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## A Review on HPLC Method Development and Validation



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

### ABSTRACT

HPLC is the most commonly used separation technique for detecting, separating, and quantifying drugs. To optimize the method, several chromatographic parameters were investigated, including sample pretreatment, mobile phase selection, column selection, and detector selection. The purpose of this article is to go over the method development, optimization, and validation processes. Because of its advantages such as rapidity, specificity, accuracy, precision, and ease of automation, the HPLC method can be used to analyze the majority of drugs in multicomponent dosage forms. HPLC method development and validation are critical in new drug discovery, development, and manufacturing, as well as a variety of other human and animal studies. Validation of analytical methods is required during drug development and manufacturing to ensure that these analytical methods are fit for their intended purpose. To meet GMP requirements, pharmaceutical industries should have an overall validation policy that details how validation will be carried out. This article is primarily concerned with the optimization of HPLC conditions.

## **INTRODUCTION:**

High-Performance Liquid Chromatography has become one of the most powerful analytical chemistry tools. It is capable of separating, identifying, and quantifying the compounds present in any sample that can be dissolved in a liquid. High-performance liquid chromatography (HPLC) is one of the most accurate analytical methods for both quantitative and qualitative drug product analysis. [1] The principle is that a sample solution is injected into a porous material column (stationary phase), and a liquid (mobile phase) is pumped at high pressure through the column. The sample is separated based on differences in migration rates through the column caused by different partitioning of the sample between the stationary and mobile phases. Elution occurs at different times depending on the partition behavior of different components. [2] A sample compound with a higher affinity for the stationary layer will travel slower and for a shorter distance than a compound with a lower affinity, which will travel faster and for a longer distance. [3] The purpose of the HPLC method is to separate and quantify the main drug, any reaction impurities, all available synthetic intermediates, and any degradants. High-Performance Liquid Chromatography has become one of the most powerful analytical chemistry tools. It is capable of separating, identifying, and quantifying the compounds present in any sample that can be dissolved in a liquid. It is one of the most accurate analytical methods for quantitative and qualitative drug product analysis, as well as determining drug product stability. [4]

## **HPLC principle:**

The distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase is the basis for the separation principle of HPLC (packing material of the column). The molecules are retarded while passing through the stationary phase depending on the chemical structure of the analyte.

## **Types of HPLC:**

The phase system used in the process determines the type of HPLC used in the analysis. Normal phase chromatography (NP-HPLC): This method separates analytes based on polarity and is also known as normal phase HPLC (NP-HPLC). In NP-HPLC, a polar stationary phase and a non-polar mobile phase are used. The polar stationary phase interacted with the polar analyte and retained it. The interaction between the polar analyte and the polar stationary phase prolongs elution time as analyte polarity increases. [5,6]

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

- I. Preparative HPLC and analytical HPLC (based on the scale of operation)
- II. Affinity chromatography, adsorption chromatography, size exclusion chromatography,
- III. Ion exchange chromatography, chiral phase chromatography (based on the principle of separation)
- IV. Gradient separation and isocratic separation, (based on elution technique)
- V. Normal phase chromatography and reverse-phase chromatography (based on modes of operation). [9]

**1. Size exclusion chromatography:**

SEC, also known as gel permeation or gel filtration chromatography, is a technique for separating particles based on their size. It is also capable of determining the tertiary and quaternary structures of proteins and amino acids. This method is commonly used to calculate polysaccharide molecular weight.

**2. Ion exchange chromatography:**

Retention in ion-exchange chromatography is based on the attraction of solute ions to charged sites bound to the stationary phase. Ions with the same charge are not allowed. This chromatography technique is widely used in water purification, ligand-exchange chromatography, protein ion-exchange chromatography, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides, and other applications. [5,6]

**3. Bio-affinity chromatography:**

Separation based on reversible protein-ligand interactions. Ligands are covalently attached to solid support on an abio-affinity matrix, which keeps proteins that interact with the column-bound ligands in place. [5]

**4. Normal phase chromatography:**

The mobile phase in normal phase chromatography is non-polar, while the stationary phase is polar. As a result, the polar analyte is retained by the station phase. The increased polarity of solute molecules increases adsorption capacity, resulting in a longer elution time. In this chromatography, a stationary phase of chemically modified silica (cyanopropyl, aminopropyl,

