



## A REVIEW ON METHOD DEVELOPMENT AND VALIDATION OF HPLC METHOD

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### ABSTRACT

Validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. Chromatographic methods play significant role in the pharmaceutical industry from the drug discovery, development, formulations and quality control. These methods are essential for a number of purposes, including testing for quality control release, testing of stability samples, testing of reference materials and to provide data to support specifications. A validated analytical method ensures that it provides consistent, reliable and accurate data. So these methods help pharmaceutical analyst to ensure quality products are released for market. This review describes general approach towards validation process and validation parameters to be considered during validation of a HPLC method. It also refers to various regulatory

requirements. The parameters described here are according to ICH guidelines and include accuracy, precision, specificity and limit of detection, limit of quantitation, linearity, range and robustness.

**KEYWORDS:** Method Development, Validation, HPLC.

### INTRODUCTION

Analytic method development and validation are key elements of any pharmaceutical development program. HPLC analysis method is developed to identify, quantity or purifying compounds of interest. This technical brief will focus on development and validation activities as applied to drug products.

## 1. Method development

Effective method development ensures that laboratory resources are optimized, while methods meet the objectives required at each stage of drug development. Method validation, required by regulatory agencies at certain stages of the drug approval process, is defined as the “process of demonstrating that analytical procedures are suitable for their intended use”. Understanding of the physical and chemical characteristics of drug allows one to select the most appropriate high performance liquid chromatography method development from the available vast literature. Information concerning the sample, for example, molecular mass, structure and functionality, pKa values and UV spectra, solubility of compound should be compiled. The requirement of removal of insoluble impurities by filtration, centrifugation, dilution or concentration to control the concentration, extraction (liquid or solid phase), derivatization for detection etc. should be checked. For pure compound, the sample solubility should be identified whether it is organic solvent soluble or water soluble, as this helps to select the best mobile phase and column to be used in HPLC method development.

Method development in HPLC can be laborious and time consuming. Chromatographers may spend many hours trying to optimize a separation on a column to accomplish the goals. Even among reversed phase columns, there is astonishing diversity, owing to differences in both base silica and bonded phase characteristics. Many of these show unique selectivity. What is needed is a more informed decision making process for column selection that may be used before the chromatographer enters the laboratory. The method of column selection presented here involves a minimal investment in time initially, with the potential of saving many hours in the laboratory.

Analytic methods are intended to establish the identity, purity, physical characteristics and potency of the drugs that we use. Methods are developed to support drug testing against specifications during manufacturing and quality release operations, as well as during long-term stability studies. Methods that support safety and characterization studies or evaluations of drug performance are also to be evaluated. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light in order to evaluate the potential degradation of the API in the presence of formulation excipients.

The three critical components for a HPLC method are: sample preparation (% organic, pH, shaking/sonication, sample size, sample age) analysis conditions (%organic, pH, flow rate, temperature, wavelength, and column age), and standardization (integration, wavelength,

standard concentration, and response factor correction). During the preliminary method development stage, all individual components should be investigated before the final method optimization. This gives the scientist a chance to critically evaluate the method performance in each component and streamline the final method optimization. The percentage of time spent on each stage is proposed to ensure the scientist will allocate sufficient time to different steps. In this approach, the three critical components for a HPLC method (sample preparation, HPLC analysis and standardization) will first be investigated individually.

The degraded drug samples obtained are subjected to preliminary chromatographic separation to study the number and types of degradation products formed under various conditions. Scouting experiments are run and then conditions are chosen for further optimization. Resolving power, specificity, and speed are key chromatographic method attributes to keep in mind during method development. Selectivity can be manipulated by combination of different factors like solvent composition, type of stationary phase, mobile phase, buffers and pH. Changing solvents and stationary phases are the most comfortable approaches to achieve the separation. The proper range of pH is an important tool for separation of ionizable compounds. Acidic compounds are retained at low pH while basic compounds are more retained at higher pH. The neutral compounds remain unaffected. The pH range 4-8 is not generally employed because slight change in pH in this range would result in a dramatic shift in retention time. However, by operating at pH extremes (2-4 or 8-10), not only is there a 10-30 fold difference in retention time that can be exploited in method development but also the method can be made more robust which is a desirable outcome with validation in minutes.

