

Review Article

Method development by liquid chromatography with validation

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Abstract

HPLC method development and validation plays an important role in the discovery, manufacturing, development of pharmaceutical products. HPLC methods are able to separate, detect and quantify various drugs their related substances, degraded products and impurities that may be introduced during the synthesis of drug substance. Here in this review various strategies for method development and validation on HPLC are well described. Along with this systematic approach for method validation is also described. Method validation establishes the performance characteristics and limitations of the developed method. Optimization of chromatographic conditions includes fixation of parameters like mobile phase, stationary phase, detection wavelength, elution mode that must affords to system suitability as well as stability of drugs, degradants, impurities. Force degradation studies helpful in the development and validation of stability indicating assay. They also demonstrates the specificity while developing stability indicating assay.

Keywords: HPLC, Method development, Method validation, force degradation studies, Analytical chemistry.

1. Introduction

Analytical chemistry is an art and science of recognizing the different substances in a particular matrix and determining their constituents. Analytical chemistry is divided into 2 parts qualitative and quantitative analysis. Qualitative is used for identification of material while quantitative analysis is used for the determination of quantity of material. The instrumental methods of analysis are based upon measurement of physical properties of material using some instrument to determine its composition. The instrumental methods are simple, precise, reproducible compare to classical methods of analysis therefore now day's analytical methods are developed using sophisticated instruments like UV, HPLC, HPTLC and GC¹. Analytical method development and validation are continuous and interconnected activities throughout the drug development process it is very much trial and error basis approach. Here in this review the method development on RP-HPLC has been described and its further validation. The techniques high performance liquid chromatography is so called because of its improved performance over classical column chromatography and it is the most important tools of analytical chemistry today. 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.

- High resolution
- Small diameter, Stainless steel, Glass column
- Rapid analysis
- Relatively higher mobile phase pressure
- Controlled flow rate of mobile phase²

Method development on HPLC: Steps involved in method development of HPLC is as follows

- a) Understanding the Physicochemical properties of drug molecule.
- b) Selection of chromatographic conditions.
- c) Developing the approach of analysis.
- d) Sample preparation
- e) Method optimization
- f) Method validation

a) Understanding the physicochemical properties of drug molecule^{1,2}: Physicochemical properties of a drug molecule play an important role in method development. For Method development one has to study the physical properties like solubility, polarity, pKa and pH of the drug molecule. Polarity is a physical property of a compound. It helps an analyst, to decide the solvent and composition of the mobile phase. 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. $pH = -\log_{10}[H_3O^+]$. 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.

b) Selection of chromatographic conditions:

Selection of column: Selection of the stationary phase/column is the first and the most important step in method development. The development of a rugged and reproducible method is impossible without the availability of a stable, high performance column. To avoid problems from irreproducible sample retention during method development, it is important that columns be stable and reproducible. A C8 or C18 column made from specially purified, less acidic silica and designed specifically for the separation of basic compounds is generally suitable for all samples and

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is strongly recommended³. The separation selectivity for certain components vary between the columns of different manufacturer as well as between column production batches from the same manufacturer. Column dimensions, silica substrate properties and bonded stationary phase characteristics are the main ones. The use of silica-based packing is favored in most of the present HPLC columns due to several physical characteristics. Silica substrates are available in spherical or irregular shapes and can be prepared with different surface areas, pore sizes and particle sizes, which make them suitable for most HPLC applications. @-MS phases are stable over the pH range of 1-10. Totally porous silica particles with 5 µm diameter provide the desired characteristics for most HPLC separations. Zirconia-based columns are revolutionary HPLC phases. Zirconia particles are mechanically stable, and have a porous structure similar to that of silica. However, zirconia's main advantage over silica is that it is very stable in a wide range of eluent pH; indeed the ZirChrom®-EZ and ZirChrom.⁴

Selection of column oven 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 nature of the mixture components. The overall separation can be improved by simultaneous changes in column temperature and mobile phase composition.⁴