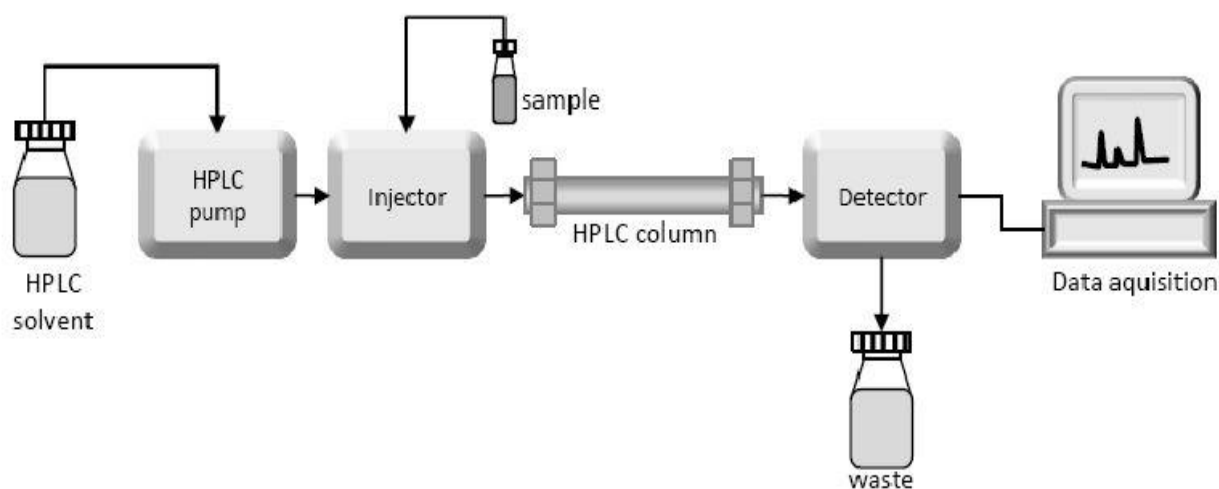
and diol) is used. [7] As an example, A typical column has an internal diameter of approximately 4.6 mm and a length ranging from 150 to 250 mm. Polar compounds in the mixture that are passed through the column will stick to the polar silica for a longer period than non-polar compounds. As a result, the non-polar ones will move faster through the column. [10]

### 5. RP-HPLC (Reversed-Phase HPLC):

The stationary phase of RP-HPLC is non-polar, and the mobile phase is polar or moderately polar. The principle of hydrophobic interaction underpins RP-HPLC [9]. The non-polar stationary phase will retain analytes that are relatively less polar in a mixture of components for a longer period than those that are relatively more polar. As a result, the most polar component elutes first. [11]

### Instrumentation of High-Performance Liquid Chromatography (HPLC):

In analytical separation, a high-pressure flow of a liquid through a column containing the stationary phase is used. Stationary phase: This can be a solid (LSC) or a liquid (L) (LLC). A mixture of compounds injected at one end separates as the compounds pass through the column. Separated compounds are detected electronically as they elute at the other end of the column. High-Performance Liquid Chromatography is a more versatile technique than gas chromatography. There is a greater variety of mobile and stationary phases to choose from.[7]



**Figure No. 1:** Flow Diagram of HPLC Instrumentation

**The HPLC technique has the characteristics listed below:**

- ❖ High resolution, small diameter, stainless steel, and glass column
- ❖ Quick analysis
- ❖ Significantly higher mobile phase pressure
- ❖ Mobile phase flow rate control.

