faster, more efficient separation with improved resolution and sensitivity due to the use of high-pressure pumps that drive the mobile phase through closely packed stationary phase columns.

#### Importance Of Method Development

HPLC method development is critical for achieving dependable and reproducible analytical results. The purpose of method development is to identify the best chromatographic conditions for

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appropriate separation, accurate quantification, and consistent reproducibility. This requires fine-tuning numerous parameters, such as:

- Mobile phase: composition entails selecting suitable solvents and pH for the sample's solubility and interaction with the stationary phase.
- Column selection: influences separation efficiency and resolution.
- Detection Method: Depending on the analyte properties, different detectors (UV, fluorescence, and refractive index) provide different sensitivity and selectivity levels.

Creating an HPLC technique is frequently an iterative process that necessitates trial and tweaking of conditions until the desired performance is obtained. When working with complicated sample matrices or multi-component systems, overlapping peaks or contaminants can make proper analysis difficult.

#### **Industry And Regulatory Compliance :**

Method development and validation are crucial in highly regulated industries such as pharmaceuticals, not only to ensure product quality but also to meet regulatory requirements. Regulatory authorities such as the FDA, ICH, and WHO require rigorous validation processes before a method may be employed for quality control, release testing, or stability research. Adherence to these standards reduces the likelihood of product recalls, ensures the safety of medications and medical equipment, and promotes customer confidence. With the growing complexity of current medicine formulations and biopharmaceutical products, HPLC technique development and validation have gotten increasingly complicated. Advances in HPLC technology, such as ultra-high-performance liquid chromatography (UHPLC) and the use of mass spectrometry as a detection instrument, have increased the technique's capabilities.

#### **CONCLUSION:**

development and validation of HPLC techniques is critical to ensure that analytical results are accurate, reliable, and reproducible. Properly developed and verified methodologies are critical for quality control and regulatory compliance in a variety of sectors. Dealing with complex samples or attaining appropriate separation are examples of method development issues that can be handled by thorough chromatographic condition optimization. Successful validation ensures that the method performs consistently under typical testing conditions, meeting both industry and regulatory requirements. development and validation of HPLC techniques is critical to ensure that analytical results are accurate, reliable, and reproducible. Properly developed and verified methodologies are critical for quality control and regulatory compliance in a variety of sectors. Dealing with complex samples or attaining appropriate separation are examples of method development issues that can be handled by thorough chromatographic condition optimization. Successful validation ensures that the method performs consistently under typical testing conditions, meeting both industry and regulatory requirements. development and validation of HPLC techniques is critical to ensure that analytical results are accurate, reliable, and reproducible. Properly developed and verified methodologies are critical for quality control and regulatory compliance in a variety of sectors. Dealing with complex samples or attaining appropriate separation are examples of method development issues that can be handled by thorough chromatographic condition optimization. Successful validation ensures that the method performs consistently under typical testing conditions, meeting both industry and regulatory requirements.

#### **HPLC System:**

Typical HPLC system consists of the following main components:

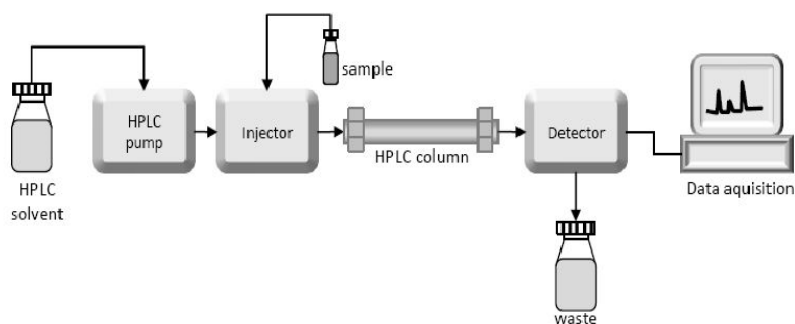
**Solvent reservoirs:** store enough HPLC solvents to ensure the system runs continuously. Could be equipped featuring an online degassing system and unique using filters to separate the solvent from environmental influence. **Pump:** The pump ensures continuous flow of the mobile phase through the system. Most current pumps support this function. Controlled mixtures of several solvent from different reservoirs. **injector:** introduces the analyte mixture into the mobile phase stream prior to its entry. Most current injectors are column-mounted. **Autosamplers,** which allow programmed injections of various sample quantities. which are taken from the vials in autosampler tray. **column :** The HPLC technology relies on the column to separate analytes from the mixture. A column is the space where the mobile phase comes in touch with the stationary phase from a contact with the enormous surface. Most of the chromatography