Selection of Mobile Phase: The mobile phase effects resolution, selectivity and efficiency. Mobile phase composition (or solvent strength) plays an 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^{2,4}. Selection of the mobile-phase and gradient conditions is dependent on the ionogenic nature of the analyte and the hydrophobicity of the analytes in the mixture respectively. The aqueous buffer serves several purposes. At low pH, the mobile phase protonates free silanols on the column and reduces peak tailing. At sufficiently low pH basic analytes are protonated; when ionized the analyte will elute more quickly but with improved peak shape. Acidic analytes in buffers of sufficiently low pH will remain uncharged, increasing retention. Conversely, at higher pH neutral basic compounds will be more retained, and ionized acidic compounds will elute earlier. Peak splitting may be observed if the pKa of a compound is similar to the pKa of the buffer, and the analyte elutes as both a charged and uncharged species. The pH of a buffer will not greatly affect the retention of non-ionizable sample components. The most commonly employed buffer is phosphate buffer. The pH of a phosphate buffer is easily adjusted by using mono-, di-, or tribasic phosphate salts. However, when phosphate salts are used the solution should be filtered to remove insoluble particles with 0.22µm filter paper^{1,2}. Acetonitrile and Methanol are 2 most widely employed organic modifiers. Isopropanol (2-propanol) can be employed because of its strong eluting properties, but is limited by its high viscosity which results in lower column efficiencies and higher backpressures. Both acetonitrile and methanol are less viscous than isopropanol. All three solvents are essentially UV transparent. This is a crucial property for reversed phase chromatography since column elution is typically monitored using UV detectors. Acetonitrile is used almost exclusively when separating peptides. Most peptides only absorb at low wavelengths in the ultra-violet spectrum (typically less than 225 nm) and acetonitrile provides much lower background absorbance than other common solvents at low wavelengths.⁵

Selection of elution mode: There are basically 2 modes of elution employed: Isocratic and Gradient. Isocratic, constant eluent composition means equilibrium conditions in the column and the actual velocity of compounds moving through the column are constant; analyte-eluent and analyte-stationary-phase interactions are also constant throughout the whole run. This makes isocratic separations more predictable, although the separation power (the number of compounds which could be resolved) is not very high. The peak capacity is low; and the longer the component is retained on the column, the wider is the resultant peak. Gradient separation significantly increases the separation power of a system mainly because of the dramatic increase of the apparent efficiency (decrease of the peak width). Gradient elution is employed for complex multicomponent samples since it may not be possible to get all components eluted between k (retention factor) 1 and 10 using a single solvent strength under isocratic conditions. When a gradient method is used, the column must be allowed to equilibrate at the starting mobile-phase conditions prior to the next sample injection and the start of the next gradient run.^{1,2,5}

Selection of detector: Detector is a very important part of HPLC. Selection of detector depends on the chemical nature of analytes, 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. Refractive Index (RI) Detector offers high sensitivity, stability and reproducibility, which make 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. Characteristics that are to be fulfilled by a detector to be used in HPLC determination are:

- ✓ High sensitivity, facilitating trace analysis.
- ✓ Negligible baseline noise to facilitate lower detection.
- ✓ Low drift and noise level.
- ✓ Wide linear dynamic range (this simplifies quantitation).
- ✓ Low dead volume (minimal peak broadening).
- ✓ Cell design that eliminates remixing of the separated bands.
- ✓ Insensitivity to changes in type of solvent, flow rate and temperature.
- ✓ Operational simplicity and reliability.
- ✓ Tunability so that detection can be optimized for different compounds.^{1,2,5,4}

c) Developing the approach for analysis: While developing the analytical method on RP-HPLC the first step which is followed is the selection of various chromatographic parameters like selection of mobile phase, selection of column, selection of flow rate of mobile phase, selection of pH of mobile phase. All of these parameters are selected on the basis of trials and followed by considering the system suitability parameters. Typical parameters of system suitability are e.g. retention time should be more than 5 min, the theoretical plates should be more than 2000, the tailing factor should be less than 2, resolution between 2 peaks should be more than 5, % R.S.D. of the area of analyte peaks in standard chromatograms should not be more than 2.0 % like other. Detection wavelength is usually isobestic point in the case of simultaneous estimation of 2 components. 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^{7,13-20}.