**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. [8]

**HPLC Method Development:**

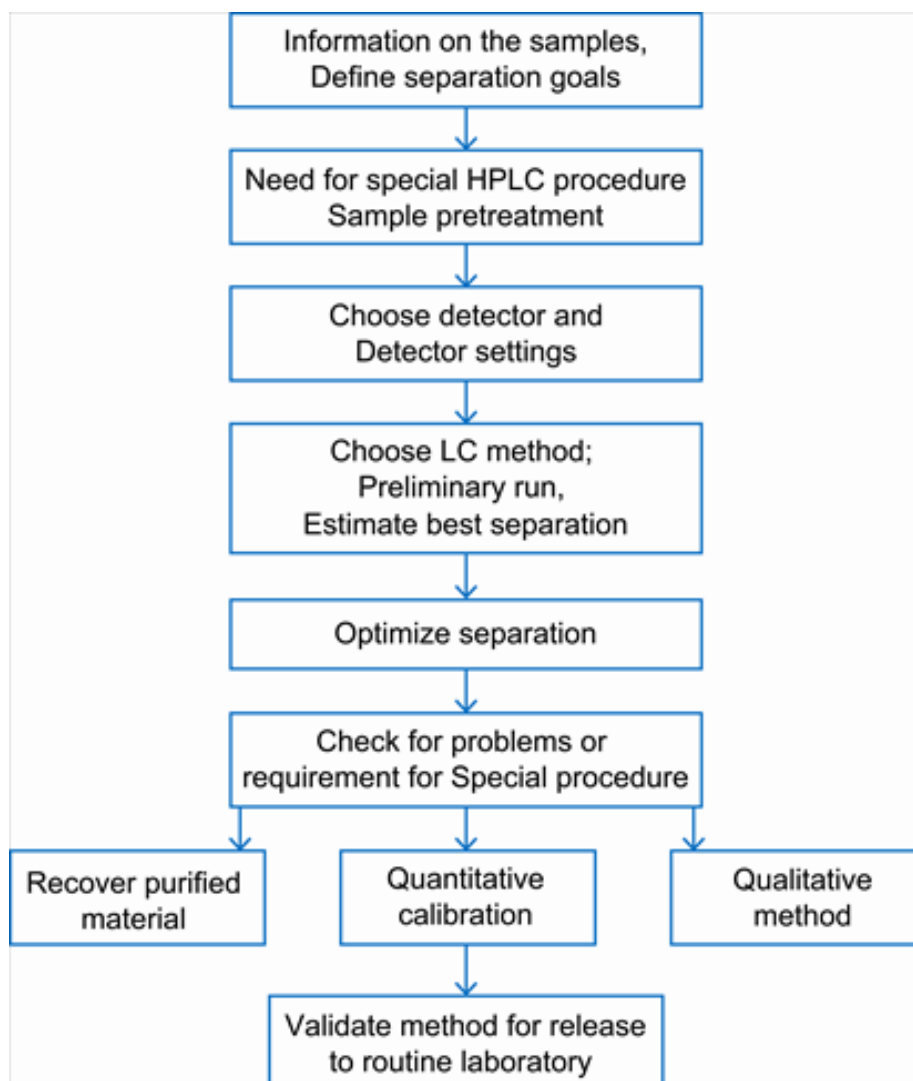
Analytical method development and validation are critical steps in the discovery, development, and manufacturing of pharmaceuticals. These techniques are used to ensure the identity, purity, potency, and performance of pharmaceutical products. When developing methods, there are numerous factors to consider. In the case of UV detection, they first gather information about the analyte's physicochemical properties (pKa, log P, solubility) and determine which mode of detection would be suitable for analysis. The majority of the analytical development effort is spent validating an HPLC–method for indicating stability. The purpose of the HPLC method is to separate and quantify the main active drug, any reaction impurities, all available synthetic intermediates, and any degradants. [12,13]

**HPLC method development:**

**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:**

The physicochemical properties of a drug molecule are critical in method development. To develop a method, one must first investigate the physical properties of the drug molecule, such as solubility, polarity, pKa, and pH. A compound's polarity is a physical property. It

assists an analyst in determining the solvent and mobile phase composition. The polarity of molecules can be used to explain molecular solubility. Polar solvents, such as water, and nonpolar solvents, such as benzene, do not mix. In general, like dissolves like, which means that materials with similar polarities dissolve in each other. The solubility of the analyte influences the choice of mobile phase or diluents. The analyte must be soluble in diluents and not react with any of its constituents. The pH and pKa values are important in the development of HPLC methods. The pH value is defined as the inverse of the logarithm to base 10 of the hydrogen ion concentration.

$$\text{pH equals } -\log_{10}[\text{H}_3\text{O}^+].$$

Choosing an appropriate pH for ionizable analytes frequently results in symmetrical and sharp peaks in HPLC. In quantitative analysis, sharp, symmetrical peaks are required to achieve low detection limits, low relative standard deviations between injections, and reproducible retention times. [14,15]

## **2. Choosing Chromatographic Conditions:**

During initial method development, a set of initial conditions (detector, column, mobile phase) is chosen to generate the sample's first "scouting" chromatograms. In most cases, reversed-phase separations on a C18 column with UV detection are used. At this point, a decision should be made whether to develop an isocratic or a gradient method.

