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Review Article

A Review: HPLC Method Development and Validation

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Abstract

HPLC is the dominant separation technique in modern pharmaceutical and biomedical analysis because it results in highly efficient separations and in most cases provides high detection sensitivity. Most of the drugs in multi component dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC methods development and validation play important roles in new discovery, development, manufacture of pharmaceutical drugs and various other studies related to humans and animals. An analytical procedure is developed to test a defined characteristic of the drug substance or drug product against established acceptance criteria for that characteristic. This review gives information regarding various stages involved in development and validation of HPLC method. Validation of HPLC method as per ICH Guidelines covers all the performance characteristics of validation, like Accuracy, precision, specificity, linearity, range and limit of detection, limit of quantification, robustness and system suitability testing.

Keywords: HPLC, Method development, Validation.

1.0 Introduction:

High Performance Liquid Chromatography (HPLC) was derived from the classical column chromatography and, is one of the most important tools of analytical chemistry today.[1]In the modern pharmaceutical industry, high-performance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development, and production.[2] HPLC is the method of choice for checking peak purity of new chemical entities, monitoring reaction changes is in synthetic procedures or scale up, evaluating new formulations and carrying out quality control / assurance of the final drug products.[3] The Goal of HPLC method is to try & separate, quantify the main drug, any reaction impurities, all available synthetic intermediates and any degradants. [4]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. HPLC is the most accurate analytical methods widely used for the quantitative as well as qualitative analysis of drug product and used for determining drug product stability. [5] HPLC principle is the solution of sample is injected into a column of porous material (stationary phase) and liquid phase (mobile phase) is pumped at higher pressure through the column. The principle of separation followed is the adsorption of solute on stationary phase based on its affinity towards stationary phase. (Figure-1) The technique of HPLC has following features.[6]

- High resolution
- Small diameter, Stainless steel, Glass column
- Rapid analysis
- Relatively higher mobile phase pressure
- Controlled flow rate of mobile phase

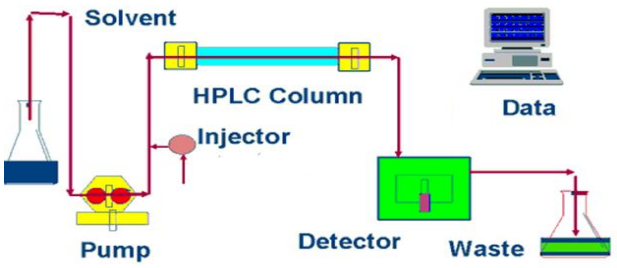

Fig-1: Flow Diagram of HPLC

2.0 HPLC Method Development:

Methods are developed for new products when no official methods are available. Alternate method for existing (Non-Pharmacopoeial) products are to reduce the cost and time

for better precision and ruggedness. When alternate method proposed is intended to replace the existing procedure comparative laboratory data including merit/demerits are made available. The goal of the HPLC-method is to try & separate, quantify the main active drug, any reaction impurities, all available synthetic inter-mediates and any degradants.[7]

Steps involved in Method development are. [6,7]

- Understanding the Physicochemical properties of drug molecule.
- Selection of chromatographic conditions.
- Developing the approach of analysis.
- Sample preparation
- Method optimization
- Method validation (figure-2)

