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Analytical Method Development by High Performance Liquid Chromatography

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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 plays an important role in new discovery, development, manufacture of pharmaceutical drugs and various other studies related to humans and animals. 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, Specificity, Precision*

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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 and 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. The technique of HPLC has following features. [6, 46]

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

ANALYTICAL METHOD DEVELOPMENT

Analytical method development and validation play important roles in the discovery development and manufacture of pharmaceuticals. These methods used to ensure the identity, purity, potency and performance of drug products. There are many factors to consider when developing methods. The initially collect the information about the analyte's physicochemical properties (pka,log solubility) and determining which mode of detection would be

suitable for analysis (i.e. suitable wavelength in case of UV detection).[7] The majority of the analytical development effort goes into validating a stability indicating HPLC method. The goal of the HPLC method is to separate quantify the main active drug, any reaction impurities, all available synthetic inter-mediate and any degradants. Steps involve in method development are:

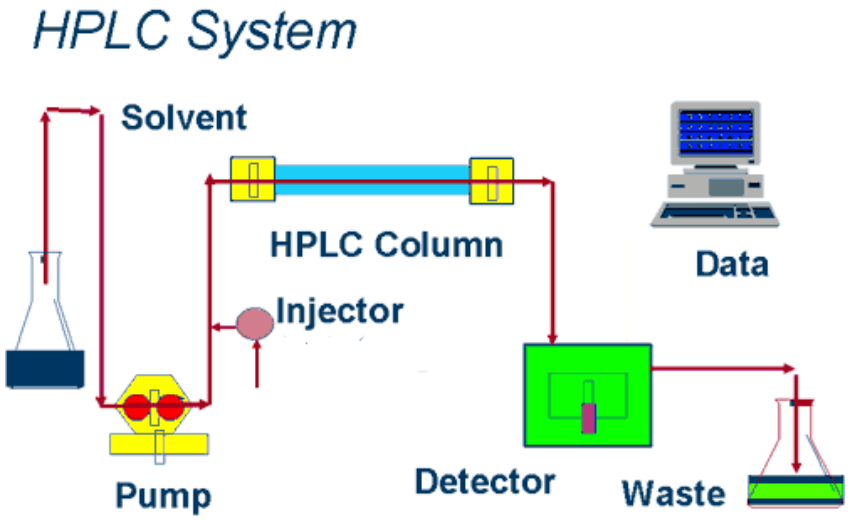
1. Qualified and calibrated instrument
2. Documented methods
3. Reliable reference standards
4. Qualified analysts
5. Sample selection and integrity
6. The analysis should take a minimal time and should be economical.
7. The accuracy of the analyst must accept the guidelines of pharmacopoeia.
8. The chosen method should be precise and selective.
9. Set up HPLC conditions.
10. Preparation of sample solution for method development.
11. Method optimisation.
12. Development and validation of method.[8]

PURPOSE OF ANALYTICAL METHOD DEVELOPMENT:

Drug analysis reveals the identification, characterisation and determination of the medication in mixtures like indefinite quantity forms and biological fluids. Throughout producing method and drug development the most purpose of analytical ways is to produce info regarding efficiency (which is directly associated with the need of a notable dose), impurity (related to safety profile of the drug), bioavailability (includes key drug characteristics a like crystal type, drug uniformity and drug release), stability (which indicated the degradation products), and impact of producing parameters to make sure that the assembly of drug merchandise in consistent.[9]

The idea of internal control is meant to look at and establish a real and right product by series of measures designed to avoid and find eliminate errors at varied stages in production. To require a choice to unharness or discard a product relies on one or lot of forms of management action. Proving easy and analytical method for varied complicated formulation may be a subject material of utmost importance. Fast increase in pharmaceutical industries and constant production of drug in varied components of the globe has brought a fast rise in demand for a brand new analytical techniques within the pharmaceutical industries as a consequence; analytical methodology development has become the essential activity of study during internal control laboratory.[9]

METHOD DEVELOPMENT ON HPLC:



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

1. Understanding the Physiochemical properties of drug molecule.
2. Selection of chromatographic condition
3. Developing the approach of analysis
4. Sample preparations
5. Method optimization
6. Method validation

Understanding the Physiochemical properties of drug molecule

Physiochemical 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 n 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 ionizable analytes 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.[10-11]

1. Selection of chromatographic condition :

Selection of column: Selection of the stationary phase/column is the first and the most important step in method development. The development of 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.