### **2.2.1 Selection of Column:**

A column is, of course, the first and most important component of a chromatograph. A properly chosen column can produce a good chromatographic separation, resulting in accurate and reliable analysis. An incorrectly used column can frequently produce confusing, insufficient, and poor separations, resulting in invalid or difficult-to-interpret results. [9] The column is the heart of an HPLC system. During method development, changing a column will have the greatest impact on analyte resolution. The best column for an application must take into account stationary phase chemistry, retention capacity, particle size, and column dimensions. The hardware, matrix, and stationary phase are the three main components of an HPLC column. Silica, polymers, alumina, and zirconium are some of the matrices used to support the stationary phase. The most common matrix for HPLC columns is silica. Silica matrices are strong, easily derivatized, produced in consistent sphere sizes, and do not compress under pressure. The best column for an application must take into account

stationary phase chemistry, retention capacity, particle size, and column dimensions. The hardware, matrix, and stationary phase are the three main components of an HPLC column. Silica, polymers, alumina, and zirconium are some of the matrices used to support the stationary phase. The most common matrix for HPLC columns is silica. Silica matrices are strong, easily derivatized, produced in consistent sphere sizes, and do not compress under pressure. Most organic solvents and low pH systems are chemically stable to silica. One disadvantage of a silica solid support is that it dissolves above pH 7. In recent years, silica-supported columns for use at high pH have been developed. The silica's nature, shape, and particle size aid in effect separation. A smaller particle results in an increased number of theoretical plates. The type of stationary phase determines whether a column can be used for normal or reverse-phase chromatography. A polar stationary phase and a non-polar mobile phase are used in normal phase chromatography. Polar compounds elute later than non-polar compounds in general. The most common reverse phase columns and their applications are listed below. Propyl (C3), Butyl (C4), and Pentyl (C5) phases are useful for ion-pairing chromatography (C4) and peptides containing hydrophobic residues, as well as other large molecules. When compared to C8 or C18 phases, C3–C5 columns generally retain non-polar solutes less well. Zorbax SB-C3, YMC-Pack C4, and Luna C5 are a few examples. These columns are less resistant to hydrolysis in general than columns with longer alkyl chains. Octyl (C8, MOS) phases have a wide range of applications. This phase is less retentive than the C18 phases, but it is still beneficial for pharmaceuticals, nucleosides, and steroids. The first and most important step in method development is the selection of the stationary phase/column. It is impossible to develop a robust and reproducible method without the availability of a stable, high-performance column. Columns must be stable and reproducible to avoid problems caused by irreproducible sample retention during method development. Separation selectivity for specific components varies between columns manufactured by different manufacturers as well as between column production batches manufactured by the same manufacturer. The main ones are column dimensions, silica substrate properties, and bonded stationary phase characteristics. Due to a variety of physical properties, silica-based packing is preferred in the majority of today's HPLC columns. [16]

### **2.2.2 Selection of Chromatographic mode:**

Chromatographic modes are determined by the molecular weight and polarity of the analyte. All case studies will concentrate on reversed-phase chromatography (RPC), which is the most commonly used mode for small organic molecules. Ionizable compounds (acids and bases)



are frequently separated by RPC using buffered mobile phases (to keep the analytes from becoming ionized) or ion-pairing reagents. [17]

### 2.2.3 Optimization of Mobile phase:

#### ❖ Buffer Selection:

Various buffers, including potassium phosphate, sodium phosphate, and acetate, were tested for system suitability parameters and overall chromatographic performance. Following sequential trials with various buffers, it was determined that potassium dihydrogen phosphate was suitable for the effective separation of all peaks. Potassium dihydrogen phosphate buffer concentrations of 0.02M, 0.05M, and 0.1 M were tested. The change in buffer concentration did not result in significant changes in the elution pattern or resolution, but the 0.05M concentration increased the method's sensitivity. [18]

#### ❖ Effect of pH:

If analytes are ionizable, the appropriate mobile-phase pH must be chosen based on the analyte pKa so that the target analyte is either ionized or neutral. The ability to change the pH of the mobile phase is one of the most powerful tools in the "chromatographer's toolbox," allowing for simultaneous changes in retention and selectivity between critical pairs of components. [18]

#### ❖ Effect of organic modifier:

In reverse phase HPLC, selecting the organic modifier type is relatively simple. The most common options are acetonitrile and methanol (rarely THF). Gradient elution is typically used with complex multicomponent samples because it may be impossible to elute all components using a single solvent strength between  $k$  (retention factor) 1 and 10 under isocratic conditions. [18]

