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Review Article

A Comprehensive Overview Of HPLC Method Development And Validation

Sayali V. Ganjiwale*, A. P. Dewani, A. V. Chandewar

Department of Pharmaceutical Quality Assurance, P. Wadhwani College of Pharmacy, Yavatmal, Maharashtra, India

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ABSTRACT

HPLC has become the workhorse of analytical separations due to its versatility, sensitivity, and precision. However, optimizing and validating an HPLC method for specific analytes requires an intricate adjustment of parameters. This review provides a comprehensive overview of the key steps involved in developing and validating a robust HPLC method. Initially, we delve into the critical factors influencing method development, including analyte properties, sample preparation strategies, column selection, mobile phase optimization, and detector choice. We detail the importance of resolution, peak shape, and retention time control in achieving optimal separation. Next, we dissect the validation process, highlighting essential parameters like linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, specificity, robustness, and system suitability. We discuss established protocols and regulatory guidelines for each parameter, emphasizing the principles behind their evaluation. Furthermore, we explore advanced method development approaches, such as hyphenation with mass spectrometry (MS) for enhanced analyte identification and

INTRODUCTION

High-Performance Liquid Chromatography (HPLC) is an analytical technique employed for the separation of solutes based on their differential rates of elution within a chromatographic column. This separation method hinges on the distribution of solutes between the mobile phase and the stationary phase. The HPLC instrumentation comprises eight fundamental components: a mobile phase reservoir, solvent delivery system, sample introduction device, column, detector, waste reservoir, connective tubing, and a computer, integrator, or recorder [1]. Achieving successful outcomes with HPLC involves optimizing various operating conditions, including the type of column packing and mobile phase, column length and diameter, mobile phase flow rate, column temperature, and sample size [1]. In

***Corresponding Author:** Sayali V. Ganjiwale
Address: *Student (M.Pharm QA) P. Wadhwani College of Pharmacy, Yavatmal, Maharashtra, India*
Email ✉: ganjiwalesayali124@gmail.com

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contemporary HPLC applications, reversed-phase chromatography stands out as the predominant separation technique due to its wide-ranging applicability. Remarkably, an estimated 65% to possibly 90% of all HPLC separations are conducted using the reversed-phase mode. This prevalence is attributed to the simplicity, versatility, and broad scope of the reversed-phase method, allowing it to effectively handle compounds with diverse polarity and molecular mass [2-4].

PRINCIPLE

Isocratic HPLC involves the propulsion of the analyte through a column packed with small round particles of specific surface chemistry, utilizing a liquid mobile phase under high pressure. The sample, introduced in a small volume into the mobile phase stream, undergoes retardation due to specific chemical or physical interactions with the stationary phase as it traverses the column. The extent of retardation is influenced by the nature of the analyte, the composition of the stationary phase, and the mobile phase. The time at which a particular analyte elutes from the column, known as retention time, serves as a unique identifier for that analyte. To enhance resolution in the resulting chromatogram, pressure is employed to increase the linear velocity, reducing the time for components to diffuse within the column. Common solvents include miscible combinations of water or various organic liquids, with methanol and acetonitrile being the most prevalent. Water may contain buffers or salts to aid in analyte component separation [4]. A refinement to HPLC involves varying the mobile phase composition during the analysis, known as gradient elution. In reverse-phase chromatography, a typical gradient may start at 5% methanol and linearly progress to 50% methanol over 25 minutes, depending on the analyte's hydrophobicity. The gradient separates analyte mixtures based on their affinity for the current mobile phase composition relative to the stationary phase, resembling a continuous partitioning process. Solvent choice, additives, and gradient design depend on the stationary phase and analyte nature. Optimization of the HPLC method for a specific analyte often involves a series of tests and generic runs to achieve the best peak separation.