C8 or C18 column made from specially purified, less acidic silica and designed specially for the separation of basic compounds is generally suitable for all samples and is strong recommended. [12] Column dimension, silica substrate properties and bonded stationary phase characteristics are the main ones. The use of silica based packing is favoured in the most of present HPLC columns due to several physical characteristics.[13]

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 accelerate the dissolution of silica and severely shortness 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 hours. The pH of this mobile phase tends to become more basic due to to the release of carbon dioxide.
8. After buffers are prepare, they should be filtered through a 0.2micrometer filter.
9. Mobile phase should be degassed.[14]

Buffer concentration: Generally, a buffer concentration of 10-50 mm is adequate for small molecule. 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 salt are the most common buffer system for reversed-phase HPLC. Sulfonate buffers can replace phosphate buffers when analysing organophosphate compounds.[15]

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 increase the separation bower of the 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 on isocratic would be required and initial gradient run is performed and ratio between the total gradient time and the difference in the gradient time between the first and last components are calculated. The calculated ratio is <0.25 isocratic is adequate. When the ratio is >0.25 gradient would be adequate.[16]

Internal Diameter: The internal diameter (ID) of an HPLC column is important parameter that influence 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 micro meter beads being the most commonly used. The smaller particles usually provide more surface area and better separation but pressure required for the optimum linear velocity increases by the inverse of the particles dimeter squared. Larger particles are used in preparative HPLC where column diameters are in range of 5 cm to >30 cm and for non-HPLC such as solid-phase extractions.[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 and 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, CAN, 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/Buffers	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 delivers high chromatographic and spectral sensitivity. Refractive index chromatographic and spectral sensitivity, stability and reproducibility, which makes 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 component can be detected
UV visible and 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 planar system.
Conductivity detector	Charged compounds, such as inorganic ions and organic acids.
Electrochemical detector	For easily oxidized compounds like quinines or amines.
Refractive index detector and Evaporative light scattering detector	Compounds that do not show characteristics usable by the other detectors, eg. polymers, saccharides.

2. **Developing the approach for analysis:** While developing the analytical method on RP-HPLC the first step 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 5min, 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, 45]
3. **Sample preparation:** Sample preparation is an essential part of HPLC analysis, intended to provide a reproducible and homogeneous 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]
4. **Method optimization:** Identify the "weakness" 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]
5. **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 requirements. In essence, it knows what your method is capable of delivering, particularly at low concentrations.[32]

Need of pharmaceutical validation: Validation is an integral part of quality assurance; it involves the systematic study of systems, facilities and processes aimed at determining whether they perform their intended functions adequately and consistently as specified. A valid method is one that has been incontestable to supply a high degree of assurance that uniform batches are made that meet the desired specifications and has therefore been formally approved. Validation in itself does not improve processes but confirms that the processes have been properly developed and are under control. [33]

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 substance or drug product or other selected components in the drug product.[34]

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

1. Accuracy
2. Precision
3. Linearity
4. Limit of Detection
5. Limit of Quantification
6. Specificity
7. Range
8. Robustness

Accuracy

Accuracy is defined as the nearness of a measured value to the true accepted value. Practically accuracy indicates the deviation between the mean value found and the true value. It is determine by applying the method