### 2.2.4 Selection of detector and wavelength:

Following the chromatographic separation, the analyte of interest is detected using appropriate detectors. UV detectors [32], fluorescence detectors, electrochemical detectors, refractive index (RI) detectors, and mass spectrometry (MS) detectors are examples of commercial detectors used in LC. The detector used is determined by the sample and the purpose of the analysis. In the case of multicomponent analysis, the absorption spectra may have shifted to longer or shorter wavelengths than the parent compound. As a result, the UV

spectra of the target analyte and impurities must be taken and overlaid, and the spectra must be normalized due to the different amounts present in the mixture. A wavelength must be chosen so that an adequate response is obtained for the majority of analytes. [18,19]

### **3. Creating an analytic approach:**

The first step in developing an analytical method for RP-HPLC is to select various chromatographic parameters such as mobile phase, column, a flow rate of mobile phase, and pH of the mobile phase. All of these parameters are chosen based on trials, and they are then compared to the system suitability parameters. Typical system suitability parameters include, for example, a retention time of more than 5 minutes, a theoretical plate count of more than 2000, a tailing factor of less than 2, a resolution of more than 5, and a percent R.S.D. of the area of analyte peaks in standard chromatograms of no more than 2.0 percent. In the case of simultaneous estimation of two components, the detection wavelength is usually an isobestic point. Following that, the drug's linearity is investigated to determine the concentration range at which the drug follows the linear pattern. The laboratory mixture is also analyzed to determine the practicability of the developed method for simultaneous estimation. Following that, the marketed formulation is analyzed by diluting it up to the concentration range of linearity.[20,21]

### **4. Sample preparation:**

Sample preparation is an important part of HPLC analysis because it ensures that the solution is reproducible and homogeneous enough to be injected onto the column. The goal of sample preparation is to create a sample aliquot that is relatively free of interferences, will not damage the column, and is compatible with the intended HPLC method, which means that the sample solvent will dissolve in the mobile phase without affecting sample retention or resolution. Sample preparation begins with sample collection and continues with sample injection onto the HPLC column. [22]

### **5. Method optimization:**

Identify the method's "weaknesses" and optimize the method using experimental design. Understand how the method performs under different conditions, with different instrument setups, and with different samples. [23]

## 6. Validation:

Validation is the examination and provision of objective evidence that the specific requirements for specific intended use are met. A method of assessing method performance and demonstrating that it meets a specific requirement. In other words, it understands what your method is capable of delivering, especially at low concentrations. [24]

### Method Validation:

The process of validating an analytical method is the process of establishing, through laboratory studies, that the method's performance characteristics meet the requirements for the intended analytical application. Any new or modified method must be validated to ensure that it is capable of producing reproducible and reliable results when used by different operators using the same equipment in the same or different laboratories. The type of validation program required is entirely dependent on the specific method and its proposed applications. 13 Method validation results can be used to assess the quality, reliability, and consistency of analytical results; it is an essential component of any good analytical practice. The use of equipment that is within specification, working properly, and properly calibrated is critical to the method validation process. Validation or revalidation of analytical methods is required. [25]

- Before they are put into routine use;
- When the conditions for which the method has been validated change;
- When the method is changed,

The following are typical parameters recommended by the FDA, USP, and ICH. [25,26]

1. Specificity
2. Linearity & Range
3. Precision
  - I. Method precision (Repeatability)
  - II. Intermediate precision (Reproducibility)
4. Accuracy (Recovery)
5. Solution stability
6. Limit of Detection (LOD)

7. Limit of Quantification (LOQ)

8. Robustness

9. Range

10. System suitability

### **1. Specificity:**

In strategy approval, selectivity and specificity are sometimes used interchangeably to represent the same concept. Specificity is the ability to evaluate the analyte unequivocally in the vicinity of parts that may be required to be displayed. The specificity of a test system is controlled by contrasting test results obtained from an investigation of tests containing contaminations, debasement items, or placebo fixings with those obtained from an investigation of tests without contaminations, debasement items, or placebo fixings. [27,28]