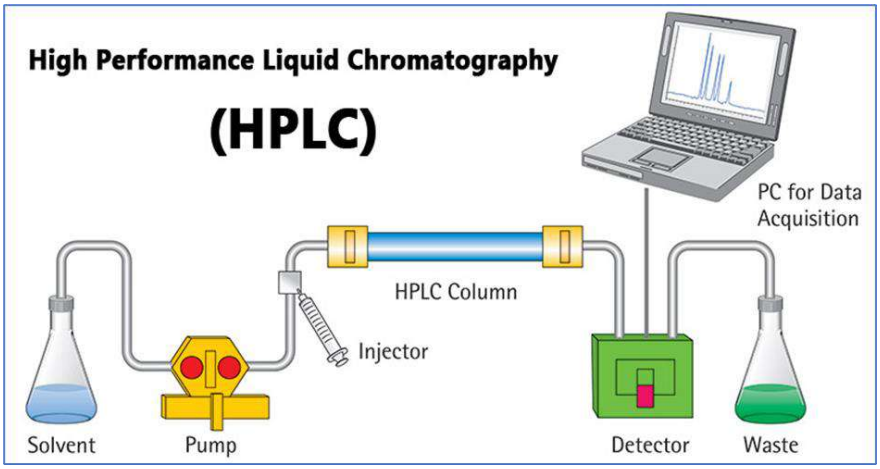

Figure 1: Instrumentation of High-Performance Liquid Chromatography (HPLC)

APPLICATION

Preparative HPLC refers to the process of isolation and purification of compounds. Important is the degree of solute purity and the throughput, which is the amount of compound produced per unit of time. This differs from analytical HPLC, where the focus is to obtain information about the sample compound. The information that can be obtained includes identification, quantification, and resolution of a compound. Chemical Separations

can be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase. Thus, the chromatographer can separate compounds (more on chiral separations) from each other using HPLC; the extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase. Identification of compounds by HPLC is a crucial part of any HPLC assay. To identify any compound by HPLC a detector must first be selected. Once the detector is selected and is set to optimal detection settings, a separation assay must be developed. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels at which the assay will be performed. To alter the retention time of a compound, several parameters can be manipulated. The first is the choice of column, another is the choice of mobile phase, and the last is the choice of flow rate. All of these topics are reviewed in detail in this document[5].

Identifying a compound by HPLC is accomplished by researching the literature and by trial and error. A sample of a known compound must be utilized to assure identification of the unknown compound. Identification of compounds can be assured by combining two or more detection methods.

Types of HPLC methods

1. **Reverse Phase HPLC**

Reversed-phase chromatography has found both analytical and preparative applications in biochemical separation and purification. Molecules that possess some degree of hydrophobic character can be separated by reversed-phase chromatography with excellent recovery and resolution [5]. Uses water-organic as a mobile phase, columns may be C18 (ODS), C8, phenyl, Trimethyl Silane (TMS), and Cyano as a stationary phase. It is the first choice for most samples especially neutral or non-ionized compounds, that dissolve in water organic mixtures [6].

2. **Normal Phase HPLC**

In normal-phase chromatography, the stationary phase is polar and the mobile phase is nonpolar. In the reversed phase we have just the opposite; the stationary phase is nonpolar and the mobile phase is polar. Typical stationary phases for normal-phase chromatography are silica or organic moieties with cyano and amino functional groups. In this, the mixtures of organic solvents for the mobile phase and columns i.e. cyano, diol and amino silica can be used as stationary phase. It is the first choice for mixtures of isomers and for preparative scale HPLC and the second choice for lipophilic samples that cannot dissolve well in water-organic mixtures [6].

Steps for HPLC Method Development

The wide variety of equipment, columns, eluant and operational parameters involved makes high-performance liquid chromatography (HPLC) method development seem complex.

The process is influenced by the nature of the analytes and generally follows the following steps:

Step 1 - Selection of the HPLC method and initial system

Step 2 - Selection of initial conditions

Step 3 - Method optimization

Step 4 - Method validation

Steps for HPLC Method Development

A. Step 1 - Selection of the HPLC method and initial system

When developing an HPLC method, the first step is always to consult the literature to ascertain whether the separation has been previously performed and if so, under what conditions - this will save time doing unnecessary experimental work. When selecting an HPLC system, it must have a high probability of actually being able to analyze the sample; for example, if the sample

includes polar analytes then reverse phase HPLC would offer both adequate retention and resolution, whereas normal phase HPLC would be much less feasible [7].

