WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.074
Volume 8, Issue 3, 352-363. Review Article ISSN 2277– 7105

A REWIEW ON METHAOD DEVELOPMENT AND VALIDATION BY HPLC

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Article Received on
31 Dec. 2018,

Revised on 21 Jan. 2019,
Accepted on 10 Feb. 2019

DOI: 10.20959/wjpr20193-13860

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ABSTRACT

HPLC methods development and validation play important roles in new discovery, development, manufacture of pharmaceutical drugs and various other studies related to humans and animals. Analytical method validation required during drug development and manufacturing and these analytical methods are fit for their intended purpose. Many different strategies of high performance liquid chromatographic method development are used today. HPLC is an analytical tool which can detect, separate and quantify the drug, its various impurities and drug related degradants that can form on synthesis or storage. It involves the understanding of chemistry of drug substance and facilitates the development of analytical method. Most of the drugs in multi component dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. A number of chromatographic parameters were evaluated in order to optimize the method. An appropriate mobile phase, column, column temperature, wavelength and gradient must be found that affords suitable compatibility and stability of drug as well as degradants and impurities.

KEYWORDS: High Pressure Liquid Chromatography (HPLC), Method validation, Method development.

INTRODUCTION

Analytical method development and validation play important roles in the discovery, development and manufacturing of pharmaceutical products. Among various analytical methods, HPLC (High Performance Liquid Chromatography) - dominant and most powerful analytical technique in todays modern pharmaceutical world, have been widely practiced for

over five decades because of its wide applicability to innumerous analytes in very wide variety of matrices.[R8]

Chromatography is defined as a set of techniques which is used for the separation of constituents in a mixture. This technique involves 2 phases stationary and mobile phases. The separation of constituents is based on the difference between partition coefficients of the two phases.[R13] Has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid. High performance liquid chromatography (HPLC) is the most accurate analytical methods widely used for the quantitative as well as qualitative analysis of drug product. HPLC is the method of choice for checking peak purity of new chemical entities, monitoring reaction changes is in synthetic procedures or scale up, evaluating new formulations and carrying out quality control / assurance of the final drug products.[3] The Goal of HPLC method is to try & separate, quantify the main drug, any reaction impurities, all available synthetic intermediates and any degradants.[R4]] High Performance Liquid Chromatography (HPLC) was derived from the classical column chromatography and, is one of the most important tools of analytical chemistry today. The principle is that a solution of the sample is injected into a column of a porous material (stationary phase) and a liquid (mobile phase) is pumped at high pressure through the column. The separation of sample is based on the differences in the rates of migration through the column arising from different partition of the sample between the stationary and mobile phase. Depending upon the partition behaviour of different components, elution at different time takes place.[R6]

Component Involve In HPLC System HPLC System In Their Overall Instrumentation Have Following Components As: Solvent reservoir, Pump module, Injector, Column compartment and column, Detector, Data integration system.

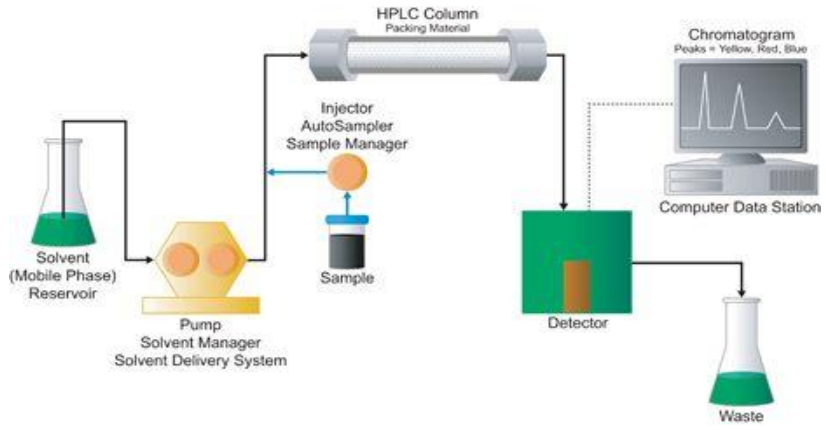

Figure.