development in recent years went toward the design of many different ways to enhance this interfacial contact. Detector: This is a device for continuous registration of specific physical (sometimes chemical) properties of the column effluent. The most common detector used in pharmaceutical analysis is UV (ultraviolet) which allows monitoring and continuous registration of the UV absorbance at a selected wavelength or over a span of wavelengths (diode array detection). registration of specific physical (sometimes chemical) properties of the column effluent. The most common detector used in pharmaceutical analysis is UV (ultraviolet) The presence of an

analyte in the detector flow-cell can induce the Change of the absorbance. If the analyte absorbs more Compared to the background (mobile phase), A favorable signal is obtained.

The Data Acquisition and Control System is a computer-based system that regulates all parameters of the HPLC apparatus (eluent). composition (the combination of various solvents); Temperature, injection sequence, and so on. and collects data from the detector and monitors System performance (continuous monitoring) of the mobile-phase composition, Temperature, back pressure, and so on. What is knew before beginning technique development?



### HPLC CONDITION:

- Buffer: To achieve a desirable peak shape for your analytes, choose the appropriate mobile phase buffer. Buffers enhance the peak form of basic substances, alter the band spacing, and retain acidic

molecules Basic compounds. We strongly propose 10 - 20 mM of an ammonium salt of your choice as a starting buffer (i.e. acetate, formate, carbonate, or phosphate salts) for electrolyte solutes.

buffer	pH range	LC-MS compatible
phosphate ( $pK_1$ )	1.1 – 3.1	X
phosphate ( $pK_2$ )	6.2 – 8.2	X
phosphate ( $pK_3$ )	11.3 – 13.3	X
acetate <sup>1</sup>	3.8 – 5.8	YES
citrate ( $pK_1$ )	2.1 – 4.1	X
citrate ( $pK_2$ )	3.7 – 5.7	X
citrate ( $pK_3$ )	4.4 – 6.4	X
trifluoroacetic acid (0.1%)	2.0	YES
phosphoric acid (0.1%)	2.0	X
formic acid (0.1%)	2.7	YES
ammonium formate	2.7 – 4.7	YES
ammonium bicarbonate	6.6 – 8.6	YES
borate	8.3 -10.3	YES

The section on method development for ionizable chemicals provides additional guidance on selecting and manipulating buffer systems. A buffer keeps the pH stable when a little amount of acid or base is applied. (10) Some of the additives are listed below. Buffers are most efficient when utilized within  $\pm 1$  pH unit of its  $pK_a$ , but may offer sufficient buffering  $\pm 2$  pH units from  $pK_a$  [10]. The most commonly used buffers for HPLC with UV detection are phosphate and acetate. Phosphate and acetate are especially beneficial. Buffers can be utilized at wavelengths below 220 nm.

- **Buffering Capacity:** Buffering capacity refers to the maximum amount of strong acid or base that can be applied prior to a "A significant pH change will occur." Buffering capacity increases with the concentration (molarity) of the buffer salt/acid solution. The buffering capacity increases as the buffered pH approaches the  $pK_a$  level. expressed as the molarity of sodium hydroxide. It is necessary to elevate pH by 1.0.
- **Buffer Selection:** Buffer solutions consist of a weak acid and its conjugate base, or a weak base and its conjugate acid. They reduce the influence of Hydrogen/hydronium and hydroxide ions subsequently reduce pH fluctuations, even upon diluting. Reversed phase on silica-based packaging typically has a pH range of 2-8. Choosing a buffer is often controlled by the intended pH. The buffer must have a  $pK_a$  approaching the target pH level. Buffers effectively control pH at their  $pK_a$ . A rule of thumb is to select a buffer with a  $pK_a$  value less than 2 units of the desired mobile phase pH
- **Detectors Of HPLC:** HPLC separations are monitored using various detectors, similar to gas chromatography. The majority of HPLC detectors are stand-alone or modified equipment, not specific to the procedure.

