MANTECH Publications

Journal of Pharmacovigilance and Quality Assurance
Volume 3, Issue 1, January-June, 2021

Review on Analytical Method Development and Validation of HPLC

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Abstract

Chromatography is the important technique of separation science and used in all pharmaceutical industries and research laboratories. High Performance Liquid Chromatography is the most important technique used to identify, detect, separate and quantify the drug. There are various chromatographic parameters were analyzed like physiochemical properties of drug, chromatographic conditions, sample preparation, detection of analyte etc. Review the method development and validation of HPLC system is the main objective of these articles. Validation of HPLC method contains system suitability, specificity, linearity, range, accuracy, precision, robustness, LOD, LOQ. Validation of HPLC system is done as per ICH guidelines.

Keywords: *Chromatography, HPLC, Validation, ICH guidelines.*

INTRODUCTION

Analytical chemistry is the science of obtaining, processing and communicating information about the composition and structure matters. In other words, it is the art and science of determining what matter is and how much of it exists.[1]

High Performance Liquid Chromatography (HPLC) is a process of separating components in a liquid mixture. A liquid sample is injected into a stream of solvent (mobile phase) flowing through a column packed with a separation medium (stationary phase). Sample components separate from one another by a process of

differential migration as they flow through the column.[2]

TYPES OF HPLC TECHNIQUE

1. **Based on Modes of Chromatography**
   a) Normal phase chromatography
   b) Reversed phase chromatography

2. **Based on Principle of Separation**
   a) Adsorption chromatography
   b) Chiral phase chromatography
   c) Size exclusion or Gel permeation chromatography
   d) Ion exchange chromatography
   e) Ion pair chromatography
   f) Affinity chromatography

3. **Elution Technique**
   a) Isocratic separation
   b) Gradient separation

4. **Based on Scale of Operation**
   a) Analytical HPLC
   b) Preparative HPLC

5. **Based on Type of Analysis**
   a) Qualitative analysis
   b) Quantitative analysis

METHOD DEVELOPMENT ON HPLC

1. **Understanding the Physiochemical Properties of Drug Molecule: [4]**

Study of physiochemical properties of drug molecule plays an important role in method development. For method development one has to study physical properties like solubility, polarity, pka and PH of drug molecule. Polarity is a physical property of a compound. It helps an analyst, to decide solvent and composition of the mobile phase. The solubility of molecules can be explained on the basis of polarity of molecules. Polar e.g., water, Nonpolar e.g., benzene, solvents do not mix. In general, like dissolves like i.e. materials with similar polarity are soluble in each other. The selection of mobile phase or diluents is based on solubility of analyte. The analyte must be soluble in diluents and must not react with any of its component. PH, Pka plays an important role in HPLC method development. The PH value is defined as negative of the logarithm to base 10 of concentration of hydrogen ion.

$$PH = -\log 10[H_3O_+]$$

Selecting a proper PH for ionizable analytes often leads to symmetrical and sharp peak in HPLC. Sharp, symmetrical peaks are necessary in quantitative

analysis in order to achieve low detection limits, low relative standard deviation between injections and reproducible retention times.

2. Selection of chromatographic conditions: [5]

Selection of column*:* Selection of the stationary phase/column is the first and the most important step in method development. The development of a rugged and reproducible method is impossible without the availability of a stable, high performance column. To avoid problems from irreproducible sample retention during method development, it is important that columns be stable and reproducible. A C8 or C18 column made from specially purified, less acidic silica and designed specifically for the separation of basic compounds is generally suitable for all samples and is strongly recommended3. The separation selectivity for certain components varies between the columns of different manufacturer as well as between column production batches from the same manufacturer. Column dimensions, silica substrate properties and bonded stationary phase characteristics are the main ones. The use of silica-based packing is favored in most of the present HPLC columns due to several physical characteristics. Silica substrates are available in spherical or irregular shapes and can be prepared with different surface areas, pore sizes and particle sizes, which make them suitable for most HPLC applications. ®-MS phases are stable over the pH range of 1-10. Totally porous silica particles with 5 μm diameter provide the desired characteristics for most HPLC separations. Zirconia-based columns are revolutionary HPLC phases. Zirconia particles are mechanically stable, and have a porous structure similar to that of silica. However, zirconia's main advantage over silica is that it is very stable in a wide range of eluent pH; indeed, the ZirChrom®-EZ and ZirChrom.

Selection of column oven temperature:

Separation of many samples can be enhanced by selecting the right column temperature. Higher column temperature reduces system backpressure by decreasing mobile phase viscosity, which in turn allows use of longer columns with higher separation efficiency. However, an overall loss of resolution between mixtures components in many samples occurs by increasing column temperature.The optimum temperature is dependent upon nature of the mixture components. The overall separation can be improved by simultaneous changes in column temperature and mobile phase composition.