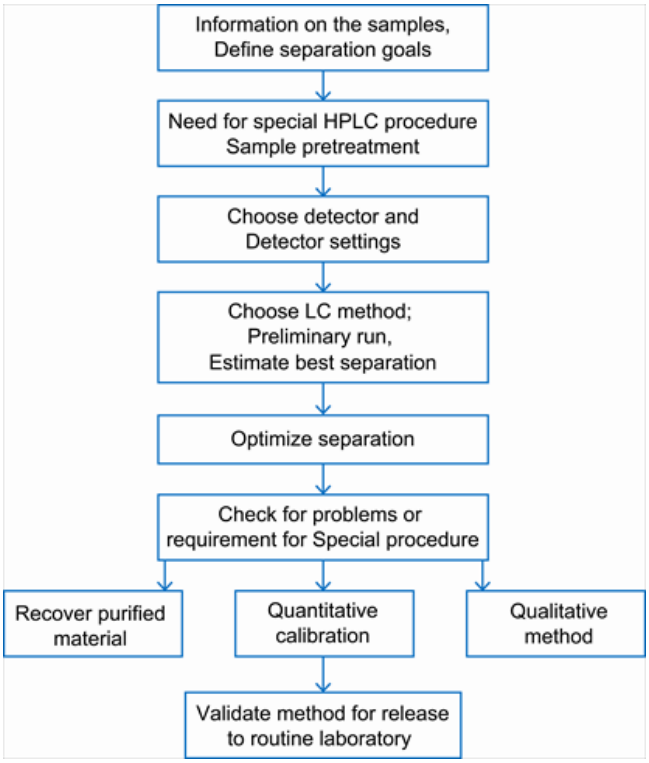

Figure -2: Steps involved in HPLC Method development

2.1 Understanding the physicochemical properties of drug molecules:

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. [6] 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. Selection of diluents is based on the solubility of analyte. The acidity or basicity of a substance is defined most typically by the pH value. Selecting a proper pH for ionizable analytes often leads to symmetrical and sharp peaks in HPLC.[7]

2.2 Selection of chromatographic conditions

During initial method development, a set of initial conditions (detector, column, mobile phase) is selected to obtain the first "scouting" chromatograms of the sample. In most cases, these are based on reversed-phase separations on a C18 column with UV detection. A decision on developing either an isocratic or a gradient method should be made at this point.

2.2.1 Selection of Column:

A column is of course, the starting and central piece of a chromatograph. A appropriately selected column can produce a good chromatographic separation which provides an accurate and reliable analysis. An improperly used column can often generate confusion, inadequate, and poor separations which can lead to results that are invalid or complex to interpret.[9]The heart of a HPLC system is the column. Changing a column will have the greatest effect on the resolution of analytes during method development. Choosing the best column for application requires consideration of stationary phase chemistry, retention capacity, particle size, and column dimensions. The three main components of an HPLC column are the hardware, the matrix, and the stationary phase. There are several types of matrices for support of the stationary phase, including silica, polymers, alumina, and zirconium. Silica is the most common matrix for HPLC columns. Silica matrices are robust, easily derivatized, manufactured to consistent sphere size, and does not tend to compress under pressure. Silica is chemically stable to most organic solvents and to low pH systems. One short coming of a silica solid support is that it will dissolve above pH 7. In recent years, silica supported columns have been developed for use at high pH. The nature, shape and particle size of the silica support effects separation. Smaller particle results in a greater number of theoretical plates, or increased.The nature of the stationary phase will determine whether a column can be used for normal phase or reverse phase chromatography. Normal phase chromatography utilizes a polar stationary phase and a non-polar mobile phase. Generally, more polar compounds elute later than non-polar compounds. Commonly used reverse phase columns and their uses are listed below. Propyl (C3), Butyl (C4), and Pentyl (C5) phases are useful for ion-pairing chromatography (C4) and peptides with hydrophobic residues, and other large molecules. C3–C5 columns generally retain non-polar solutes more poorly when compared to C8 or C18 phases. Examples include Zorbax SB-C3, YMC-Pack C4, and Luna C5. These columns are generally less stable to hydrolysis than columns with longer alkyl chains. Octyl (C8, MOS) phases have wide applicability. This phase is less retentive than the C18 phases, but is still quite useful for pharmaceuticals, nucleosides, and steroids.[10]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. The separation selectivity for certain components vary between the columns of different