Versions of the same. Spectroscopic detectors The most common HPLC detectors rely on spectroscopic measurements, such as UV/Vis. Absorption and fluorescence. Detectors might be basic designs that use filters to select the analysis wavelength or modified spectrophotometers with flow cells. Using a UV/Vis detector produces a chromatogram that plots elution time. A diode array spectrophotometer records full spectra, resulting in a three-dimensional chromatogram demonstrating absorbance as a function of wavelength and elution time.

- **Column Selection:** Choosing the right column is crucial. The column significantly impacts analyte resolution during the procedure development. Modern reverse phase HPLC columns are typically manufactured by filling the column container with spherical silica gel beads covered with a hydrophobic stationary phase. The stationary phase is introduced into the matrix by reacting chlorosilane with the hydroxyl groups on the silica gel surface. Generally speaking, the stationary phase has the most effect on elution, capacity factor, efficiency, and selectivity. The stationary phase is supported by a variety of matrices such as silica, polymers, and alumina. Silica is the most frequent matrix used in HPLC columns. Silica matrices are durable, easily derivatized, and may be manufactured. It has a regular size and form and does not compress under pressure. Silica is chemically stable in most organic solvents and low pH environments. One drawback of silica solid support is that it will dissolve at pH levels above 7. During recent years, silica-supported columns were created for use at high pH. The shape and particle size of the silica support affect separation.

Smaller particles result in more theoretical plates and higher separation efficiency. Using smaller particles increases backpressure during chromatography, making the column more susceptible to clogging. In reverse phase chromatography, the stationary phase is non-polar while the mobile phase is polar, resulting in polar peaks that generally elute earlier than non-polar peaks. To construct a stationary phase for reverse phase chromatography on silica, free silanol are combined with a chlorosilane with hydrophobic activity to create a non-polar surface. Steric restrictions limit derivatization to around 1/3 of surface silanol. Free silanol can interact with analytes, resulting in peak tailing. After derivatizing a column to get the desired stationary phase, it is often treated with chlorotrimethyl silane to end cap any leftover free silanols and improve column

efficiency. Common stationary phases include C4 (butyl), C8 (octyl), C18 (octadecyl), nitrile (cyanopropyl), and phenyl propyl. columns. In general, longer alkyl chains have higher phase loading, and greater carbon loads offer increased retention of non-polar analytes. Mobile Phase:

Choosing the right mobile phase composition and type is crucial for effective separation. However, the type of stationary phase used in the column limits the available choices. The primary distinction is between reversed and normal phase chromatography. In normal phase systems. Nonpolar solvents like hexane or iso-octane are used, but reversed phase requires polar. Solvents include water, acetonitrile, and methanol. The solvent's physicochemical characteristics determine which mobile phase is best. Considerations include polarity and miscibility with other chemical inertness, solvents, and UV cutoff wavelength as well as poisoning.

Mobile Phase	Polarity Index (P')	UV Cutoff (nm)
cyclohexane	0.04	210
n-hexane	0.1	210
carbon tetrachloride	1.6	265
diethyl ether	2.4	220
toluene	2.4	286
diethyl ether	2.8	218
tetrahydrofuran	4.0	220
methanol	4.3	210
ethyl acetate	4.4	255
chloroform	4.8	215
acetonitrile	5.1	210
acetonitrile	5.8	190
water	10.2	—

#### Practical Tips For Handling Mobile Phase:

- a) Detector consider the purity of solvents and only use HPLC grade materials:
  - noisy baselines, such as UV detection at low wavelengths, are caused by impurities;
  - Water that has been demineralized ought to be used for mobile phases.

- b) Make sure there is no dust in the mobile phase, then attach a stainless steel filter element to the tube's end that extends from the reservoir. to the power source.
  - C) Eliminate dissolved air as this may result in erratic pumping behavior and variable signals from the

through carrying out one or additional of the following :

- Use helium to degas the mobile phase.
- Put a vacuum on the mobile phase.
- Agitate the mobile phase in an ultrasonic bath.