Solvent reservoir

Solvent Reservoir Mobile phase storage glass container used in HPLC known as solvent reservoir. There are four channels or mobile phase lines as A, B, C and D are connected to system for mobile phase supply. These mobile phase channels are dipped into mobile phase which store in the solvent reservoir bottles made up of glass.

Pump Module

As we know in HPLC mobile phase play an important role for the separation so to force up the mobile phase into the stationary phase pumps are used. In most of the HPLC system pumps can operate about 5000psi pressure but now ultimate HPLC system launched by dionex can operate 8000psi pressure which is the latest advancement in HPLC pump modules.

Sample Injector

To inject a sample into HPLC system there is a sample injector. In most of the HPLC system Auto samplers are used which controlled by software programming but in some models of HPLC we have manual samplers too in which we inject the sample by the help of injection or syringe. In pharmaceutical industries auto sampler are used so for this purpose different sample solutions are filled in a different vials and load into sampler plate then set the sequence programming by the software. According to sequence set by the user sample get injected automatically.

Columns

Columns are the heart for HPLC so there is a great impact of column packing configuration on separation of drug components. In HPLC columns are made up of stainless steel. Columns are available in different sizes as between 50-300 mm in length having an internal diameter between 2-5mm. The packing materials used in columns are generally having particle size between 3-10 µm. In HPLC, column compartment play an essential role to maintain the temperature of mobile phase and column as temperature affects Detector In HPLC, detectors are used to generate electric signals in form of absorbance with respect to time (retention time of components). A separate compartment is situated at the bottom of HPLC system for detector. To observe an accurate and error less signals, detectors should sensitive to analyte with lower detector noise. UV-visible, photo diode array, fluorescence, mass spectroscopic are the most common HPLC detectors but UV-visible detector is the most common detector used for analytical methods of most of the pharmaceutical drug components.[R9]

Data integration system: Computer operating systems to integrate and store the data obtain in form of signals, used in HPLC system. Various software are used to integrate the data as per the requirement like „Empower" is the most common software used to integrate the data which is developed by the popular HPLC brand known as „WATERS". On the other hand „Chromeleon" is software used to integrate data in HPLC system made by the brand „DIONEX". [Thammana M. A review on high performance liquid chromatography. Research & Reviews: Journal of Pharmaceutical Analysis, 2016; 5(2): 22.]

The technique of HPLC has following features

1. High resolution
2. Small diameter, Stainless steel, Glass column
3. Rapid analysis
4. Relatively higher mobile phase pressure
5. Controlled flow rate of mobile phase[R4]

Analytical Method Development

Analytical chemistry deals with methods for identification, separation, and quantification of the chemical components of natural and artificial materials.[R18] When there are no authoritative methods are available, new methods are being developed for analysis of novel products. To analyze the existing either pharmacopoeial or non-pharmacopoeial products novel methods are developed to reduce the cost besides time for better precision and ruggedness. These methods are optimized and validated through trial runs. Alternate methods are proposed and put into practice to replace the existing procedure in the comparative laboratory data with all available merits and demerits.

Steps involve in method development are

1. Understanding the Physicochemical properties of drug molecule.
2. Selection of chromatographic conditions.
3. Sample preparation
4. Method optimization
5. Method validation[R4]

1. Understanding the Physicochemical properties of drug molecule

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. 6 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. Selection of diluents is based on the solubility of analyte. The acidity or basicity of a substance is defined most typically by the pH value. Selecting a proper pH for ionizable analytes often leads to symmetrical and sharp peaks in HPLC.(R4).