Various

**Steps for HPLC method development are given below.**

Steps for HPLC method development

1. Information on sample
2. Defining separation goals
3. Special procedure requirement, sample pretreatment, if any
4. Detector selection and setting
5. Separation conditions optimization
6. Checking for problems or special procedure requirements
7. Recovery of purified material
8. Quantitative calibration/ Qualitative method

9. Method validation for release to laboratories

## 2. Requirements for good method development

### 2.1 Choosing the appropriate HPLC column

C18 columns are the commonly used columns in HPLC method analysis. C8 or Octyl bonded phases are also used occasionally. Like C18, they are non-polar, but not as hydrophobic. Therefore, retention times for hydrophobic compounds are typically shorter. Also, they may show somewhat different selectivity than C18 due to increased base silica exposure unique selectivity results in proton interaction of the bonded phase with electron deficient functional groups of solute molecules. The chemical structure of C18 column is shown in Figure-1.

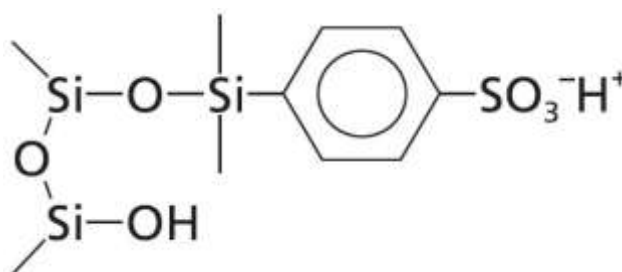


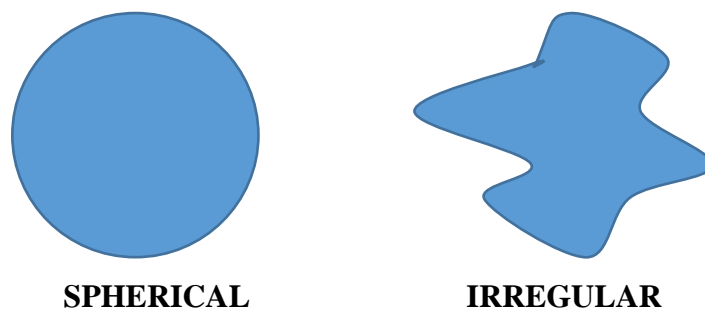
Figure-1: Chemical structure of C18 column

### 2.2 Column Dimensions

This refers to the length (Figure-3.3) and internal diameter of the packing media bed within the column tube. Short columns (30-50mm) offer short run times, fast equilibration, low back pressure and high sensitivity. Long columns (250-300mm) provide higher resolving power, but create more backpressure, lengthen analysis times and use more solvent. Narrow column (2.1mm and smaller) beds inhibit sample diffusion and produce narrower, taller peaks and a lower limit of detection. They may require instrument modification to minimize distortion of the chromatography. Wider columns (10-22mm) offer the ability to load more sample.

### 2.3 Particle shape

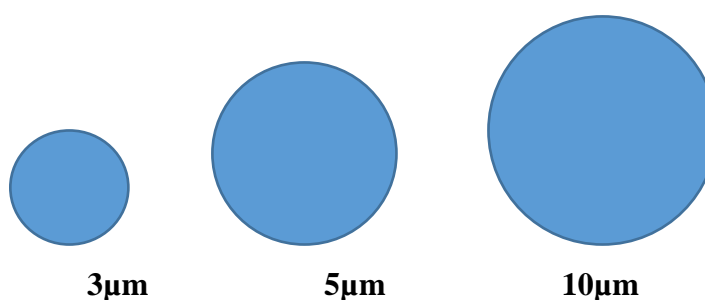
Most modern chromatographic packings have spherical particles (Figure-3.4), but some are irregular in shape. Spherical particles offer reduced back pressures and longer column life when using viscous mobile phases like 50:50 MeOH: H<sub>2</sub>O.



**Figure 2: Particles with different shapes.**

#### **2.4 Particle Size**

This refers to the average diameter (Figure-3.5) of the packing media particles. Standard particle sizes range from 3 $\mu\text{m}$  (high efficiency) to 15-20 $\mu\text{m}$  (preparative). A 5 $\mu\text{m}$  particle size offers a good compromise between efficiency and back pressure. Smaller particles pack into columns with a higher density, allowing less diffusion of sample bands between particles and causing narrower, sharper peaks. However, smaller particles also cause higher solvent back pressures. As a rule of thumb, 1.5 or 3 $\mu\text{m}$  particle sizes are to chosen for resolving complex, multi-component samples. Otherwise, 5 or 10 $\mu\text{m}$  packings should be considered.