### **2. Linearity and range:**

The ability of an analytical procedure to produce test results that are directly proportional to the concentration of analyte in the sample (within a given range) is referred to as linearity. A linear relationship should be evaluated across the analytical procedure's range. The proposed procedure is used to demonstrate it directly on the drug substance by dilution of a standard stock solution of the drug product components. Linearity is typically expressed as the confidence limit around the regression line's slope. 16-18 The ICH guidelines recommend a minimum of five concentrations for the establishment of linearity. 19 The interval between the upper and lower levels that have been demonstrated to be determined with precision, accuracy, and linearity using the method is referred to as the range of an analytical method.[29]

### **3. Precision:**

It denotes the degree of agreement (degree of scattering) between a set of measurements obtained from multiple samplings of the same homogeneous sample under the specified conditions. Precision is a measure of how reproducible the entire analytical method is. [30] It is made up of two parts: repeatability and intermediate precision. The variation experienced by a single analyst on a single instrument is referred to as repeatability. It does not distinguish between variation caused by the instrument or system and variation caused by the sample preparation process. Repeatability is determined during validation by analyzing multiple

replicates of an assay composite sample using the analytical method. The value of recovery is computed. The variation within a laboratory, such as different days, different instruments, and different analysts, is referred to as intermediate precision. [31] The relative standard deviation is then used to express the precision.

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

#### 4. Accuracy:

The closeness of a measured value to the true or accepted value is defined as accuracy. In practice, accuracy denotes the difference between the mean value discovered and the true value. It is calculated by applying the method to samples containing known amounts of analyte. To ensure that there is no interference, these should be compared to standard and blank solutions. The accuracy is then calculated as a percentage of the analyte recovered by the assay based on the test results. It is frequently expressed as the recovery of known, added amounts of analyte by assay.[32]

#### 5. Solution stability:

The stability of standards and samples is established during validation under normal conditions, normal storage conditions, and sometimes in the instrument to determine if special storage conditions, such as refrigeration or light protection, are required.[33]

#### 6. Limit of Detection (LOD):

The detection limit of a single explanatory method is the most basic measure of analyte in an example that can be recognized but not quantitated as an accurate quality.[34]

#### 7. Limit of Quantification (LOQ):

The quantitation limit of an individual expository system is the smallest amount of analyte in an example that can be quantitatively determined with appropriate accuracy and exactness [35,36]. The quantitation limit is a quantitative test parameter for low levels of mixtures in test lattices, and it is particularly useful for determining polluting influences and/or corruption items.[37]

## 8. Robustness:

The robustness of an analytical procedure is a measure of its ability to remain unaffected by small but deliberate variations in method parameters, and it indicates its dependability under normal conditions. [38]

## 9. Range:

The method's range is the range of an analyte's upper and lower levels that have been determined with acceptable precision, accuracy, and linearity. It is normally expressed in the same units as the test results and is determined on a linear or nonlinear response curve (i.e. where more than one range is involved, as shown below). [39]

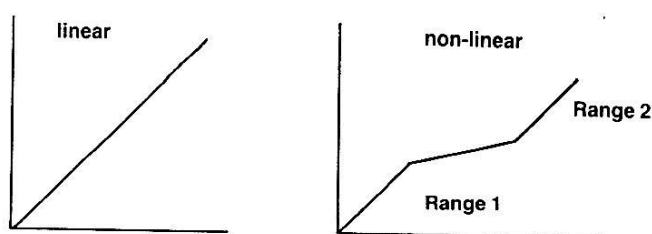


Figure No. 3: Range determination

## 10. System Suitability:

Liquid chromatographic methods include system suitability tests as a standard procedure. They are used to ensure that the chromatographic system's detection sensitivity, resolution, and reproducibility are adequate for the analysis. The tests are based on the idea that the equipment, electronics, analytical operations, and samples to be analyzed are all part of a whole that can be evaluated as a whole. To determine the suitability of the used method, factors such as peak resolution, the number of theoretical plates, peak tailing, and capacity were measured.[40]

## CONCLUSION:

The development of analytical methods for drug identification, purity evaluation, and quantification has received a lot of attention in the field of pharmaceutical analysis in recent years. This review provides a general overview of HPLC method development and validation. A general and very simple approach to developing HPLC methods for compound separation was discussed. Before developing an HPLC method, it is critical to understand the

physicochemical properties of the primary compound. The composition of the buffer and mobile phase (organic and pH) has a significant impact on separation selectivity. Finally, the gradient slope, temperature, and flow rate, as well as the type and concentration of mobile-phase modifiers, can be optimized. The optimized method is validated using various parameters (e.g., specificity, precision, accuracy, detection limit, linearity, and so on) following ICH guidelines.

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