Sample collection and preparation

The sample should ideally be dissolved in the initial mobile phase. If this is not possible due to stability or solubility problems, formic acid, acetic acid or salt can be added to the sample to increase solubility. These additives do not usually affect the separation so long as the volume of the sample loaded is small compared to the column volume. The only effect when large sample volumes are applied may be an extra peak or two eluting in the void volume after sample injection. Sample preparation is an essential part of HPLC analysis, intended to provide a reproducible and homogenous solution that is suitable for injection into the column. The aim of sample preparation is a sample aliquot that, Is relatively free of interferences, Will not damage the column, and Is compatible with the intended HPLC method that is, the sample solvent will dissolve in the mobile phase without affecting sample retention or resolution. Sample preparation begins at the point of collection, and extends to sample injection onto the HPLC column. All of these operations form an important part of sample preparation and have a critical effect on the accuracy, precision, and convenience of the final method [8].

B. Step 2 - Selection of initial conditions

This step determines the optimum conditions to adequately retain all analytes; that is, ensures no analyte has a capacity factor of less than 0.5 (poor retention could result in peak overlapping) and no analyte has a capacity factor greater than 10– 15 (excessive retention leads to long analysis time and broad peaks with poor detectability) [7].

C. Step 3 - Method optimization

The experimental conditions should be optimized to get the desired separations and sensitivity after getting appropriate separations. Stability indicating assay experimental conditions will be achieved through planned/systemic examination of parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, temperature, sample amounts, Injection volume and diluents solvent type [9].

D. Step 4 - Validation of method

Validation of an analytical method is the process which is established by laboratory studies to evaluate the performance uniqueness of the procedure meet the requirements for its intended use. The validation process for analytical procedures begins with planned and systematic collection by the applicant of the validation data to support analytical procedures [10].

Components of Method Validation

The following are typical analytical performance characteristics that may be tested during methods validation:

Specificity, Linearity, Range, Precision, Accuracy, Limits of Detection and Quantitation, Robustness, Ruggedness System suitability.

Selectivity and Specificity

Selectivity of the analytical method is defined as the degree to which a method can quantify the analyte in the presence of interferents [11]. The other components which may be present include impurities, degradants, matrix, etc. The term specificity and selectivity is often used interchangeably. The term specific generally refers to a method that produces a response for a single analyte only, while the term selective refers to a method that responds to a number of chemical entities that may or may not be distinguished from each other. The International Union of Pure and Applied Chemistry (IUPAC) has expressed the view that "Specificity is the ultimate of Selectivity'. The IUPAC discourages the use of the term specificity and instead encourages the use of the term selectivity [15]. Specificity study of the chromatographic method is performed by the separation of the analyte from the other potential

components such as impurities, degradants, excipients, etc. In addition, forced degradation studies are carried out to challenge the method. The forced degradation studies are of particular importance when the impurities are not available. During forced degradation studies, the sample is subjected to the stressed conditions of light, heat, humidity, acid/base hydrolysis, and oxidation. The scheme which is generally used for forced degradation studies for drug substances and drug products is summarized in Table 1 below [12]. The selectivity of chromatographic methods may be assessed by examination of peak homogeneity or peak purity test. The peak purity test shows that there is no co-elution of any sample component. For this, peak purity assessment is done by using PDA or MS detectors. Representative chromatograms with peaks labeled should be included with the resolution, plate count, and tailing factor reported in the validation report.

Linearity

The linearity of a method is its ability to obtain test results that are directly proportional to the sample concentration over a given range. For HPLC methods, the linear relationship between detector response (peak area and height) and sample concentration is determined. The relationship can be demonstrated directly on drug substance by dilution of standard stock or by separate weighing of the sample components, using the proposed procedures [17]. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by regression analysis. Data from the regression line is helpful to provide mathematical estimates of the degree of linearity. It is generally expressed in terms of variance around the slope of the regression line. In some cases, the analytical responses should be described by the appropriate function of the analyte concentration. The widely used linearity ranges and acceptance criteria for various pharmaceutical methods are listed in table 2 [13].

Precision

The precision of an analytical method expresses the closeness of agreement 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.

Repeatability is the precision under the same operating conditions over a short interval of time. It is also termed intra-assay precision. It is assessed by making six sample determinations at 100% concentration or by preparing three samples at three concentrations in triplicates covering the specified range for the procedure. It involves the repeated determination of the same sample.