Selection of Mobile Phase: The mobile phase effects resolution, selectivity and efficiency. Mobile phase composition (or solvent strength) plays an important role in RP-HPLC separation. 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 initial choice for the mobile phase during method development. Selection of the mobile-phase and gradient conditions is dependent on the ionogenic nature of the analyte and the hydrophobicity of the analytes in the mixture respectively. The aqueous buffer serves several purposes. At low pH, the mobile phase protonates free silanols on the column and reduces peak tailing. At sufficiently low pH basic analytes are protonated; when ionized the analyte will elute more quickly but with improved peak shape. Acidic analytes in buffers of sufficiently low pH will remain uncharged, increasing retention. Conversely, at higher pH neutral basic compounds will be more retained, and ionized acidic compounds will elute earlier. Peak splitting may be observed if the pKa of a compound is similar to the pKa of the buffer, and the analyte elutes as both a charged and uncharged species. The pH of a buffer will not greatly affect the retention of non-ionizable sample components. The most commonly employed buffer is phosphate buffer The pH of a phosphate buffer is easily adjusted by using monodi-, or tribasic phosphate salts. However, when phosphate salts are used the solution should be filtered to remove insoluble par ticles with 0.22µm filter paper1 2. Acetonitrile and Methanol are 2 most widely employed organic modifiers Isopropanol (2-propanol) can be employed because of its strong eluting properties, but is limited by its high viscosity which results in lower column efficiencies and higher backpressures. Both acetonitrile and methanol are less viscous than isopropanol. All three solvents are essentially UV transparent. This is a crucial property for reversed phase chromatography since column elution is typically monitored using UV detectors. Acetonitrile is used almost exclusively when separating peptides. Most peptides only absorb at low wavelengths in the ultra-violet spectrum (typically less than 225 nm) and acetonitrile provides much lower background absorbance than other common solvents at low wavelengths.

Selection of elution mode: There are basically 2 modes of elution are employed Isocratic and Gradient. Isocratic, constant

eluent composition means equilibrium conditions in the column and the actual velocity of compounds moving through the column are constant; analyte-eluent and analyte-stationary-phase interactions are also constant throughout the whole run. This makes isocratic separations more predictable, although the separation power (the number of compounds which could be resolved) is not very high. The peak capacity is low; and the longer the component is retained on the column, the wider is the resultant peak. Gradient separation significantly increases the separation power of a system mainly because of the dramatic increase of the apparent efficiency (decrease of the peak width). Gradient elution is employed for complex multicomponent samples since it may not be possible to get all components eluted between k (retention factor) 1 and 10 using a single solvent strength under isocratic conditions. When a gradient method is used, the column must be allowed to equilibrate at the starting mobile-phase conditions prior to the next sample injection and the start of the next gradient run.

Selection of detector: Detector is a very important part of HPLC. Selection of detector depends on the chemical nature of analytes, potential interference, limit of detection required, availability and/or cost of detector. UV-Visible detector is versatile, dual-wavelength absorbance detector for HPLC. This detector offers the high sensitivity required for routine UV-based applications to low-level impurity identification and quantitative analysis. Photodiode Array (PDA) Detector offers advanced optical detection for Waters analytical HPLC, preparative HPLC, or LC/MS system solutions. Refractive Index (RI) Detector offers high sensitivity, stability and reproducibility, which make this detector the ideal solution for analysis of components with limited or no UV absorption. Multi-Wavelength Fluorescence Detector offers high sensitivity and selectivity fluorescence detection for quantitating low concentrations of target compounds. Characteristics that are to be fulfilled by a detector to be used in HPLC determination are:

- ✓ High sensitivity, facilitating trace analysis.
- ✓ Negligible baseline noise to facilitate lower detection.
- ✓ Low drift and noise level.
- ✓ Wide linear dynamic range (this simplifies quantitation).
- ✓ Low dead volume (minimal peak broadening).

- ✓ Cell design that eliminates remixing of the separated bands.
- ✓ Insensitivity to changes in type of solvent, flow rate and temperature.
- ✓ Operational simplicity and reliability.

DEVELOPING APPROACH OF ANALYSIS

The analytical method on RP-HPLC the first step performed is the selection of various chromatographic parameter like selection of mobile phase, selection of column, selection of PH of mobile phase, flow rate of mobile phase in developing method. All of these parameters selected on the basis of trials and followed by considering system suitability parameters.

Detection of wavelength is usually isobestic point in the case simultaneous estimation of two components. After this the linearity of the drug is studied in order to know the range of concentration up to which drug follows linear pattern.

Analysis of laboratory mixture is also carried out in order to know predictability of developed method for simultaneous estimation. After that analysis of marketed formulation is carried out by diluting the marketed formulation up to concentration range of linearity.

SAMPLE PREPARATION

The prepared sample should be an adiquot relatively free of interferences that is suitable with HPLC method and that will not damage column.

