Development of Validated method by HPLC: A Review

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Abstract- **HPLC is the most widely used separation technique for classifying, quantifying, and identifying medicines. A number of chromatographic parameters were changed to enhance the procedure. The mobile stage and sample pretreatment are examined. choosing a column and a detector. The development and validation of analytical methods are crucial for the development and discovery of new drugs. The numerous steps necessary in creating and establishing the validity of an HPLC technology are covered in this paper. The chemistry of the molecules, the synthetic route, solubility, polarity, pH and pKa values, and the activity of functional groups are a few more elements that affect the development of an HPLC technique. According to ICH Guidelines, validating an HPLC process includes testing for accuracy, specificity, linearity, range, limit of detection, limit of quantification, robustness, and system suitability.**

Keywords: **High-Pressure Liquid Chromatography (HPLC), Method validation, Method development.**

INTRODUCTION:
In order to separate, identify, and quantify active compounds, high-performance liquid chromatography, also known as high-pressure liquid chromatography, is a type of column chromatography that is frequently employed in biochemistry and analysis. It is a well-liked analytical method for distinguishing, locating, and quantifying each component of a mixture. [1] The qualitative and quantitative composition of the sample under investigation is ascertained using analytical chemistry. To comprehend the sample content, one must be aware of both of these factors. The two branches are quantitative and qualitative analytical chemistry.By determining the existence or absence of specific components, a qualitative analysis informs us about the sample's nature. An analysis using numbers can quantify the relative amounts of one or more of these components. Different analytical methods are frequently employed to analyse drug materials in bulk, pharmaceutical formulations, and biological fluids. [2]
High-performance liquid chromatography's guiding principle is to partition solute on stationary phase based on affinity towards stationary phase, ion exchange, and absorption to separate components. Typically, gravity helps the solvent move through the column. However, in HPLC procedures, a high pressure of up to 400 atmospheres will be applied to the solvent. [3]
Types of HPLC:
HPLC can be classified as follows:
1) Based on a scale of operation
Preparative HPLC and analytical HPLC
2) Based on the principle of separation
Affinity chromatography, adsorption chromatography, size
Exclusion chromatography, ion-exchange Chromatography, chiral phase chromatography
3) Based on the elution technique
Gradient separation and isocratic separation
4) Based on modes of operation
Normal phase chromatography and reverse-phase Chromatography. [4]
1.Normal phase chromatography:
The stationary phase in normal phase chromatography is polar, whereas the mobile phase is non-polar. The stationary phase as a result holds onto the polar analyte. Longer elution times are caused by the Solute molecules' enhanced adsorption ability due to their higher polarity. A stationary phase of chemically modified silica (cyanopropyl, aminopropyl, and diol) is employed in this chromatography. As a result, the normal phase mode of separation is rarely used in the pharmaceutical industry because the majority of medication molecules are polar in nature and take longer to elute.[5]
2. RP **Reversed Phase Chromatography:**
In RP-HPLC, the mobile phase is polar or moderately polar whereas the stationary phase is non-polar. -HPLC is based on the idea of hydrophobic contact. The non-Polar stationary phase will retain analytes that are Significantly more Polar for a shorter period than those that are Comparatively less Polar in a mixture of components. As a result, the First is eluted by the most polar component.[6]
3. Adsorption Chromatography:
 When the mobile phase is a liquid or gaseous phase and the stationary phase is a solid, adsorption chromatography is employed.. Examples include gas-solid chromatography, thin-layer chromatography, and column chromatography. [7]
4. Ion Exchange Chromatography:

The attraction of solute ions to charged sites bound to the stationary phase underlies retention in ion-exchange chromatography. The use of identically charged ions is prohibited. The ligand-exchange chromatography, protein ion-exchange chromatography, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides, and additional uses of this chromatography technology are all common. [8]

5. Ion Pair Chromatography:

Ion exchange, or the reversible interchange of functional groups, is the basis for separation. In ion exchange chromatography, a mixture of similarly charged ions is separated using an ion exchange resin. The attraction between the charged ions bound to the stationary phase and the solute ions serves as the basis for retention. Where opposite charge ions are kept and similar ions are excluded, cationic and anionic exchange resins are utilised. Therefore, this is only utilised to separate charged molecules. Elution time is regulated by pH and ionic strength.[9]

6. Size exclusion Chromatography.

SEC is a method for separating particles according to their size. It is often referred to as gel permeation or gel filtration chromatography. It can also figure out how proteins and amino acids are structured at the tertiary and quaternary levels. This approach is frequently used to determine the molecular weight of polysaccharides. [10]