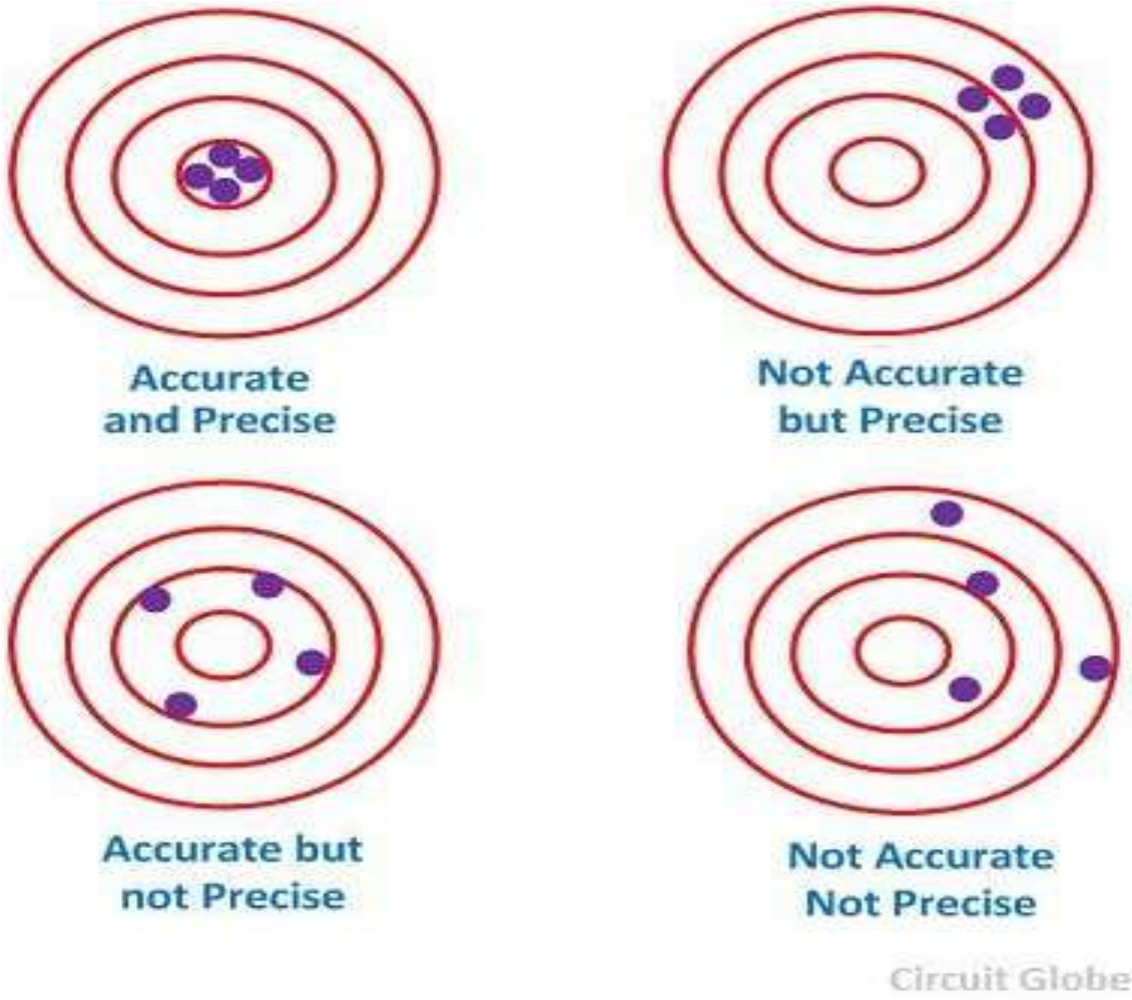
to samples to which known amounts of analyte have been added. These should be analysed against standard and blank solutions to ensure that no interferences exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay. It may often be expressed as the recovery by the assay of known, added amounts of analyte.[35]

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.[36] 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 analysing 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.[37-38] The precision then expressed as the relative standard deviation.

%RSD = Standard deviation X 100 / 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

The Linearity of an analytical method may be defined as "Its ability to elicit tests that are directly or by well defined mathematical transformations proportional to the concentration of analyte in samples within a given range.[39]

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.[40-42,47]

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 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.[41-42]

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.[43]

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) and is normally expressed in the same units as the test results. [44]

Robustness

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 the analysis of samples.[40-41)

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