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Review Article

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An Overview on Analytical Method Development and Validation by Using HPLC

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Abstract
HPLC is an analytical technique widely used for identification, separation, detection and quantification of various drugs and its related degradants. HPLC process development is important in case of drug discovery, drug development and in analysis of pharmaceutical products. It is also employed to separate the manufactured drugs and its related impurities. It also involves the understanding of physicochemical properties of drug substances; hence facilitate the development of analytical method. The article mainly focuses on optimization of HPLC method during process development and validation of that method according to ICH guidelines. It also involves some important parameters required for determining the efficiency of the method.

Keywords HPLC, method development, drugs, impurities, validation, degradants.

Introduction

HPLC was derived from the classical column chromatography and, is one of the most important tools of analytical chemistry today. Chromatographic technique was originally developed by the Russian botanist Tswett in 1903 [1].
Chromatographic technique is basically divided into quantitative and qualitative analysis. A qualitative analysis gives the information regarding nature of sample by knowing about the presence of certain compound. A quantitative analysis provides numerical information to the relative amount of one or more of this component [2]. Basically, this technique relies on differences in the rate at which component of a mixture moves through a porous medium *i.e.* Stationary phase under the influence of some solvent i.e. mobile phase under high pressure [3]. The molecules in the sample will have distinct affinities for the stationary phase, results in separation of components. The component of sample which shows strong interactions with the stationary phase will move slowly through the column than the component shows weaker interactions [4]. RP-HPLC has both analytical and preparative applications in the area of biochemical separation and purification. Molecules that possess some degree of hydrophobic character, such as proteins, peptides and nucleic acids, can also be separated and recovered by reversed phase chromatography [5].

Types of HPLC

Normal phase chromatography

In NP-HPLC the nature of polar stationary phase is polar and mobile phase is non-polar [6]. In a mixture of components to be separated those analytes which are relatively more polar will be retained by the polar stationary phase longer than those which are relatively less polar. Therefore the least polar component will elute first. The attractive forces which exist are mostly dipole-dipole and hydrogen bonding interaction [7].

Reversed phase chromatography

RP-HPLC has a non-polar stationary phase and polar or moderately polar mobile phase. RP-HPLC is based on the principle of hydrophobic interaction [6]. In a mixture of components those analytes which are relatively less polar

will be retained by the non-polar stationary phase longer than those which are relatively more polar. Therefore the most polar component will elute first [7].

Size exclusion chromatography

SEC, also called as gel permeation chromatography or gel filtration chromatography mainly separates particles on the basis of size.

Ion exchange chromatography

In Ion-exchange chromatography, retention is based on the attraction between solute ions and charged sites bound to the stationary phase.

Bio-affinity chromatography

Separation based on specific reversible interaction of proteins with ligands [6].

- **Separation Techniques**

Isocratic

Isocratic mode of separation includes constant eluent composition; means equilibrium conditions in the column and the actual velocity of compounds moving through the column are constant. The peak capacity is low and the longer the component is retained on the column the wider is the resultant peak.

Gradient

Gradient mode of separation includes significantly increases the separation power of a system mainly due to increase of the apparent efficiency (decrease of the peak width). Peak width varies depending on the rate of the eluent composition variation.

In deciding whether a gradient or isocratic would be required an initial gradient run is performed and the ratio between the total gradient time and the difference in the gradient time between the first and last component are calculated. The calculate ratio is <0.25 isocratic is adequate. When the ratio is >0.25 gradient would be adequate [2].

- **Theory**

Basic criteria for analytical method development by HPLC are

1. If the drug or drug combination is not be added in any pharmacopoeias,
2. If a proper analytical procedure is not available due to patent problems,
3. Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients or stability problems,
4. Analytical methods for the quantification of the drug in biological fluids may not be available,
5. Analytical methods for a drug in combination with other drugs may not be available [3].

- **Parameters**

1. Internal Diameter

The internal diameter (ID) of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution. It also determines the quantity of analyte that can be loaded into a column.

2. Particle size

The silica particles used for packing of HPLC column are available in different sizes, that provide more surface area and better separations but the pressure increases inversely.