**Figure-3: Particles with different diameters.**

#### **2.5 Surface area**

Expressed in  $\text{m}^2/\text{gram}$ , the total surface area of a particle is the sum of the outer particle surface and the interior pore surface (Figure-4). Solute retention is greater on packing's that have a high surface area. High surface areas generally provide longer retention, greater capacity and higher resolution. As a rule of thumb, a base material with maximum surface area is to be used for resolving complex and multi-component samples.



**Figure-4: Different surface area particles.**

## 2.6 Pore size

This refers to the average size of the pores or cavities present in porous packing particles (Figure-5). Pore sizes range from 60Å on the low end to greater than 10,000Å on the high end. Larger pores allow larger solute molecules to be retained longer through maximum exposure to the surface area of the particles. A pore size of 150Å or less is chosen for sample  $MW \leq 2000$ . For sample with molecular weight greater than 2000, columns with a pore size of 300Å or greater are to be used.



**Figure-5: Particles with different pore size.**

## 2.7 Detectors

Various detectors used in HPLC instrument include UV-Visible detector, photodiode array detector, fluorescence detector, conductivity detector, refractive index detector, electrochemical detector, mass spectrometer detector and evaporative light scattering detector. UV-Visible detectors are typical in many laboratories as they can detect a wide array of compounds

## 2.8 pH range

Method development within the different pH ranges from 1 to 12 for better chromatographic resolution between two or more peaks of an analyte depends upon three main factors, column efficiency, selectivity and retention time. The ionizable analytes are either bases or acids and they affect the above three factors dramatically with change in pH. Retention time can be improved by changing the pH that will lead to easy separation of ionizable analytes from non-ionized forms. Change in the mobile phase pH can also improve column efficiency because it alters both the ionization of the analyte and the residual silanols and it also minimizes secondary interactions between analytes and the silica surface that lead to poor

peak shape. To achieve optimum resolution, it requires change in the pH of mobile phase. Method development can proceed by investigating parameters of chromatographic separations first at low pH and then at higher pH until optimum results are achieved. The correlation between pH and retention factor is shown in the Figure-6.

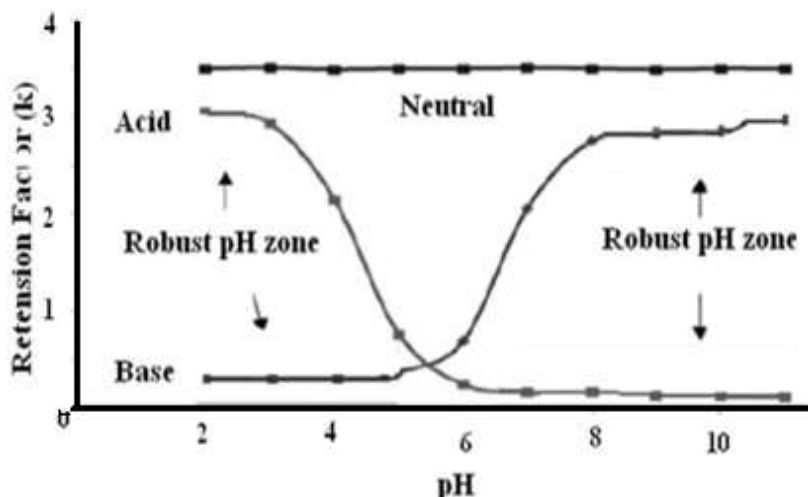


Figure-6: Correlation between retention factor and pH.

## 2.9 Mobile phase composition

Mobile phase composition (or solvent strength) plays an important role in RPHPLC 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.

## 2.10 Column temperature

Separation of many samples can be enhanced by selecting the right column temperature. Higher column temperature reduces system backpressure by decreasing mobile phase viscosity, which in turn allows use of longer columns with higher separation efficiency. However, an overall loss of resolution between mixture components in many samples occurs by increasing column temperature. The optimum temperature is dependent upon the nature of the mixture components. The overall separation can be improved by the simultaneous changes in column temperature and mobile phase composition.

Recently, normal phase HPLC is back popular with the birth of hydrophilic interaction liquid chromatography (HILIC) technology that proved to improve reproducibility in separating polar and hydrophilic compounds such as peptides, carbohydrates, vitamins, polar drugs and



metabolites. In order to develop a HPLC method effectively, most of the effort should be spent in method development and optimization as this will improve the final method performance.

### 3. Method validation

The validation of an analytical method demonstrates the scientific soundness of the measurement or characterization. It is required to provide validation data throughout the regulatory submission process. The validation practice demonstrates that an analytical method measures the correct substance, in the correct amount, and in the appropriate range for the intended samples. It allows the analyst to understand the behavior of the method and to establish the performance limits of the method.<sup>[20,21]</sup> The goal is to identify the critical parameters and to establish acceptance criteria for method system suitability.