2. Set up HPLC conditions

A buffer is a partially neutralised acid which resists changes in pH. Salts such as Sodium Citrate or Sodium Lactate are normally used to partially neutralise the acid. Buffering Capacity is the ability of the buffer to resist changes in pH. (i) Buffering Capacity increases as the molar concentration (molarity) of the buffer salt/acid solution increases. (ii) The closer the buffered pH is to the pKa, the greater the Buffering Capacity. (iii) Buffering Capacity is expressed as the molarity of Sodium Hydroxide required to increase pH by 1.0. Consideration of the affect of pH on analyte retention, type of buffer to use, and its concentration, solubility in the organic modifier and its affect on detection are important in reversed-phase chromatography (RPC) method development of ionic analytes. An improper choice of buffer, in terms of buffering species, ionic strength and pH, can result in poor or irreproducible retention and tailing in reverse-phase separation of polar and ionizable compounds. Buffer selection Choice of buffer is typically governed by the desired pH. The typical pH range for reversedphase on silica-based packing is pH 2 to 8. It is important that the buffer has a pKa close to the desired pH since buffer controls pH best at their pKa. A rule is to choose a buffer with a pKa value <2 units of the desired mobile phase Ph.

General considerations during buffer selection

1. Phosphate is more soluble in methanol/water than in acetonitrile/water or THF/water.
2. Some salt buffers are hygroscopic. This may lead to changes in the chromatography (increased tailing of basic compounds, and possibly selectivity differences).
3. Ammonium salts are generally more soluble in organic/water mobile phases·
4. TFA can degrade with time, is volatile, absorbs at low UV wavelengths.

5. Microbial growth can quickly occur in buffered mobile phases that contain little or no organic modifier. This growth will accumulate on column inlets and can damage chromatographic performance.
6. At pH greater than 7, phosphate buffer accelerates the dissolution of silica and severely shortens the lifetime of silica-based HPLC columns. If possible, organic buffers should be used at pH greater than 7.
7. Ammonium bicarbonate buffers usually are prone to pH changes and are usually stable for only 24 to 48 hours. The pH of this mobile phase tends to become more basic due to the release of carbon dioxide.
8. After buffers are prepared, they should be filtered through a 0.2-µm filter.
9. Mobile phases should be degassed. Buffer selection Generally, a buffer concentration of 10-50 mM is adequate for small molecules. Generally, no more than 50% organic should be used with a buffer. This will depend on the specific buffer as well as its concentration. Phosphoric acid and its sodium or potassium salts are the most common buffer systems for reversed-phase HPLC. Phosphonate buffers can be replaced with sulfonate buffers when analyzing organophosphate compounds.[R3]

Selection of Detector and Detection Wavelength

Following consideration must be given while selecting detector for the method, Do the analytes have chromophores to enable UV detection? - Will the sample require chemical derivatization to enhance detectability and/or improve the chromatography? - What detection limits are necessary? - Is more selective/sensitive detection required?

Different types of detectors are available such as- Ultraviolet(UV), most commonly used in pharmaceutical industry - Photo Diode Array(PDA), is very well suited for method development - Fluorescence - Electrochemical - Light Scattering - Refractive Index(RI) - Flame Ionization Detection(FID) - Evaporative Light Scattering Detection(ELSD) - Corona Aerosol Detection(CAD) - Mass Spectrometric(MS) - NMR, and others.

UV spectra of analytes must be taken and overlaid with each other, normalized the spectra due to different amounts present in the mixture and finally select a wavelength which is most common and give higher response for all analytes. For the greatest sensitivity λmax that gives adequate response for all the analytes should be used.[26] UV wavelength below 200 nm should be avoided because detector noise increases in this region, while higher wavelength give greater sensitivity[R8]