Intermediate precision expresses within laboratories variation: different days, different analysts, different equipment, etc. It is the term synonymous with the term ‘ruggedness’, defined by USP. The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. To study intermediate precision, the use of an experimental design is encouraged. The intermediate precision is generally studied by multiple preparations of sample and standard solution. Reproducibility is the precision obtained by analysis between laboratories. It is generally assessed during collaborative studies at the time of technology or method transfer. It is assessed using an inter-laboratory trial [18]. The precision data is generally expressed in the form of standard deviation, relative standard deviation, and confidence intervals. To ensure the precision of the method for major analytes, RSD should be ≤ 2 %. For low-level impurities, RSD of 5-10 % is usually acceptable [14].

Range

The range of an analytical method is the interval between the upper and lower concentration 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. The range is normally derived from the linearity studies and depends on the intended application of the procedure. The following minimum specified ranges should be considered [10]:

1. For the assay method, normally covers from 80 to 120 percent of the test concentration.
2. For content uniformity, covering a minimum of 70 to 130 percent of the test concentration, based on the nature of the dosage form.
3. For dissolution testing, ±20 % over the specified range.
4. For impurity determination, from reporting level of impurity to 120 % of the specification.

The range of a method is confirmed when linearity, accuracy, and precision criteria are fulfilled [2].

Accuracy

The accuracy of an analytical method expresses the closeness of agreement between the value accepted either as a conventional true value or an accepted reference value and the value found. Practically no measurement process is ideal, therefore, the actual value cannot be exactly known in any particular measurement. The accepted true value for accuracy assessment can be assessed by analyzing a sample with known concentration. The accuracy studies are usually carried out by determining the recovery of the spiked sample of analyte into the matrix of the sample (a placebo) or by comparing the result to the results of a certified reference material of known purity. If the placebo of the sample is not available, the technique of standard addition is used. In the case of methods for quantitation of impurities, the sample with a known amount of impurities is assessed. Accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range (e.g., three concentrations/ three replicates each of the total analytical procedure) [19]. Accuracy should be reported as percent recovery by the assay of a known added amount of analyte in the sample or as the difference between the means and the accepted true value together with the confidence intervals. The concentration should cover the range of concern. The expected recovery depends on the sample matrix, the sample processing procedure, and the analyte concentration. The reported limits for accuracy for drug substances and products are 98.0 – 102.0 % and 97.0 – 103.0 % respectively. For the impurity determination, a range from 50 - 150 % of average recovery may be accepted [2-4].

Limit of Detection

The limit of detection of an individual analytical procedure is the lowest amount of analyte in the sample which can be detected but not necessarily quantified as an exact value. The detection limit can be determined in different ways. The simplest approach is based on the signal-to-noise ratio. The signal-to-noise ratio is determined by comparing measured signals from samples with known low concentrations of analyte with those of blank samples. The concentration showing a signal-to-noise ratio between 3:1 and 2:1 is generally considered an acceptable detection limit. The other approach is based on the standard deviation of the response and the slope. The detection limit may be expressed as:

Where, σ = the standard deviation of the response, S = the slope of the calibration curve

The slope may be estimated from the calibration curve of the analyte. The σ can be estimated as the standard deviation of the blank. The value of σ can also be estimated based on the calibration curve.

For this the specific calibration curve should be studied using a sample containing analyte in the range of detection limit. The residual standard deviation of a regression line or the standard deviation of the y-intercept of regression lines may be used as standard deviation. Another approach for the estimation of the detection limit is based on visual evaluation. This method applies to non-instrumental methods but may be applied to instrumental methods. The LOD is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. The relevant chromatograms are sufficient for the justification of the detection limit.

Limit of Quantitation[20]

The Quantitation limit of an individual analytical procedure is the lowest amount of analyte in the sample which can be quantitatively determined with suitable precision and accuracy. It is mainly affected by the detector sensitivity and accuracy of sample preparation. The Quantitation limit can be determined in a similar way as that of the detection limit. It is the concentration showing signal to noise ratio of 10:1. Based on the standard deviation of the response and the slope it is calculated by the formula:

Where, σ = the standard deviation of the response
S = the slope of the calibration curve

The values of S and σ are estimated as for the detection limit. The LOQ can also be established from the visual evaluation as the LOD. The analyte concentration should be quantifiable with acceptable accuracy and precision at LOQ level. Typical acceptance criteria for LOQ are mean recovery at this level between 50 – 150 % with % RSD of ≤ 25 %.