Validation is defined by the International Organization for Standardization (ISO) as “verification, where the specified requirements are adequate for an intended use”, where the term verification is defined as “provision of objective evidence that a given item fulfills specified requirements”. The applicability and scope of an analytical method should be defined before starting the validation process. It includes defining the analytes, concentration range, description of equipment and procedures, validation level and criteria required. The validation range is defined by IUPAC as “the interval of analyte concentration within which the method can be regarded as validated”. This range is not the highest and lowest possible levels of the analyte that can be determined by the method. Instead, it is defined on the basis of the intended purpose of the method. The method can be validated for use as a screening (qualitative), semi-quantitative (5-10ppm) or quantitative method. It can also be validated for use on single equipment, different equipment’s in the laboratory, different laboratories or even for international use at different climatic and environmental conditions. The criteria of each type of validation will of course be different with the validation level required.<sup>[27]</sup> The various validation parameters include linearity, accuracy, precision, ruggedness, robustness, LOD, LOQ and selectivity or specificity.

#### 3.1. Linearity

Linearity of a method is its ability to obtain test results that are directly proportional to the sample concentration over a given range. For HPLC methods, the linear relationship between detector response (peak area and height) and sample concentration is determined. The relationship can be directly on drug substance by dilution of standard stock or by separate



weighing of the sample components, using the proposed procedures. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is linear relationship, test results should be evaluated by appropriate statistical methods, for example, by regression analysis. Data from the regression line is helpful to provide mathematical estimates of the degree of linearity. It is generally expressed in terms of variance around the slope of regression line. In some cases, the analytical responses should be described by the appropriate function of the analyte concentration. The widely used linearity ranges and acceptance criteria for various pharmaceutical methods are listed in the table.

**Table: Linearity ranges and Acceptance criteria for various pharmaceutical methods.**

Test	Linearity levels and ranges	Acceptance criteria
Assay	Five levels, 50-150% of label claim	Correlation coefficient, $R \geq 0.999$
Dissolution	Five to eight levels, 10-150% of label claim	% y intercept NMT 2.0% $R \geq 0.99$
Related substances	Five levels, LOQ to acceptance criteria	% y intercept NMT 5.0% $R \geq 0.99$

### 3.2. Accuracy

Accuracy is defined by ISO as “closeness of agreement between a measured quantity value and a true quantity value”. It is a qualitative characteristic that cannot be expressed as a numerical value. It has an inverse relation to both random and systematic errors, where higher accuracy means lower errors. Accuracy is evaluated by analyzing test drug at different concentration levels. Typically, known amounts of related substances and the drug substance in placebo are spiked to prepare an accuracy sample of known concentration of related substance. Samples are prepared in triplicate. ICH recommends accuracy evaluation using a minimum of nine determinations over a minimum of three concentration levels covering the range specified. It is determined by comparing the found concentration with the added concentration. The methods of determining accuracy include analysis of known purity (reference material), comparison of results of the proposed analytical procedure with those of a second well characterized procedure and standard addition method. The accuracy may also be inferred once precision, linearity and specificity have been established.

### 3.3. 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 may be considered at three levels: repeatability, intermediate precision and reproducibility. Repeatability is also referred to as intra-assay precision. It is a measure of precision of analysis in one laboratory by one operator using one piece of equipment over a relatively short time-span. It is degree of agreement of results when experimental conditions are as constant as possible, and expressed as RSD of replicate values. ICH recommends a minimum of nine determinations covering the specified range for the procedure (e.g., three concentrations/three replicates as in the accuracy experiment), or a minimum of six determinations at 100% of the test concentration for evaluation of repeatability which should be reported as standard deviation, relative standard deviation (coefficient of variation) or confidence interval. ICH defines intermediate precision as long-term variability of the measurement process and is determined by comparing the results of a method run within a single laboratory over a number of weeks. It is also called as intraday precision. It reflects discrepancies in results obtained by different operators, from different instruments, with standards and reagents from different suppliers, with columns from different batches or a combination of these but in the same laboratory. The objective of intermediate precision validation is to verify that the method will provide same results in the same laboratory once the development phase is over.