Sample Preparation

Nature of sample as physicochemical characteristics of a drug molecule shows an important aspect in method development process. It should necessary to know the physicochemical characteristics of sample for which we develop a method. As we know HPLC separation highly depends on the polarity of the mobile phase, stationary phase as well as sample which we want to separate. So the first thing which we have to know is that what is the nature of sample as it is hydrophobic or hydrophilic. After that we can easily select the solvents or mobile phase for the sample, as well as the choice of stationary phase can also be made. Partition coefficient value for sample should also know that‟s how we determine the distribution of drug within stationary and mobile phase. pH of sample in all aspects should also determine for separation purpose because pH can also affects the retention time of analyte. In general, before selection of column and mobile phase we know the all chemistry of the molecule which we want to separate. The first step during method development is that to select diluents in which a drug dissolve completely so it can be easily determine if know the solubility of drug substance in each solvent as it may be organic solvent as acetonitrile, methanol or non- organic solvent as water. Diluents which select to dissolve the drug should not have any interference with the drug substance as well.[R9]

4. Method optimization

While performing optimization, one parameter is changed at a time and a set of conditions are isolated, before utilizing trial and error approach. The said work need to be accomplished basing on a systematic methodical plan duly observing all steps and documented with regard to dead ends[R3]

Validation of an analytical procedure

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use. The methods validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to support analytical procedures.[21] All analytical methods that are intended to be used for analyzing any clinical samples will need to be validated. The validation of analytical methods is done as per ICH guidelines.

Typical parameters recommended by FDA, USP, and ICH are as follow

1. Selectivity and 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. System suitability[R4]

1. 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 measure and such that the values of each measure and are independent of other measure ands 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(s).[R6]

Linearity and range: The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of analyte in the sample. A linear relationship should be evaluated across the range of the analytical procedure. It is demonstrated directly on the drug substance by dilution of a standard stock solution of the drug product components, using the proposed procedure. Linearity is usually expressed as the confidence limit around the slope of the regression line.[16-18] For the establishment of linearity, minimum of five concentrations are recommended by ICH guideline.[19] The range of an analytical method is the interval between the upper and lower

levels that have been demonstrated to be determined with precision, accuracy and linearity using the method.[R4]

3. Precision

The precision of an analytical procedure expresses the closeness of agreement(degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision may be considered at three levels, - Repeatability: Repeatability expresses the precision under the same operating conditions over a short interval of time. It is also termed as intra-assay precision. Repeatability should be assessed using: a) a minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each); or b) a minimum of 6 determinations at 100% of the test concentration. - Intermediate Precision: Intermediate precision expresses within-laboratories variations like different days, different analysts, different equipments, etc. - Reproducibility: Reproducibility expresses the precision between laboratories.For establishment of linearity, a minimum of 5 concentrations is recommended(r8) A linear relationship should be evaluated across the range of analytical procedure. Linearity is performed to demonstrate that the analytical method is capable for obtaining the test results within a selected specified range of concentration. As we know results obtain in chromatogram are in form of absorbance so these results should directly proportional to the concentration range as selected for analysis. For chromatographic purity methods linearity can be obtain within a range of LOQ% to 120% of specified limits for each impurity and main component individually. Three replicates of LOQ, 50, 80, 100 and 120% are injected and plot a graph between these levels of concentration and area obtain. Regression line equation is used to determine the correlation coefficient, slope of the regression line and y- intercept. Detect ability of linear range which should obey Beer-Lambert law is dependent on the detector which used in process and on the compound nature. The relation who obeys Beer-lambert law is acceptable as according to this law absorbance is directly proportional to the concentration of analyte. A perfect relation between concentration and absorbance is shown by the value of correlation coefficient which should near 1 as not less than 0.999 for assay methods and not less 0.99 for chromatographic purity methods. The range of an analytical procedure is the upper and lower interval levels of concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.[(r9)

The accuracy of an analytical method expresses the closeness of agreement between the value accepted either as a conventional true value or an accepted reference value and the value found.