d) Sample preparation: The drug substance being analyzed should be stable in solution (diluent). During initial method development, preparations of the solutions in amber flasks should be performed until it is determined that the active component is stable at room temperature and does not degrade under normal laboratory conditions. The sample solution should be filtered; the use of a 0.22 or 0.45 µm pore-size filter is generally

recommended for removal of particulates. Filtration is a preventive maintenance tool for HPLC analyses. Sample preparation is a critical step of method development that the analyst must investigate. The sample should ideally be dissolved in the initial mobile phase. If this is not possible due to stability or solubility problems, formic acid, acetic acid or salt can be added to the sample to increase solubility. These additives do not usually affect the separation so long as the volume of the sample loaded is small compared to the column volume. The only effect when large sample volumes are applied may be an extra peak or two eluting in the void volume after sample injection. Sample preparation is an essential part of HPLC analysis, intended to provide a reproducible and homogenous solution that is suitable for injection onto the column. The sample preparation encompasses the following steps:

- Sample collection
- Sample storage and preservation
- Preliminary sample processing
- Weighing or volumetric dilutions
- Alternative sample processing methods
- Removal of particulates
- Sample extraction
- Derivatization^{2,5}

e) Method optimization: The experimental conditions should be optimized to get desired separations and sensitivity after getting appropriate separations. This will be achieved through planned/systemic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, temperature, sample amounts, Injection volume and diluents solvent type.^{1,2}

f) Method validation^{2,11,12,16}: The following are typical analytical performance characteristics which may be tested during methods validation:

- Accuracy
- Precision
- Repeatability
- Intermediate precision
- Linearity
- Detection limit
- Quantitation limit
- Specificity
- Range
- Robustness
- System suitability determination
- Forced degradation studies
- Solution stability studies

Accuracy is the nearness of a measured value to the true or accepted value. Accuracy indicates the deviation between the mean value found and the true value. It is determined by applying the method to samples to which known amounts of analyte have been added. These should be analysed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay. It may often be expressed as the recovery by the assay of known, added amounts of analyte

Precision of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample. Precision is a measure of the reproducibility of the whole analytical method²³. 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 analyzing 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. The precision is then expressed as the relative standard deviation.

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

Linearity is the ability of analytical procedure to obtain a response that is directly proportional to the concentration (amount) of analyte in the sample. If the method is linear, the test results are directly or by well-defined mathematical transformation proportional to concentration of analyte in samples within a given range. Linearity is usually expressed as the confidence limit around the slope of the regression line.

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.^(book) 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.

Limit of Quantitation (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. For analytical procedures such as HPLC that exhibit baseline noise, the LOQ is generally estimated from a determination of S/N ratio (10:1) and is usually confirmed by injecting standards which give this S/N ratio and have an acceptable percent relative standard deviation as well.

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as impurities, degradation products, and excipients. Specificity measures only the desired component without interference from other species that might be present; separation is not necessarily required.

Range is defined as the interval between the upper and lower concentrations of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity.

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

System Suitability Determination is the evaluation of the components of an analytical system to show that the performance of a system meets the standards required by a method. These parameters can be calculated experimentally to provide a quantitative system suitability test report: number of theoretical plates (efficiency), capacity factor, separation (relative retention), resolution, tailing factor, relative standard deviation (precision). These are measured on a peak or peaks of known retention time and peak width.