7. Affinity Chromatography:

Based on reversible interactions between ligands and proteins, separation. Proteins that interact with the column-bound ligands are kept in place by ligands being covalently bonded to solid support on an abio-affinity matrix.[11]

Instrumentation of High-Performance Liquid Chromatography (HPLC):

The fundamentals of HPLC technology entail injecting a solution (the stationary phase) into a porous material column, and pumping a liquid phase (the mobile phase) through the column at a higher pressure. [12]
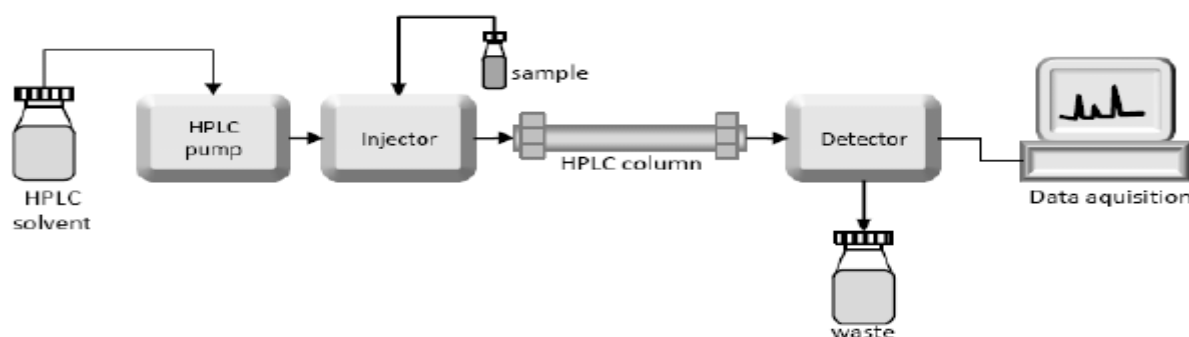

Figure No.1: Flow Diagram of HPLC Instrumentation

The technique of HPLC has following features.
- ❖ High resolution
- ❖ Small diameter, Stainless steel, Glass column
- ❖ Rapid analysis
- ❖ Relatively higher mobile phase pressure
- ❖ Controlled flow rate of mobile phase

HPLC has many advantages, including:
- ❖ Simultaneous Analysis
- ❖ High Resolution
- ❖ Extreme Sensitivity
- ❖ Excellent repeatability
- ❖ Limited sample size
- ❖ The analysis condition is moderate.
- ❖ It is simple to fractionate and purify the sample. [13]

HPLC method development:

The discovery, development, and production of pharmaceuticals depend on the development and validation of analytical methods. Pharmaceutical items' identification, purity, potency, and effectiveness are ensured using these methods. There are several things to think about when constructing methods. The physiochemical characteristics of the analyte (pKa, log P, solubility), in the case of UV detection, are first gathered, and it is then decided which mode of detection would be best for analysis. Validating an HPLC-method to indicate stability takes up the majority of the analytical development effort. The basic objective of the HPLC approach is to quantify and separate the primary active substance, any reactive contaminants, all readily available synthesis intermediates, and any degradation products.[14]

The following is a step in HPLC method development:
- ❖ Recognizing the Physicochemical Properties of Drug Molecules
- ❖ Choosing chromatographic conditions
- ❖ Creating an analytic approach.
- ❖ Preparation of Samples
- ❖ Method Improvement

❖   Validation of methods
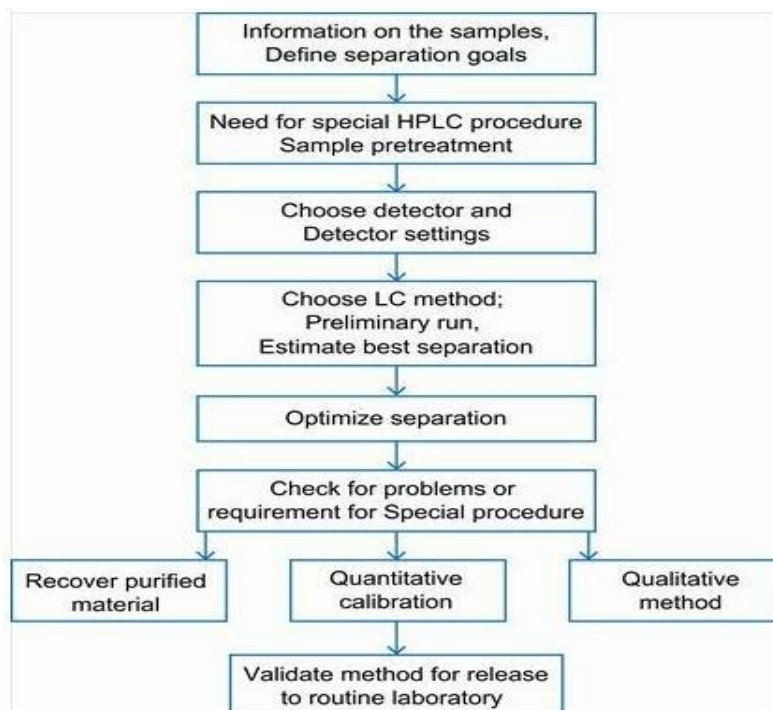
Figure No. 2: Steps involved in HPLC Method development