Fugire-3 : Method validation parameters

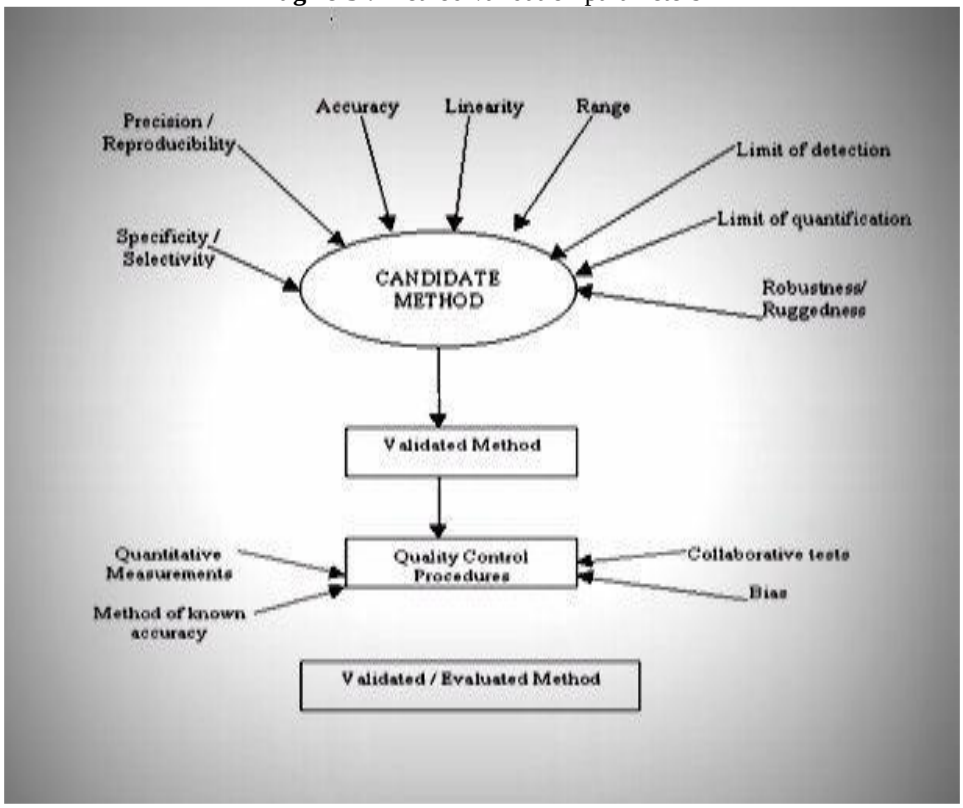

Table 1: Important Information concerning sample composition and properties

Number of Compounds present
Chemical Structures (functionality) of compounds
Molecular weight of compounds
PKa values of compounds
UV Spectra of compounds
Concentration range of compound in sample of interest
Sample Solubility

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.[6]

2.2.2 Selection of Chromatographic mode: chromatographic modes based on the analyte's molecular weight and polarity. All case studies will focus on reversed-phase chromatography (RPC), the most common mode for small organic molecules. Ionizable compounds (acids and bases) are often separated by RPC with buffered mobile phases (to keep the analytes in a non-ionized state) or with ion-pairing reagents.[8]

2.2.3 Optimization of Mobile phase:

Buffer Selection: Different buffers such as potassium phosphate, sodium phosphate and acetate were evaluated for system suitability parameters and overall chromatographic performance.

Effect of pH.:- If analytes are ionisable, the proper mobile-phase pH must be chosen based on the analyte p*Ka* so the target analyte is in one predominate ionization state, ionized or neutral. Alteration of the mobile-phase pH is one of the greatest tools in the "chromatographer's toolbox" allowing simultaneous change in retention and selectivity between critical pair of components**.**[12]

- **Effect of organic modifier: -**Selection of the organic modifier type is relatively simple in reverse phase HPLC, The usual choice is between acetonitrile and methanol (rarely THF). Gradient elution is usually employed with 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. [12]

2.2.4 Selection of detector and wavelength:

After the chromatographic separation, the analyte of interest is detected by using suitable detectors. Some commercial detectors used in LC are: ultraviolet (UV) detectors, fluorescence detectors, electrochemical detectors, refractive index (RI) detectors and mass spectrometry (MS) detectors. The choice of detector depends on the sample and the purpose of the analysis. In case of multicomponent analysis the absorption spectra may have been shifted to longer or shorter wavelengths compared to the parent

compound. Therefore the UV spectra of target analyte and impurities must be taken and overlaid with each other, and the spectra should be normalized due to different amounts present in the mixture. A wavelength must be chosen such that adequate response is for most of the analytes can be obtained.[12,13]

2.3. Developing the approach for analysis:

While developing the analytical method on RP-HPLC the first step which is followed, 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.[6]

2.4 Sample preparation:

Sample preparation is a critical step of method development that the analyst must investigate. For example, the analyst should investigate if centrifugation (determining the optimal rpm and time) shaking and/or filtration of the sample is needed, especially if there are insoluble components in the sample. The objective is to demonstrate that the sample filtration does not affect the analytical result due to adsorption and/or extraction of leachable. The effectiveness of the syringe filters is largely determined by their ability to remove contaminants/insoluble components without leaching undesirable artifacts (i.e., extractable) into the filtrate. The sample preparation procedure should be adequately described in the respective analytical method that is applied to a real in-process sample or a dosage form for subsequent HPLC analysis. The analytical procedure must specify the manufacturer, type of filter, and pore size of the filter media. [12]The purpose of sample preparation is to create a processed sample that leads to better analytical results compared with the initial sample. The prepared sample should be an aliquot relatively free of interferences that is compatible with the HPLC method and that will not damage the column**.**[13,15]

