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International Journal of Pharmaceutical Research & Analysis

e-ISSN: 2249 – 7781
Print ISSN: 2249 – 779X

www.ijpra.com

HPLC METHOD DEVELOPMENT AND VALIDATION: AN OVERVIEW

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ABSTRACT

High performance liquid chromatography (HPLC) is an analytical technique which is proficient to separate, detect and quantify various drugs and its related degradants. An appropriate mobile phase, column, column temperature, wavelength and gradient must be found that affords suitable compatibility and stability of drug as well as degradants and impurities. This review covers the importance of RP-HPLC in analytical method development and their strategies along with brief knowledge of critical chromatographic parameters need to be optimized for an efficient method development.

Keywords: Method validation, Method development, High PressureLiquid Chromatography (HPLC).

INTRODUCTION

HPLC is an analytical technique in which solutes are resolved by differential rates of elution as they pass through a chromatographic column. The method of separation by this instrument is governed by distribution between the mobile phase and stationary phase. The instrumentation is made-up of eight basic components, mobile phase reservoir, solvent delivery system, sample introduction device, column, detector, waste reservoir, connective tubing and computer, integrator or recorder. The successful use of HPLC for the possible problem requires the right combination of variety of operating conditions such as the type of column packing and mobile phase, column length and diameter, mobile phase flow rate , column temperature and sample size [1]. Now a day reversed-phase chromatography is the most commonly used separation technique in HPLC due to its broad application range. It is estimated that over 65% (possibly up to 90%) of all HPLC separations are carried out in the reversed phase mode. The reasons for this include the simplicity, versatility and scope of the reversed-phase method as it is able to handle compounds of a diverse polarity and molecular mass [2-4].

PRINCIPLE

In isocratic HPLC the analyte is forced through a column of the stationary phase (usually a tube packed with small round particles with a certain surface chemistry) by pumping a liquid (mobile phase) at high pressure through the column. The sample to be analyzed is introduced in a small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase as it traverses the length of the column. The amount of retardation depends on the nature of the analyte, stationary phase and mobile phase composition. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time and is considered a reasonably unique identifying characteristic of a given analyte. The use of pressure increases the linear velocity (speed) giving the components less time to diffuse within the column, leading to improved resolution in the resulting chromatogram. Common solvents used include any miscible combinations of water or various organic liquids (the most common are methanol and acetonitrile). Water may contain buffers or salts to assist in the separation of the analyte components.

A further refinement to HPLC has been to vary the mobile phase composition during the analysis, this is known as gradient elution. A normal gradient for reverse phase chromatography might start at 5% methanol and progress linearly to 50% methanol over 25 minutes,

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depending on how hydrophobic the analyte is. The gradient separates the analyte mixtures as a function of the affinity of the analyte for the current mobile phase composition relative to the stationary phase. This partitioning process is similar to that which occurs during a liquid-liquid extraction but is continuous, not step-wise. In this example, using a water/methanol gradient, the more hydrophobic components will elute (come off the column) under conditions of relatively high methanol; whereas the more hydrophilic compounds will elute under conditions of relatively low methanol. The choice of solvents, additives and gradient depend on the nature of the stationary phase and the analyte. Often a series of tests are performed on the analyte and a number of generic runs may be processed in order to find the optimum HPLC method for the analyte - the method which gives the best separation of peaks.

APPLICATIONS

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 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. In order 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 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 last is the choice in flow rate. All of these topics are reviewed in detail in this document.

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 in order 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 the area of 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 mobile phase, columns may be C_{18} (ODS), C_8, phenyl, TrimethylSilane (TMS), cyano as a stationary phase. It is first choice for most samples especially neutral or non ionized compounds, that dissolve in water organic mixtures.

2. Normal Phase HPLC

In normal-phase chromatography, the stationary phase is polar and the mobile phase is nonpolar. In 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 mobile phase and columns i.e. cyano, diol and amino silica can be used as stationary phase. It is first choice for mixtures of isomers and for preparative scale HPLC and 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

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 analyse 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].

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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 effect 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 onto 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, 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].

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].

Step 3 - Method optimization

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

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 which 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 inerferents [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 provides responses for a number of chemical entities that may or may not be distinguished from each other. The International Union of Pure and Applied Chemistry (IUPAC) have expressed the view that "Specificity is the ultimate of Selectivity'. The IUPAC discourages 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 or 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 are summarized in table 1 below [12]. The selectivity of chromatographic methods may be assessed by examination of peak homogeneity or peak purity test. 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 labelled should be included with resolution, plate count and tailing factor reported in the validation report.

Linearity

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.

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is 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

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linearity. It is generally expressed in terms of variance around the slope of 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 the table 2 [13].

Precision

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 as 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 repeated determination of same sample.

Intermediate precision expresses within laboratories variation: different days, different analyst, different equipments, 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, 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 by means of an inter-laboratory trial.

The precision data is generally expressed in the form of standard deviation, relative standard deviation and confidence intervals. To ensure precision of method for major analytes, RSD should be $\leq$ 2 %. For low level impurities, RSD of 5-10 % is usually acceptable [14].

Range

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 covering from 80 to 120 percent of the test concentration.
2. For content uniformity, covering 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 true or 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 case of methods for quantitation of impurities, the sample with known amount of impurities is assessed. Accuracy should be assessed using minimum of nine determinations over a minimum of three concentration levels covering the specified range (for e.g., three concentrations/ three replicates each of the total analytical procedure).

Accuracy should be reported as percent recovery by the assay of 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, range from 50 - 150 % of average recovery may be accepted [2].

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 concentration of analyte with those of blank samples. The concentration showing signal to noise ratio between 3:1 or 2:1 is generally considered as acceptable detection limit.

The other approach is based on the standard

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deviation of the response and the slope. The detection limit may be expressed as:

$$LOD = {}^{3.3}\sigma/s$$

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 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 base on visual evaluation. This method is applicable to non-instrumental methods but may be applied to the 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

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 the 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:

$$LOQ = {}^{10}\sigma/s$$

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

The value 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 products	
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	Limits
Resolution (R_s)	>2.0
Repeatability (RSD)	<1.0% for five replicates
Plate count (N)	>2000
Tailing factor (T_f)	≤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 method development stages. The aim of the robustness study is 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 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 which significantly affects the method.

Stability of the analytical solution and extraction time are other parameters which are also evaluated as additional parameters during robustness study. Stability of analytical solution is determined by assessing the results obtained by subjecting the analytical solution to the method parameters for longer period of time e.g. 4 hrs, 12 hrs, 24 hrs, 48 hrs, etc. The acceptance criteria are based on relative difference between initial value and the value at 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 solution 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 test 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 case of HPLC methods, system suitability tests ensure the adequacy for performing the intended application on daily basis. The primary SST parameters considered are resolution (R_s), repeatability (% RSD of peak response and retention time), column efficiency (N), and tailing factor (T_f). The other SST parameters include retention factor (k) and separation factor (α). The limits which are considered for the SST parameters are listed table 3 [15].

CONCLUSION

The method development and validation are continuous and interrelated processes that are conducted throughout the drug development process. The analytical validation verifies that a given method measures a parameter as intended and establishes the performance limits of the measurement. Reproducible quality HPLC results can only be obtained if proper attention has been paid to the method development, validation and system's suitability to carry out the analysis. The validated methods produce results within known uncertainties that are helpful to continuing drug development and provide emerging knowledge supporting the product. The time and effort that is devoted into developing scientifically sound and robust analytic methods should be aligned with the drug development stage. The resources that are constantly used during method development and validation must be balanced with regulatory requirements and the probability for product commercialization.

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