D) When mixing solvents to form mobile phases:

- A common statement for the ingredients of the mobile phase is 75 /25 v/v methanol / water, which denotes a volume measurement (75 mL of methanol + 25 mL of water, for example). The analyst needs to be familiar with the language used to define these components.
- The majority of systems allow the analyst to simply set the percentages; they can also combine solvents from various reservoir bottles.  
such as 75% methanol and 25% water.
- If a mixed composition mobile phase is to be made by hand, each solvent's volume needs to be measured. individually before combining them.

E) Be aware that volatile components in a mobile phase of mixed composition may evaporate.

This can be minimized by :

- Keeping the solution cool during the degassing procedure;
- keeping the bottle cork in the reservoir at all times.

f) Ensure that the sample to be analyzed is soluble in the mobile phase.

g) It is important to take the mobile phase's UV absorption into account while employing UV detectors. This is what the UV cut-off value signifies. Tetrahydrofuran, for instance, has a UV cut off of 280 nm, hence it cannot be used to analyze pyridine samples because pyridine's peak maximum is approximately 260 nm.

h) Make sure there is no reaction between the stationary phase and the mobile phase. Ammonia is one such ingredient that buffers and pH

modifiers may contain. which is capable of swapping out one of the NR groups of an amide on a stationary phase of amino propyl.

i) Additionally, it's critical to keep an eye on the mobile phases' levels and make sure they're regularly replenished, since this will ensure the The system is never permitted to empty.

j) In order to prevent the system from ever being dry, it's also critical to keep an eye on the mobile phases' levels and make sure they're regularly topped off.

Make sure the bottle labels are accurately labeled with the following information whenever there is a change in the mobile phase

- Method Optimization;

After obtaining the proper separations, the experimental conditions should be adjusted to obtain the required separations and sensitivity. Consistency indicating experimental circumstances for the assay

will be accomplished by systematic/planned analysis of variables such as gradient, flow, pH (if ionic), and mobile phase components and ratio. temperature, pace, sample size, and injection amount and type of solvent diluents. "Welcome to our validation process! This is an important step to ensure the quality and accuracy of our work. Validation involves reviewing and confirming that our output meets the required standards and specifications. Your participation in this process will help us refine our performance and provide the best possible results. Please carefully review the output and provide your feedback."

**Definition Of Validation-According To ISO** – “validation is the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled”

**According To USFDA-** “To establish documented evidence which provide high degree of assurance that a specific process will consistently produce a product meeting its



predetermined specifications and quality attributes”

### Importance Of Validation-

- Assurance of quality
- Process optimization.
- Reduction of quality cost.
- Minimal batch failure, improved efficiency and productivity.
- Reduction of rejections.
- Increased output.
- Fewer complaints about process related failures.
- Reduced testing in process and in finished goods.
- More rapid and reliable start-up of new equipment.
- Easier maintenance of equipment.
- Improved employee awareness of process.
- More rapid automation.
- Government regulation (compliance with validation requirements is necessary for obtaining approval to manufacture and to introduce new products)

The process of validation typically involves the following steps:

1. Review: Examine the output, data, or results to ensure they meet the requirements and specifications.
2. Verification: Check that the output is accurate, complete, and consistent with the expected outcome.
3. Evaluation: Assess the output against predetermined criteria, standards, or benchmarks.
4. Feedback: Provide input on the output, highlighting any errors, discrepancies, or areas for improvement.
5. Correction/Revision: Address any issues or deficiencies identified during validation.
6. Re-validation: Repeat the validation process to ensure corrections/changes meet the requirements.

7. Approval: Confirm that the validated output meets the necessary standards and is ready for use.

8. Documentation: Record the validation process, results, and any subsequent changes.

### 4.1 General Principles Of Analytical Method

**Validation Definition:** Validation of Analytical method may be defined as "The process by, which it is established, by laboratory studies, that the performance characteristics of the method, which meet the requirements for the intended analytical application"

Typical analytical characteristics can be listed as follows:

1. Accuracy
2. Precision
3. Specificity
4. Limit of Detection
5. Limit of Quantitation
6. Linearity
7. Range
8. Ruggedness
9. Robustness

#### 1. Accuracy

Definition: Accuracy of an analytical method may be defined as "The closeness of test results obtained by that method to the true value. This accuracy should be established across its range".

Accuracy of an analytical method may be determined by the assay method used on a highly pure substance like reference standards and compared it with the same material with a known and established method.