Forced degradation or stress studies are undertaken to deliberately degrade the sample. These studies are used to evaluate an analytical method's ability to measure an active ingredient and its degradation products, without interference, by generating potential degradation products. During validation of the method, drug substance are exposed to acid, base, heat, light and oxidizing agent to produce approximately 10% to 30% degradation of active substance. The studies can also provide information about the degradation pathways and degradation products that could form during storage. Reasons for carrying out forced degradation studies include: development and validation of stability-indicating methodology, determination of degradation pathways of drug substances and drug products, discernment of degradation products in formulations that are related to drug substances versus those that are related to non-drug substances.

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

2. Advantages of method validation:

- It builds up the confidence in not only the user but also in developer.
- The method validation absorbs the shocks of analytical variations and pays for more than invested on process.
- It proves inexpensive by removing frustrating repetitions.¹

Figure 1: Mechanism of gradient separations in RP-HPLC⁵

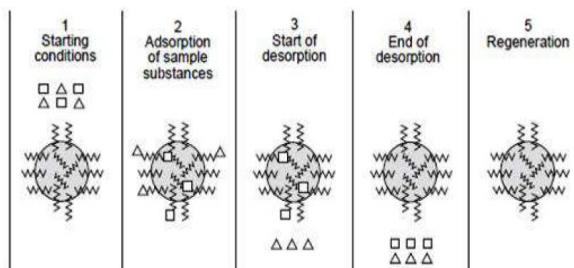


Figure 2: Typical HPLC system⁴

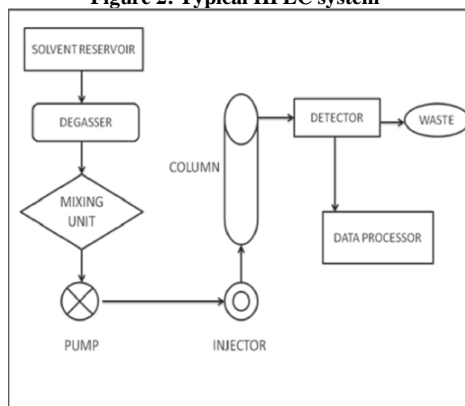


Figure 3: Typical steps involved in HPLC method development⁵

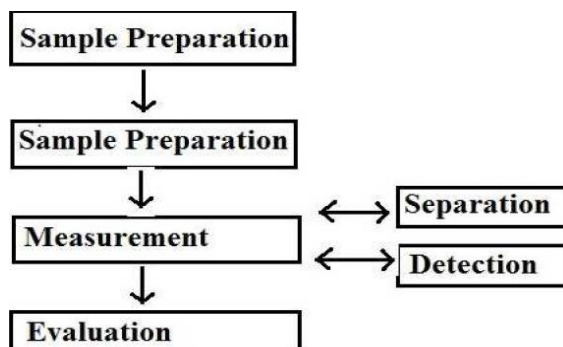
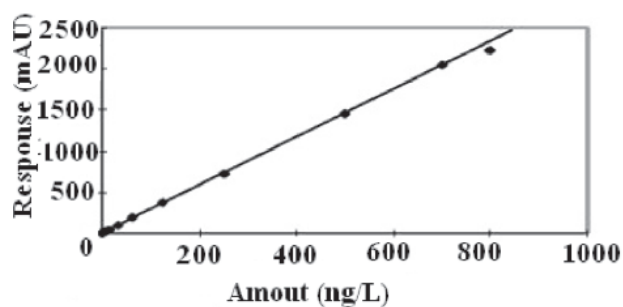


Figure 4: Linearity Pattern⁴

3. Conclusion

Analytical method development and validation are interconnected activities throughout the drug development process. This review describes the general technique of HPLC method development and validation of optimized method. The analytical validation verifies that a given method measures a parameter as intended and establishes the performance limits of the measurement. Reproducible quality HPLC results can only be obtained if proper attention has been paid to the method development, validation and system's suitability to carry out the analysis. While developing the analytical methods for pharmaceuticals by RP-HPLC, must have good practical understanding of chromatographic separation to know how it varies with the sample and with varying experimental conditions in order to achieve optimum separation.

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