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# A REVIEW ON HPLC METHOD DEVELOPMENT AND VALIDATION

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**ABSTRACT:** HPLC is the dominant separation technique to detect, separate and quantify the drug. A number of chromatographic parameters were analyzed to optimize the method like sample pretreatment, choosing mobile phase, column, detector selection. The objective of this article is to review the method development, optimization and validation. HPLC method development depends on chemical structure of the molecules, synthetic route, solubility, polarity, pH and pKa values, and functional groups activity etc. Validation of HPLC method as per ICH Guidelines gives information regarding various stages and knowing characteristics like Accuracy, specificity, linearity limit of detection, limit of quantification.

**KEYWORDS:** High Pressure Liquid Chromatography (HPLC), Method validation, Method development

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## 1.INTRODUCTION

High Performance Liquid Chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid. High performance liquid chromatography (HPLC) is the most accurate analytical methods widely used for the quantitative as well as qualitative analysis of drug product.[1] The principle is that a solution of the sample is injected into a column of a porous material (stationary phase) and a liquid (mobile phase) is pumped at high pressure through the

column. The separation of sample is based on the differences in the rates of migration through the column arising from different partition of the sample between the stationary and mobile phase. Depending upon the partition behaviour of different components, elution at different time takes place.
[2] The sample compound with the greater affinity to the stationary layer will travel slower and for a shorter distance in comparison to compounds with less affinity which travel faster and for a longer distance. [3] The High Performance Liquid Chromatography is more versatile than gas chromatography since (a) it is not limited to volatile and thermally stable samples, and (b) the choice of mobile and stationary phases is wider. [4]
HPLC has numerous advantages like
• Simultaneous Analysis
• High Resolution
• High Sensitivity
• Good repeatability
• Small sample size
• Moderate analysis condition.
• Easy to fractionate the sample and purify. [5]

**Classification of HPLC can be done as:**

- preparative HPLC and analytical HPLC (based on scale of operation)
- affinity chromatography, adsorption chromatography, size exclusion chromatography, ion exchange chromatography, chiral phase chromatography (based on principle of separation)
- gradient separation and isocratic separation, (based on elution technique)
- normal phase chromatography and reverse phase chromatography (based on modes of operation).[6]

**A. Normal phase chromatography:**

In normal phase chromatography, mobile phase is non-polar and stationary phase is polar. Hence, the station phase retains the polar analyte. An increase in polarity of solute molecules increases the adsorption capacity leading to an increased elution time. Chemically modified silica (cyanopropyl, aminopropyl and diol) is used as a stationary phase in this chromatography. [7] For example. A typical column has an internal diameter of around 4.6 mm, and a length in the range of 150 to 250 mm. Polar compounds in the mixture that are passed through the column will stick longer to the polar silica than the non-polar compounds. Therefore, the non-polar ones will pass more quickly through the column.[8]

**B. RP-HPLC (Reversed phase HPLC):**

RP-HPLC has a non-polar stationary phase and polar or moderately polar mobile phase. RP-HPLC is based on the principle of hydrophobic interaction [9].In a mixture of components those analytes which are relatively less polar will be retained by the non-polar stationary phase longer than those which are relatively more polar. Therefore the most polar component will elute first. [10]

**METHOD DEVELOPMENT ON HPLC**

A step involved in method development of HPLC is as follows:

1 Understanding the Physicochemical properties of drug molecule.

2 Selection of chromatographic conditions.

3 Developing the approach of analysis.

4 Sample preparations

5 Method optimization

6 Method validation

**1. Understanding the Physicochemical properties of drug molecule.**

Physicochemical properties of a drug molecule play an important role in method development. For Method development one has to study the physical properties like solubility, polarity, pKa and pH of the drug molecule.Polarity is a physical property of a compound. It helps an analyst, to decide the solvent and composition of the mobile phase.The solubility of molecules can be explained on the basis of the polarity of molecules. Polar, e.g. water, and 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 the solubility of analyte. The analyte must be soluble in diluents and must not react with any of its component. pH and pKa plays an important role in HPLC method development. The pH value is defined as the negative of the logarithm to base 10 of the concentration of the hydrogen ion.

$$pH = - \log10[H3O+].$$

Selecting a proper pH for ionizableanalytes often leads to symmetrical and sharp peaks in HPLC. Sharp, symmetrical peaks are necessary in quantitative analysis in order to achieve low detection limits, low relative standard deviations between injections, and reproducible retention times. [11-12]

**1.Selection of chromatographic conditions:**

**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 recommended. [13] 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. [14]

**Buffer Selection**

Choice of buffer is governed by the pH that is desired. The typical pH range for reversed phase on silica based packing is pH 2 to 8. It is important that the buffer has a pKa close to the desired pH since buffer controls pH best at their pKa. A rule is to choose a buffer with a pKa value <2 units of the desired mobile phase pH.