This can also be further evaluated by addition of known pure substance and assessing the recovery of the added substance. In case of quantitative analysis of impurities, accuracy should be assessed on samples of drug substance or product by spiking with known amounts of impurities. The ICH recommends that the accuracy should be assessed using a minimum of nine determinations over a minimum of 3 concentration levels,



covering the specified range (ie. 3 concentration and 3 replicates of each concentration).

## 2. Precision

Definition: Precision of an analytical method may be defined as "the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of a homogenous sample". The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (ie. coefficient of variation) of a series of measurement. Precision refers to degree of reproducibility of results. This can be within a single laboratory or between more than one laboratories. Precision can also be considered as repeatability of results, which refers to comparison of results of an analysis within a short time by the same analyst, in same laboratory, and using the same equipment. The precision of an analytical method is determined by assessing sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation (i.e. Coefficient of variation) The I.C.H. Documents recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e. 3 concentrations and 3 replicates of each concentration or using minimum of six determinations at 100% of test concentration).

## 3. Specificity

Definition: Specificity may be defined as "The ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities degradation products and matrix components". Specificity can be used for identification tests, purity tests, assays, etc. The ICH documents state that, when chromatographic procedures are used, representative chromatogram should be represented to demonstrate the degree of specificity (selectivity) and peaks should be appropriately labeled. Peak purity tests may be

useful to show that the analyte chromatographic peak is not attributable to more than one component.

## 4. Limit Of Detection

Definition: The limit of Detection may be defined as "The lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions". The L.O.D. is generally expressed as the concentration of the analyte sample (e.g percentage or parts per million, etc). The I.C.H. documents describe a common approach, which is to compare measured signals from samples with known low concentrations of analytes with those of blank samples. These detection limits should be subsequently validated by the analysis of a suitable number of samples of known to be near or prepared at the detection limit.

## 5. Limit Of Quantization

Definition: LO.Q. May be defined as "As characteristic of quantitative assays for low levels of compounds in sample matrices such as impurities in bulk substances and degradation products in finished pharmaceuticals. It is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions". The L.O.Q. is generally expressed as the concentration of the analyte in the sample (e.g. percentage or parts per million, etc). The I.C.H. documents describe a common approach, which is to compare measured signals from samples with known low concentrations of analytes with those of blank samples. These quantitation limits should be subsequently validated by the analysis of a suitable, number of samples known to be near or prepared at the quantitation limit.

## 6. Linearity

Definition The Linearity of an analytical method may be defined as "Its ability to elicit tests that are directly or by well defined mathematical transformations proportional to the concentration





of analyte in sample within a given range". The Linearity should be established across the range of the analytical procedure. It should be established initially by visual examination of a plot of signals as a function of analyte concentration of contact. If this appears to be a linear relationship, test results should be established by appropriate statistical methods like regression analysis, etc.

1.CH Recommends that, for the establishment of Linearity, a minimum of 5 concentrations normally be used.

### 7. Range

Definition: The range of an analytical method may be defined as "Interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with a suitable level of precision, accuracy and linearity using the method as written. The range is normally expressed in the same units as test results. (e.g. Percentage, parts per million, etc.) Obtained by the analytical method". The range of the methods validated by verifying that the analytical method provides acceptable precision, accuracy and Linearity when applied to samples containing analyte at the extremes of the range as well as within the range.

### 8. Ruggedness

Definition: The Ruggedness of an analytical method may be defined as "The degree of reproducibility of test results obtained by the analysis of the same sample under a instruments, different lots of reagents, different elapsed assay times, different away temperatures, different days, etc." Ruggedness is normally expressed as the lack of influence on test results of operational environmental variables of the analytical methods. Ruggedness is a measure of reproducibility of test results under the variation in conditions normally expected from Laboratory to Laboratory and analyst to analyst. The ruggedness of an analytical method is determined by analysis of aliquots from homogeneous lots in different laboratories, by

different analysts using operational and environmental conditions that may differ but are still within the specified parameters of the away. The degree of reproducibility of test results is then determined as a function of the assay variables. The reproducibility of test result is then determined as a function of the assay variables. This reproducibility may be compared to the precision of the assay under normal conditions to obtain a measure of the ruggedness of the analytical method.

### 9. Robustness

Definition: The robustness of an analytical method may be defined "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"

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