1.Recognizing the Physicochemical Properties of Drug Molecules:
For the development of procedures, the physicochemical properties of a drug's molecule are essential. One must research the physical characteristics of the drug molecule, such as its solubility, polarity, pKa, and pH, in order to build a method. A compound's physical characteristic of polarity. An analyst can use it to choose the mobile phase's solvent and chemical makeup. Two atoms share the electrons evenly in a nonpolar covalent link. One atom has a stronger attraction to the electrons than the other atom in a polar covalent connection. The molecules' polarity can be utilised to account for their solubility. Solvents that are nonpolar, like benzene, and polar, like water, cannot be mixed.

Like generally dissolves like, which means that substances with comparable polarities can be dissolved in one another. The choice of diluents depends on how soluble the analyte is. The diluents' components must not react with the analyte, which must be soluble in them. In the development of HPLC methods, pH and pKa are crucial factors. The pH value is defined as the negative of the hydrogen ion concentration's logarithm to base-10.

$$pH = - \log_{10}[H3O+]$$

Sharp, symmetrical peaks in HPLC are usually produced by selecting an optimum pH for ionizable analytes. To obtain low detection limits, low relative standard deviations (between injections), and predictable retention times in quantitative analysis, crisp, symmetrical peaks are necessary.[15, 16]

2.Selection of chromatographic conditions:
A set of beginning conditions (detector, column, mobile phase) are chosen during the early stages of technique development in order to get the sample's initial "scouting" chromatograms. Reversed-phase separations on a C18 column with UV detection constitute the basis for these in the majority of cases. At this point, a choice should be taken regarding whether to develop an isocratic or a gradient methodology. [17]

2.2.1 Selection of Column:
The choice of the stationary phase or Column is the first and most important step in method development. Without a solid, high-performance column, it is impossible to establish a reliable, reproducible method. To avoid problems brought on by irreproducible Sample retention throughout technique development, columns must be stable and repeatable. Particularly pure, less acidic silica prepared for a C8 or C18 column Additionally, it is generally suited for all samples and highly advised for the separation of basic Chemicals.

Column diameters, silica substrate qualities, and bonded stationary phase characteristics are among the crucial ones. The bulk of today's HPLC columns prefer silica-based packing due to a range of physical features. The three essential parts of an HPLC column are the hardware, matrix, and stationary phase.Some of the Matrices used to maintain the stationary phase are silica, polymers, alumina, and zirconium. Silica is the most popular matrix for HPLC columns. Strong, simple to derivatize, generated in constant sphere sizes, and resistant to pressure are some of the characteristics of silica matrices. Silica can withstand the majority of organic solvents and low pH solutions chemically. The fact that a silica solid support dissolves above pH 7 is a drawback. [18]

2.2.2 Selection of Chromatographic mode:
Chromatographic modes determined by the polarity and molecular weight of the analyte. Reversed-phase chromatography (RPC), the most typical method for small organic compounds, will be the main topic of all case studies. Ion-pairing reagents or buffered

mobile phases, which retain the analytes in a non-ionized state, are frequently used in RPC to separate ionizable chemicals (acids and bases). [19]

2.2.3 Optimization of Mobile phase:

❖   **Buffer Selection :**

The desired pH dictates the choice of buffer. Various buffers, including potassium phosphate, sodium phosphate, and acetate, were examined for system compatibility factors and overall chromatographic performance. For reversed phase on silica-based packing, the normal pH range is 2 to 8. Since buffers control pH best at their pKa, it is crucial that the buffer possess a pKa near to the desired pH. A general rule is to select a buffer whose pKa value is less than two units of the required mobile phase pH.