Practically no measurement process is ideal, therefore, the true or actual value cannot be exactly known in any particular measurement. The accepted true value for accuracy assessment can be assessed by analyzing a sample with known concentration. The accuracy studies are usually carried out by determining the recovery of the spiked sample of analyte into the matrix of the sample (a placebo) or by comparing the result to the results of a certified reference material of known purity. If the placebo of the sample is not available, the technique of standard addition is used. In case of methods for quantitation of impurities, the sample with known amount of impurities is assessed. Accuracy should be assessed using minimum of nine determinations over a minimum of three concentration levels covering the specified range (for e.g., three concentrations/ three replicates each of the total analytical procedure).[r12] During validation the 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.18(r4)

Limit Of Detection

The limit of detection of an individual analytical procedure is the lowest amount of analyte in the sample which can be detected but not necessarily quantified as an exact value. The detection limit can be determined in different ways. The simplest approach is based on the signal to noise ratio. The signal to noise ratio is determined by comparing measured signals from samples with known low concentration of analyte with those of blank samples. The concentration showing signal to noise ratio between 3:1 or 2:1 is generally considered as acceptable detection limit. The other approach is based on the standard deviation of the response and the slope. The detection limit may be expressed as:

$$LOD =$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The slope may be estimated from the calibration curve of the analyte. The σ can be estimated as the standard deviation of the blank. The value of σ can also be estimated based on the calibration curve. For this the specific calibration curve should be studied using sample

containing analyte in the range of detection limit. The residual standard deviation of a regression line or the standard deviation of the y-intercept of regression lines may be used as standard deviation. Another approach for the estimation of the detection limit is base on visual evaluation. This method is applicable to non-instrumental methods but may be applied to the instrumental methods. The LOD is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. The relevant chromatograms are sufficient for the justification of the detection limit. (r12)

Limit of Quantitation

The Quantitation limit of an individual analytical procedure is the lowest amount of analyte in the sample which can be quantitatively determined with suitable precision and accuracy. It is mainly affected by the detector sensitivity and accuracy of sample preparation. The Quantitation limit can be determined in the similar way as that of the detection limit. It is the concentration showing signal to noise ratio of 10:1. Based on the standard deviation of the response and the slope it is calculated by the formula:

$$LOQ =$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The value of S and σ are estimated as for the detection limit.

The LOQ can also be established from the visual evaluation as the LOD. The analyte concentration should be quantifiable with acceptable accuracy and precision at LOQ level. Typical acceptance criteria for LOQ are mean recovery at this level between 50 – 150 % with % RSD of ≤ 25 %.[r12]

ROBUSTNESS

The robustness of an analytical procedure is the measurement of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indications of its reliability during normal usage. Studied by evaluating the effect of small but deliberate variations in the chromatographic conditions.[R5]

System Suitability

System suitability testing originally believed by the industry of pharmaceuticals to decide whether a chromatographic system is being utilized day today in a routine manner in

pharmaceutical laboratories where quality of results is most important which is suitable for a definite analysis. The parameters used in the system suitability tests (SST) report are as follows: 1. Number of theoretical plates or Efficiency (N). 2. Capacity factor (K). 3. Separation or Relative retention (α). 4. Resolution (Rs). 5. Tailing factor (T). 6. Relative Standard Deviation (RSD).[r3]

System suitability tests are an integral part of liquid chromatographic methods. They are used to verify that the detection sensitivity, resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Factors, such as the peak resolution, number of theoretical plates, peak tailing and capacity have been measured to determine the suitability of the used method[r4]

CONCLUSION

HPLC is an important procedure in the pharmaceutical industry and it is utilized to ensure that quality is built in to the processes supporting drug development and manufacture.

This review describes the general technique of HPLC method development and validation of optimized method. The knowledge of the pKa, pH and solubility of the primary compound is of utmost importance prior to the HPLC method development. Final optimization can be performed by changing the temperature, gradient slope, and flow rate as well as the type and concentration of mobile-phase modifiers.

Knowledge of pH can help to discern the ionizable nature of the other impurities (i.e., synthetic byproducts, metabolites, degradation products, etc.) in the mixture. Selection of buffer and mobile phase composition (organic and pH) plays a dramatic role on the separation selectivity. Final optimization can be performed by changing the temperature, gradient slope, and flow rate as well as the type and concentration of mobile-phase modifiers.