METHOD OPTIMIZATION

After the individual components of methods are optimized LOQ use an experimental design approach to determine the experimental factors that have significant impact on the method. This is very important in determining what factors need to be investigated in the robustness testing during method validation. The streamline the method optimization process, use Placket Burmann Design or similar approach to simultaneously determine the main effects of many experimental factors. Some of the typical experimental factor that need to be investigated are:

HPLC conditions: %organic, PH, flow rate, temperature, wavelength, column age.

Sample preparation: % organic, PH, shaking/sonication, sample size and sample age.

Calculation/ standardization: Integration, wavelength, standard concentration, response factor correction.

METHOD VALIDATION

Validation means assessment of validity or action of proving effectiveness. Method validation is the process of proving that the analytical method is an acceptable for its intended purpose. For pharmaceutical methods guidelines from the United States of Pharmacopoeia (USP). International Conference of Harmonization (ICH) and Food and Drug Administration (FDA) provide a frame work for performing such validations.

PARAMETERS OF HPLC VALIDATION

a) System suitability parameter
b) Specificity
c) Linearity and Range
d) Accuracy
e) Precision
f) LOD
g) LOQ
h) Robustness
i) Forced degradation studies

a) System Suitability parameter: System suitability test is used to check the sensitivity, resolution, reproducibility of the chromatographic system as well for the analysis to be done. The factors mainly used in system suitability are tailing factor, a number of theoretical plate, retention time, resolution etc.

Table 1: Acceptance Criteria of System Suitability Parameters

Sr. No.	Parameter Name	Acceptance Criteria
1.	Tailing factor	< 2
2.	Number of theoretical plates	>2000
3.	Resolution	>1.5
4.	RSD	<2

b) Specificity: Selectivity of an analytical method as its ability to measure accurately an analyte in the presence of interferences, such as synthetic precursors, excipients, enantiomers and known (or likely) degradation products that may be excepted to be present in the sample matrix.

c) Linearity and Range: The linearity of an analytical procedure is its ability (within the given range) to obtain test results, which are directly proportional to concentration (amount) of analyte in the sample. A linear relationship should be evaluated across the range of the analytical procedure. It is demonstrated directly on the drug substance by dilution of a standard stock solution of drug product components, using the proposed procedure for the establishment of

linearity, minimum of five concentrations are recommended by ICH guidelines. The range of an analytical method is the interval between the upper and lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written.

d) Accuracy: It is calculation of empirical methods precision, or consistency of agreement between the calculated value and the true or agreed reference value.

e) Precision: The precision of an analytical procedure represents the nearness of agreement between a series of measurement got from multiple sampling of the same homogeneous sample under the similar analytical conditions and it is divided into three categories-

Repeatability: Precision under the same operating conditions, some analyst over a short period of time.

Intermediate Precision: Method is tested or multiple days, instruments, analyst etc.

Reproducibility: Inter-laboratory studies. The Ich guidelines suggest that reproducibility should be conformed duly utilizing at least nine determinations with specified range for the procedure (e.g. three concentrations/ three replicates each) or a minimum of six determinations at 100% of the test concentrations.

f) Limit of Detection (LOD): Limit of detection (LOD) of an individual procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. In analytical procedure that exhibits baseline noise, the LOD can be based on the signal to noise (S/N) ratio (3:1) which is usually expressed as the concentration of analyte in the sample.

The signal to noise ratio is determined by :

S= H/h,

Where,

H= Height of the peak to corresponding to the component.

h = Absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.

g) ***Limit of Quantitation (LOQ):*** The Limit of Quantitation (LOQ) or quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. For analytical procedures such as HPLC that exhibit baseline noise, the LOQ is generally estimated from a determination of S/N ratio and usually confirmed by injecting standard which gives this S/N ratio and have an acceptable percent relative standard deviation as well.

h) ***Robustness:*** It is characterized by the level of ability of an analytical technique, to stay similar by minute purposely change in the technique parameter. The different technique parameter which can be modified in HPLC are PH, drift rate, temperature of the column and mobile phase composition.

i) ***Forced Degradation Studies:*** Forced degradation or stress studies are undertaken to deliberately degrade the sample. These studies are used to evaluate an analytical methods ability to measure an active ingredient and its degradation products, without interference, by generating potential degradation products. During validation of the method, drug substance are supposed to acid, base, heat, light and oxidizing agent to produce approximately 10% to 30% degradation of active substance. The studies can also provide information about degradation pathway and degradation product that could be form during storage. These studies may also help in formulation development, manufacturing and packaging to improved drug product. Reasons for carrying out forced degradation studies include: Development and validation of stability indicating methodology, determination of degradation pathways of drug substances and drug products, discernment of degradation products in formulation that are related to drug substances versus those that are related to non-drug substances (i.e., excipients).

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