❖   **Effect of pH:**

If the target analyte is ionizable, the proper mobile-phase pH must be selected based on the analyte pKa such that it is either ionised or neutral. One of the "chromatographer's toolbox's" most potent tools is the capacity to alter the pH of the mobile phase. Allowing simultaneous adjustments to the selection and retention of crucial pairs of components

 Effect of organic modifier:

 In reverse phase HPLC, choosing the type of organic modifier is very straightforward. Acetonitrile and Methanol are often the options (THF is uncommon). Usually used with complex multicomponent samples, gradient elution Since not all components may be available, Eluted using a single solvent strength under isocratic conditions between k (retention factor) 1 and 10. [20]

2.2.4 Selection of detector and wavelength:

 The target analyte is identified using the right detectors after the chromatographic separation. Commercial detectors used in LC include UV detectors [32], fluorescence detectors, electrochemical detectors, Refractive index (RI) detectors, and mass spectrometry (MS) detectors. The sample and the goal of the analysis define the detector to be used. The absorption spectra may have moved to longer or shorter wavelengths than the parent chemical in the case of multicomponent analysis. Due to the various concentrations of contaminants in the combination, the UVspectra of the target analyte and the impurities must be obtained, overlaid, and the spectra must be normalised. A wavelength must be chosen to ensure that the majority of analytes respond appropriately to it. [20, 21]

3. Creating an analytic approach:

The first stage in establishing an analytical procedure for RP-HPLC is to choose several chromatographic parameters, such as the mobile phase, the column, the flow rate of the mobile phase, and the mobile phase pH. These parameters are all chosen after extensive testing, and the system suitability parameters are then taken into account. Typical Retention time should be greater than 5 minutes, and the theoretical plates should be at least The tailing factor should be less than 2 for more than 2000. Resolution between two peaks should be greater than 5%, and the relative standard deviation (RSD) of the area of analyte peaks in standard Chromatograms should not be greater than 2.0%. The typical isobestic point for the detection wavelength is the. [22]

4. Sample preparation:

 In Solution (diluent), the drug ingredient being examined need to be stable. The preparation of the solutions in amber flasks should be carried out during the earliest stages of method development until it is established that the active component is stable at room temperature and does not degrade under typical laboratory circumstances. The sample solution should be filtered, and for the removal of particles, a 0.22 or 0.45 m pore-size filter is typically advised. For HPLC analysis, filtering is a preventive maintenance tool.The analyst must look at the crucial technique development step of sample preparation.. The ability of the Syringe filters to remove contaminants and insoluble components without releasing undesirable artefacts (i.e., extractable) into the filtrate is a key factor in determining how successful they are. To establish whether a leachable component is coming from the syringe filter housing/filter, the diluents must be filtered if any further peaks are seen in the filtered samples. [23]

5. Method optimization:

 Utilising experimental design, pinpoint the method's "weaknesses" and improve it.Recognise how the approach operates in various settings, with various instrument configurations, and with various samples. [24]

6. Validation:

 Validation is the investigation and provision of unbiased proof that the particular requirements for the particular intended usage are satisfied. a way to gauge a method's effectiveness and show that it satisfies a particular need. In other words, it is aware of the potential of your method, particularly at low concentrations. [25]

Method Validation:

The process by which it is determined, by laboratory tests, that the performance characteristics of the procedure match the needs for its intended usage is known as validation of an analytical procedure. The planned and methodical collection of validation data by the applicant to support analytical Procedures is the first step in the methods validation process. All analytical techniques must be validated before they may be used to analyse any clinical samples. Analytical methods are validated in accordance with ICH criteria. [26]

COMPONENTS OF METHOD VALIDATION:

The following are typical analytical performance characterstics which may be tested during Methods validation:

1.   System Suitability
2.   Accuracy
3.   Precision
4.   Repeatability
5.   Intermediate precision
6.   Linearity
7.   Detection limit

8.   Quantitation limit
9.   Specificity
10.   Range
11.   Robustness
12.   System suitability determination
13.   Forced degradation studies
14.   Solution stability studies

1.System Suitability:

System appropriateness checks are a common practise in liquid chromatographic systems. They are used to check if the detection sensitivity, resolution, and reproducibility of the chromatographic system are sufficient for the analysis. The foundation of the tests is the notion that the equipment, electronics, analytical procedures, and test samples are all components of a Whole that may be assessed as a Whole. Peak resolution, the number of theoretical plates, peak tailing, and capacity were examined to assess the applicability of the employed approach. [27]