2.5 Method optimization :

Most of the optimization of HPLC method development has been focused on the optimization of HPLC conditions.[14] The mobile phase and stationary phase compositions need to be taken into account. Optimization of mobile phase parameters is always considered first as this is much easier and convenient than stationary phase optimization. To minimize the number of trial chromatograms involved, only the parameters that are likely to have a significant effect on selectivity in the optimization must be examined. Primary control variables in the optimization of liquid chromatography (LC) methods are the different components of the mobile phase determining acidity, solvent, gradient, flow rate, temperature, sample amounts, injection volume, and diluents solvent type. This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved. The parameters involved include column dimensions, column-packing particle size and flow rate. These parameters may be changed without affecting capacity factor or selectivity.[10]

2.6 Method Validation :

Validation of an analytical method is the process by which it is established by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application. Validation is required for any new or amended method to ensure that it is capable of giving reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories. The type of validation program required depends entirely on the particular method and its proposed applications.[13]Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice. Use of equipment that is within specification, working correctly and adequately calibrated is fundamental to the method validation process. Analytical methods need to be validated or revalidated.[16]

• Before their introduction into routine use;

• Whenever the conditions change for which the method has been validated

• Whenever the method is changed

Typical parameters recommended by FDA, USP, and ICH are as follow.[16,18]

1. Specificity
2. Linearity & Range
3. Precision
    - Method precision (Repeatability)
    - Intermediate precision (Reproducibility)
4. Accuracy (Recovery)
5. Solution stability
6. Limit of Detection (LOD)
7. Limit of Quantification (LOQ)
8. Robustness
9. System suitability

Specificity : selectivity of an analytical method as its ability to measure accurately an analyte in the presence of interference, such as synthetic precursors, excipients, enantiomers, and known (or likely) degradation products that may be expected to be present in the sample matrix.[17]

Linearity and range: The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration 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 the drug product components, using the proposed procedure. Linearity is usually expressed as the confidence limit around the slope of the regression line.[16-18] For the establishment of linearity, minimum of five concentrations are recommended by ICH guideline.[19] The range of an analytical method is the interval between the upper and lower levels that have been demonstrated to be determined with precision, accuracy

and linearity using the method.[17]

Precision: The precision of an analytical procedure expresses the 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 may be considered at three levels: repeatability, intermediate precision and reproducibility.[19]The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation of series of measurements. Precision may be either the degree of reproducibility or of the repeatability of the analytical procedure under normal conditions. Intermediate precision (also known as ruggedness) expresses within laboratories variations, as on different days, or with different analysts or equipment within same laboratory. Precision of an analytical procedure is determined by assaying a sufficient number of aliquots of a homogeneous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation.[20]

Accuracy (Recovery): The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. 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.[18,19]

Solution stability: During validation the stability of standards and samples is established under normal conditions, normal storage conditions, and sometimes in the instrument to determine if special storage conditions are necessary, for instance, refrigeration or protection from light.[18]

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 procedures that exhibit baseline noise, the LOD can be based on a 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 corresponding to the component. h = absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.[18-20]

Limit of Quantification (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 (10:1) and is usually confirmed by injecting standards which give this S/N ratio and have an acceptable percent relative standard deviation as well.[19,20]

Robustness: is defined as the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters (e.g. pH, mobile phase composition, temperature and instrumental settings) and provides an indication of its reliability during normal usage. Determination of robustness is a systematic process of varying a parameter and measuring the effect on the method by monitoring system suitability and/or the analysis of samples.[18,19]

System Suitability: System suitability tests are an integral part of liquid chromatographic methods. They are used to verify that the detection sensitivity, resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Factors, such as the peak resolution, number of theoretical plates, peak tailing and capacity have been measured to determine the suitability of the used method.[16-20]

3. Conclusion

In recent years development of the analytical methods for identification, purity evaluation and quantification of drugs has received a great deal of attention in the field of pharmaceutical analysis.This review describes HPLC method development and validation in general way. A general and very simple approach for the HPLC method development for the separation of compounds was discussed. Knowledge of the physiochemical properties of the primary compound is of utmost importance prior to the any HPLC method development. The selection of buffer and mobile phase composition (organic and pH) plays a dramatic role on the separation selectivity. 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.

Abbreviations

HPLC	High Performance Liquid Chromatography
ICH	International conference on Harmonization
Id	Internal Diameter
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
m	Meter
mm	Mili meter
MS	Mass Spectrometry
ODS	Octyl decyl silane
RI	Refractive index
THF	Tetrahydrofuran
USP	United states Pharmacopeia
µm	Micron

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