Reproducibility expresses precision of analysis of the same sample by different analysts in different laboratories using operational and environmental conditions that may differ but are still within the specified parameters of the method. The objective is to verify that the method will provide the same results despite differences in room temperature and humidity, variedly experienced operators, different characteristics of equipment's (e.g., delay Volume of an HPLC system), variations in material and instrument conditions (e.g. in HPLC, mobile phase composition, pH, flow rate of mobile phase), equipment's and consumables of different ages, columns from different suppliers or different batches and solvents, reagents and other material with different quality.

#### **3.4. Selectivity and Specificity**

Selectivity and specificity are sometimes used interchangeably to describe the same concept in method validation. Selectivity of an analytical method is defined by the ISO as property of a measuring system, used with a specified measurement procedure, whereby it provides measured quantity values for one or more measured such that the values of each measured are independent of other measured or other quantities in the phenomenon, body, or substance

being investigated". Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. The specificity of a test method is determined by comparing test results from an analysis of samples containing impurities, degradation products, or placebo ingredients with those obtained from an analysis of samples without impurities, degradation products, or placebo ingredients. Specificity can best be demonstrated by resolution between the analyte peak and the other closely eluting peak.

### **3.5. Detection limit (LOD) and Quantitation limit (LOQ)**

LOD of an analytical procedure is the lowest concentration of an analyte in a sample which can be detected but not necessarily quantitated as an exact value whereas LOQ is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. ICH guidelines describe three methods for determining LOD and LOQ that include:

#### **3.5.1 Visual evaluation**

It may be used for both non instrumental and instrumental methods. The LOD and LOQ is determined by analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected or quantified with acceptable accuracy and precision respectively.

#### **3.5.2 Signal to noise ratio approach**

This method can only be applied to analytical procedures which exhibit baseline noise. It is determined by comparing measured signals from samples of known low concentrations of analyte with those of blank samples and establishing minimum concentration at the analyte can be reliably detected. A S/N ratio of 3:1 is considered acceptable for estimating LOD (with Relative Standard Deviation (RSD)  $\leq$  10%) LOQ, a S/N ratio of 10:1 is considered appropriate (with Relative Standard Deviation (RSD)  $\leq$  3%) as illustrated

### **3.6. Range**

The range of an analytical procedure is the interval between the upper and lower concentrations of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure. The range

of an analytical method varies with its intended purpose. It is generally 80-120% of the test concentration for the assay of a drug substance or a finished (drug) product, 70-130% of the test concentration for content uniformity,  $\pm 20\%$  over the specified range for dissolution testing, reporting level of an impurity to 120% of the specification for the determination of an impurity. It should commensurate with LOD or LOQ (the control level of impurities), for impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects.

### 3.7. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. For the determination of method robustness, a number of chromatographic parameters, for example, flow rate, column temperature, injection Volume, detection wavelength and mobile phase composition are varied within a realistic range and quantitative influence of the variables is determined. If the influence of the parameter is within a previously specified tolerance, the parameter is said to be within the method's robustness range.

Obtaining data on these effects will allow to judge whether a method needs to be revalidated when one or more of parameters are changed, for example to compensate for column performance over time.<sup>[44,45]</sup> Variation in method conditions for robustness should be small and reflect typical day-to-day variation. Critical parameters are identified during the method development process. Only these critical method parameters should be investigated for robustness. Common critical method parameters can be divided into two categories. The HPLC conditions include HPLC column (lot, age, and brand), Mobile-phase composition (pH  $\pm 0.05$ , organic content  $\pm 2\%$ ) and HPLC instrument (dwell Volume, detection wavelength  $\pm 2$  nm, column temperature  $\pm 5^\circ\text{C}$  and flow rate). The sample preparation variations include sample solvent (pH  $\pm 0.05$  unit, organic content  $\pm 2\%$ ), sample preparation procedure (shaking time, different membrane filters) and HPLC solution stability. The variations in chromatographic parameters for robustness study are given in Table.

**Table: Variations in chromatographic parameters for robustness study.**

Sr. No	Robustness parameter	Change
1	Detection wavelength	$\pm 5$ nm
2	Flow rate	$\pm 0.05$ ml/min
3	Buffer pH	$\pm 0.1$ unit
4	Mobile phase	$\pm 2$ ml
5	Column	Different brand or batch number

#### 4. CONCLUSION

The growing pharmaceutical industry demands various analytical methods for various pharmaceutical products. To ensure quality of the product, it is necessary that the analytical method used for assuring quality should give accurate and predictable results. For this the method need to be validated. The HPLC methods are the preferred methods of analysis due to their responsiveness. This review guides analysts to validate chromatographic methods in order to comply with regulatory requirements.

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