General consideration for buffer selection:

1. Phosphate is more soluble in methanol/water than in acetonitrile/water or THF/water.
2. Some salt buffers are hygroscopic and this may lead to changes in the chromatography like increased tailing of basic compounds and possibly selectivity differences.
3. Ammonium salts are generally more soluble in organic/water mobile phases·
4. Trifluoroacetic acid can degrade with time. It is volatile and absorbs at low UV wavelengths.
5. Microbial growth can quickly occur in buffered mobile phases that contain little or no organic modifier at all. The growth accumulates on column inlets and can damage chromatographic performance.
6. At pH greater than 7, phosphate buffer accelerates the dissolution of silica and severely shortens the lifetime of silica-based HPLC columns. If possible, organic buffers should be used at pH greater than 7.
7. Ammonium bicarbonate buffers usually are prone to pH changes and are usually stable for only 24 - 48 hrs. The pH of this mobile phase tends to become more basic due to the release of carbon dioxide.
8. After buffers are prepared, they should be filtered through a 0.2-µm filter.
9. Mobile phases should be degassed. [15]

**Buffer Concentration**

Generally, a buffer concentration of 10-50 mM is adequate for small molecules. Generally, no more than 50% organic should be used with a buffer. This will depend on the specific buffer as well as its concentration. Phosphoric acid and its sodium or potassium salts are the most common buffer systems for reversed-phase HPLC. Sulfonate buffers can replace phosphonate buffers when analyzing organophosphate compounds. [16]

**Isocratic and Gradient Separations:**

Isocratic mode of separation includes constant eluent composition; means equilibrium conditions in the column and the actual velocity of compounds moving through the column are constant. The peak capacity is low and the longer the component is retained on the column the wider is the resultant peak. Gradient mode of separation includes significantly increases the separation power of a system mainly due to increase of the apparent efficiency (decrease of the peak width). Peak width varies depending on the rate of the eluent composition variation. In deciding whether a gradient or isocratic would be required an initial gradient run is performed and the ratio between the total gradient time and the difference in the gradient time between the first and last component are calculated. The calculate ratio is <0.25 isocratic is adequate. When the ratio is >0.25 gradient would be adequate [9]

**Internal Diameter**

The internal diameter (ID) of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution. It also determines the quantity of analyte that can be loaded into a column. **[**17]

**Particle size**

Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles. These silica particles come in many sizes with 5 μm beads being the most commonly used. The smaller particles usually provide more surface area and better separations but the pressure required for the optimum linear velocity increases by the inverse of the particle diameter squared. Larger particles are used in preparative HPLC where column diameters are in range of 5 cm to >30 cm and for non-HPLC applications such as solid – phase extraction. [18-19]

**Pore size**

Pore size of column defines an ability of the analyte molecules to penetrate inside the particle and interact with its inner surface [20]

**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. [21]

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

**Selection of detectors**

Detector is a very important part of HPLC. Selection of detector depends on the chemical nature of analyses, 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. Its integrated software and optics innovations deliver high chromatographic and spectral sensitivity. Refractive index chromatographic and spectral 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. [22-23]

| Detector | Type of compound can be detected |
|---|---|
| UV-Visible &Photodiode array | Compounds with chromophores, such as aromatic rings or multiple alternating double bonds. |
| Fluorescence detector | Fluorescent compounds, usually with fused rings or highly conjugated planer system. |
| Conductivity detector | Charged compounds, such as inorganic ions and organic acid. |
| Electrochemical detector | For easily oxidized compounds like quinines or amines |
| Refractive Index detector & Evaporative light scattering detector | Compounds that do not show characteristics usable by the other detectors, eg.polymers, saccharides. |

**3. Developing the approach for analysis:** While developing the analytical method on RP-HPLC the first step which is followed is the selections of various chromatographic parameters like selection of mobile phase, selection of column, selection of flow rate of mobile phase, selection of pH of mobile phase. All of these parameters are selected on the basis of trials and followed by considering the system suitability parameters. Typical parameters of system suitability are e.g. retention time should

be more than 5 min, the theoretical plates should be more than 2000, the tailing factor should be less than 2, resolution between 2 peaks should be more than 5, % R.S.D. of the area of analyte peaks in standard chromatograms should not be more than 2.0 %.like other. Detection wavelength is usually isobestic point in the case of simultaneous estimation of 2 components. After this the linearity of the drug is studied in order to know the range of concentrations up to which the drug follows the linear pattern. Analysis of the laboratory mixture is also carried out in order to know practicability 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. [24-29]

**4. Sample preparation:** 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.[30]

**5. Method optimization:** Identify the "weaknesses" of the method and optimize the method through experimental design. Understand the method performance with different conditions, different instrument set ups and different samples. [31]

**6. Method Validation**

Validation is the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled. A process of evaluating method performance and demonstrating that it meets a particular requirement. In essence, it knows what your method is capable of delivering, particularly at low concentrations. [32]

**Types of Analytical Procedures to be validated**

The discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures:

- Identification tests;
- Quantitative tests for impurities' content;
- Limit tests for the control of impurities;
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product. [33]

**Components of method validation:** The following are typical analytical performance characterstics which may be tested during methods validation:

1. Accuracy
2. Precision
3. Linearity
4. Detection limit
5. Quantitation limit
6. Specificity
7. Range
8. Robustness

**Accuracy**

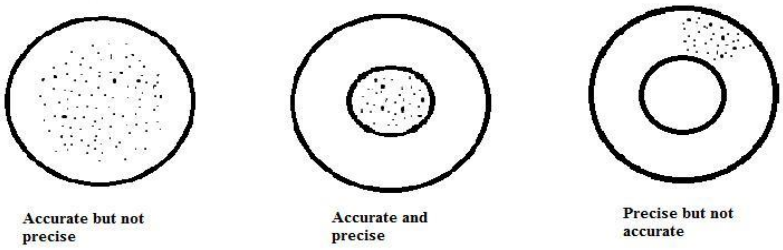
Accuracy is defined as the nearness of a measured value to the true or accepted value. Practically accuracy indicates the deviation between the mean value found and the true value. lt is determined by applying the method to samples to which known amounts of analyte have been added. These should be analysed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay. It may often be expressed as the recovery by the assay of known, added amounts of analyte. [34]

**Precision**

It expresses closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision is a measure of the reproducibility of the whole analytical method. [35] It consists of two components: repeatability and intermediate precision. Repeatability is the variation experienced by a single analyst on a single instrument. It does not distinguish between variation from the instrument or system alone and from the sample preparation process. During validation, repeatability is performed by analyzing multiple replicates of an assay composite sample by using the analytical method. The recovery value is calculated. Intermediate precision is the variation within a laboratory such as different days, with different instruments, and by different analysts. [36-37] The precision is then expressed as the relative standard deviation.

$$\%RSD = \frac{\text{std dev.} * 100}{\text{mean}}$$

Accuracy and precision are not the same, as the diagram below indicates. A method can have good precision and yet not be accurate.




**Linearity**

Linearity is the ability of analytical procedure to obtain a response that is directly proportional to the concentration (amount) of analyte in the sample. If the method is linear, the test results are directly or by well-defined mathematical transformation proportional to concentration of analyte in samples within a given range. Linearity is usually expressed as the confidence limit around the slope of the regression line. [38]

**Limits of detection and quantitation:** The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantified. LOD is expressed as a concentration at a specified signal : noise ratio, usually 3:1. The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. For LOQ, ICH has recommended a signal:noise ratio 10:1. LOD and LOQ may also be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve(s) at levels approximating the LOD according to the given below formulae.[26**,**39]

$$LOD = 3.3 \times S / SD \text{ and}$$

$$LOQ = 10 \times S / SD$$

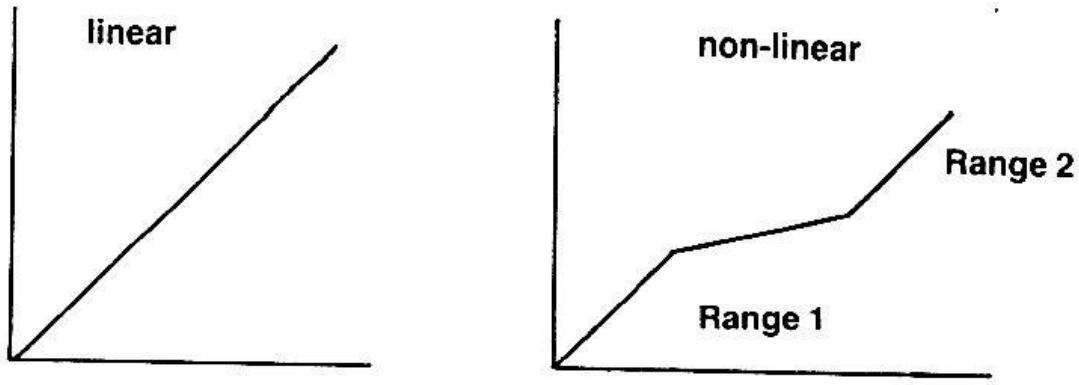
**Specificity**

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s). This definition has the following implications: Identification: to ensure the identity of an analyte. Purity Tests: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual

solvents content, etc. Assay (content or potency): to provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample. [40]

**Range**

The range of the method is the interval between the upper and lower levels of an analyte that have been determined with acceptable precision, accuracy and linearity. It is determined on either a linear or nonlinear response curve (ie where more than one range is involved, as shown below) and is normally expressed in the same units as the test results. [41]




**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. [42]

**2.CONCLUSION**

This review describes about RP-HPLC Technique. The method development and validation are continuous and interrelated processes that measure a parameter as intended and establish the performance limits of the measurement. The selection of Column, buffer, detector and wavelength and another conditions composition (organic and pH) plays a dramatic role on the separation selectivity The advantages of HPLC technique were high selectivity, sensitivity, economic, less time consuming and low limit of detection. Final optimization can be performed by changing the gradient slope, temperature and flow rate as well as the type and concentration of mobile-phase modifiers. Optimized method is validated with various parameters (e.g. specificity, precision, accuracy, detection limit, linearity, etc.) as per ICH guidelines.

**CONFLICT OF INTEREST**

The authors have no conflict of interest.

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