Table 1. Table showing different forced degradation conditions to be used for drug substances and drug products

SAMPLE	FORCED DEGRADATION STUDY
DRUG SUBSTANCES	
Solid	Photolytic, thermal, humidity
Solution/Suspension	Acid/Base hydrolysis, oxidative
DRUG PRODUCT	
Solid	Photolytic, oxidative, thermal, humidity
Semisolid	Photolytic, oxidative, thermal, humidity
Solution/Suspension	Photolytic, thermal, oxidative, hydrolysis

Table 2. Linearity ranges and Acceptance criteria for various pharmaceutical methods

Test	Linearity levels and ranges	Acceptance criteria
Assay	Five levels,	Correlation coefficient,
	50-150% of label claim	R ≥0.999
Dissolution	Five to eight levels,	% y intercept NMT 2.0%
	10-150% of label claim	R ≥0.99
Related Substances	Five levels,	% y intercept NMT 5.0%
	LOQ to acceptance criteria	R ≥0.99

Table 3. Limits for system suitability tests (SST)

SST	Limits
Resolution (Rs)	>2.0
Repeatability (RSD)	<1.0% for five replicates
Plate count (N)	>2000
Tailing factor (Tf)	≤2.0

Separation factor (α)	>1.0

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. It is partially evaluated during the method development stages. The robustness study aims to identify the critical operating parameters for the successful implementation of the method. These parameters should be adequately controlled and a precautionary statement included in the method documentation. In the case of an HPLC method, robustness study involves method parameters like pH, flow rate, column temperature, and mobile phase composition which are varied within a reasonable range. The system suitability parameters obtained for each condition are studied to check the parameter that significantly affects the method [21]. The stability of the analytical solution and extraction time are other parameters that are also evaluated as additional parameters during the robustness study. The stability of the analytical solution is determined by assessing the results obtained by subjecting the analytical solution to the method parameters for a longer period e.g. 4 hrs, 12 hrs, 24 hrs, 48 hrs, etc. The acceptance criteria are based on the relative difference between the initial value and the value at a specified solution stability time. For drug substances and products difference should be ≤ 2.0 % and for impurity determination, it should be ≤ 10 %. When filtration is done during sample preparation filter paper study can be carried out. It involves analysis by filtering sample solutions through different types of filter paper.

System suitability

System suitability testing (SST) is an integral part of many analytical procedures. The tests are based on the concept that the equipment, analytical operations, and samples are the integral part of the system that can be evaluated as such. System suitability tests provide the added assurance that on a specific occasion, the method is giving, accurate and precise results. System suitability test are run every time a method is used either before or during analysis. The results of each system suitability test are compared with defined acceptance criteria and if they pass, the method is deemed satisfactory on that occasion. In the case of HPLC methods, system suitability tests ensure the adequacy for performing the intended application on a daily basis. The primary SST parameters considered are resolution (Rs), repeatability (% RSD of peak response and retention time), column efficiency (N), and tailing factor (Tf). The other SST parameters include retention factor (k) and separation factor (α). The limits that are considered for the SST parameters are listed table 3 [15].

CONCLUSION

This comprehensive review has illuminated the intricate landscape of HPLC method development and validation, offering a holistic understanding of the pivotal techniques employed in analytical science. The discussion encompassed fundamental principles, optimization strategies, and validation parameters crucial for ensuring the robustness and reliability of HPLC methods. By understanding these critical steps, scientists can craft robust, reliable methods that ensure accurate and reproducible results. The integration of regulatory guidelines, particularly from the ICH, has emphasized the importance of adhering to stringent validation criteria in various industries. As the field continues to progress, the knowledge synthesized in this paper serves as a valuable resource, guiding researchers, analysts, and industry professionals toward the refinement of HPLC methodologies, ultimately advancing the

precision and efficacy of analytical processes in diverse applications

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