2.Accuracy:

The degree of agreement between the value recognised as a conventional true value or an authorised reference value and the value discovered40 reflects the accuracy of an analytical method. Accuracy is the degree to which a measured value is near to its real or accepted value. In actuality, accuracy refers to the discrepancy between the detected Mean value and the true value.41It is computed by using the technique on samples that include analyte levels that are known. These should be contrasted with standard and blank solutions to ensure that there is no interference On the basis of the test results, the accuracy is then calculated as a percentage of the analyte recovered by the assay. The recovery of known, additional amounts of analyte by test is a common way to represent it. Based on the results of the test, the accuracy is then calculated as a proportion of the analyte that the assay was able to recover. It is tydenpically defined as the test-based recovery of known, additional amounts of analyte. [28]

3.Precision:

It indicates the level of agreement (or scattering) between a group of measurements obtained from numerous samples taken from the same homogenous sample under the given conditions. The degree of reproducibility of the entire analytical process is measured by precision. Both repeatability and intermediate precision are components of it. Repetability is the variation that a single analyst experiences on a single piece of equipment. It does not differentiate between variation brought on by the instrument, system, or sample preparation process. Using the analytical procedure, numerous replicates of an assay composite sample are analysed to evaluate repeatability during validation. Calculated is the value of recovery. The fluctuation that takes place in a laboratory as a result of numerous conditions, such as various days, multiple instruments, and varied analysts, is referred to as intermediate precision.related benchmark The precision is then expressed using deviation.[29]

$$\%RSD = std\ dev *100/mean$$

4.Repeatability:

The variety that one analyst experiences when using a single instrument. It makes no distinction between variations caused by the equipment or system or by the method used to prepare the sample. Repeatability is assessed during validation by comparing numerous replicates of an assay Composite sample using the analytical procedure. The value of Recovery is computed. Variation within a laboratory, such as various days, different instruments, and different analyzers, is referred to as intermediate precision. The relative standard deviation is then used to express the precision.

$$\%RSD = std\ dev * 100/mean$$

5.Linearity:

The capacity of an analytical process to produce a response that is inversely proportional to the concentration (quantity) of the analyte in the sample is known as linearity.The confidence limit surrounding the slope of the regression line is how linearity is expressed. [30]

6.Detection limit:

The simplest measurement of an analyte in an example that can be recognised but not quantitated as an exact quality is the detection limit of a single explanatory method. [31]

7. Quantitation Limit:

The lowest amount (concentration) of analyte in a sample that can be quantified is known as the LOQ. ICH has suggested a signal to noise ratio of 10:1 for LOQ.

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

8.Specificity:

The capacity of an analytical method to quantify the analyte precisely in the presence of additional components is known as specificity. The following conclusions flow from this definition:

- Identification
- Purity tests
- Assay

9. Range:

The interval between an analyte's upper and lower levels that can be determined with the necessary precision, accuracy, and linearity is known as the method's range.

10.Robustness:

The robustness of an analytical procedure is a measure of its capacity to be unaffected by tiny but intentional alterations in method parameters and offers a clue as to its reliability under usual situations.

11.System suitability determination :

Tests for system compatibility are an essential component of liquid chromatographic techniques. They are used to confirm that the chromatographic system's detection sensitivity, resolution, and reproducibility are sufficient for the intended analysis.

The tests are founded on the idea that the tools, electronics, analytical procedures, and test samples together make up a whole system that can be assessed as such. Peak resolution, theoretical plate count, peak tailing, and capacity are just a few of the variables that have been measured to assess the applicability of the technique..

12.Solution stability studies:

The stability of Standards and samples is determined during validation under normal settings, normal storage conditions, and occasionally in the instrument to evaluate whether extra storage conditions, such as refrigeration or protection from light, are necessary. [32]

Conclusion:

The development and validation of the RP-HPLC Technique Method are discussed in this article. Method development and validation are ongoing, interrelated processes that measure a parameter as intended and establish the performance limits of the measurement. The buffer and mobile phase's composition (organic and pH) significantly affects the selectivity of the separation. The excellent selectivity, sensitivity, low detection limit, and low cost of HPLC technology were major advantages. Finally, it is possible to optimise the gradient slope, temperature, flow rate, and the kind and concentration of mobile-phase modifiers. According to ICH regulations, the optimised method is verified using a number of parameters, including specificity, precision, accuracy, detection limit, linearity, and other factors.

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