3. **Pump Pressure**

Modern HPLC systems works at much higher pressure and therefore smaller particle size can be used in column (<2 µm). These "Ultra Performance Liquid Chromatography" systems or UPLCs can work upto 100 MPa [8].

4. Pore size

Pore size of column defines an ability of the analyte molecules to penetrate inside the particle and interact with its inner surface [6].

5. Effect of temperature

Temperature conditions in HPLC method development present a challenge because it has unpredictable effects on selectivity. The use of elevated temperatures will results in

1. Reduce mobile phase viscosity and back-pressure.

2. Reduce retention time.
3. Improve method reproducibility [8]**.**

Method development in HPLC:

There are many steps involves in method development which are

- Physicochemical Property of the Drug
- HPLC conditions
- Sample preparation
- Method optimization
- Validation of developed method
- Re-validation [2]

1. Physicochemical property of the Drug

Physicochemical properties of a drug molecule play an important role in method development. Physical properties like solubility, polarity, pKa and pH of the drug molecule. The solubility of molecules can be explained on the basis of the polarity of molecules. pH and pKa plays an important role in HPLC method development.

$pH = - \log_{10}[H_3O^+]$ and $pKa = -\log_{10} Ka$.

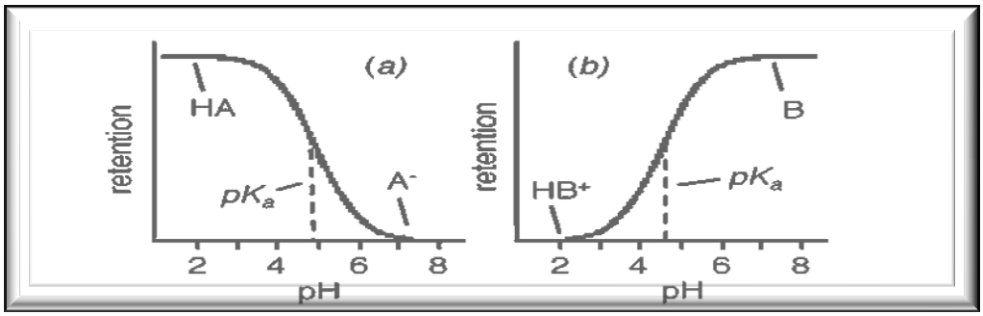

Figure 1: Relation between pH and pKa.

2. HPLC conditions

Buffering Capacity

It is the ability of the buffer to resist changes in pH (i) Buffering Capacity increases as the molarity of the buffer solution increases. (ii) The closer the buffered pH 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.

Buffer concentration

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 [1].

3. Sample preparation

The solvent in which the sample is dissolved plays an important role in terms of peak band broadening and retention time of the solute. If the sample solvent is different than the mobile phase, one or more distinct system peaks, either positive or negative, may be obtained. The best chromatographic result is obtained if sample and dilutions are prepared in mobile phase. In some cases, due to lack of solubility, it is difficult to dissolve a sample in the mobile phase. In such cases, a stronger, *i.e.*, less polar solvent may be used to dissolve the sample. The use of a less polar solvent can cause peak band broadening or distortion and reduction in retention time. It is important to inject a blank of the solvent to identify system peaks [9].

4. Method validation in HPLC

Validation means assessment of validity or action of proving effectiveness. Method validation is the process of proving that an analytical method is acceptable for its intended purpose. For pharmaceutical methods guidelines from the United States of Pharmacopeia (USP), International Conference on Harmonization (ICH) and the Food and Drug Administration (FDA) provide a frame work for performing such validations [10].

Table 1: Buffers and their useful properties

Buffer	pKa	Useful pH range	UV cut off
Ammonium acetate	4.8	3.8-5.8	205 nm (10nm)
	9.2	8.2-10.2	
Ammonium formate	3.8	2.8-4.8	205 nm (10nm)
	9.2	8.2-10.2	
Ammonium hydroxide/ Ammonia	9.2	8.2-10.2	205 nm (10nm)
KH_2PO_4 / K_2PO_4	7.2	6.2-8.2	<200 nm
KH_2PO_4 / phosphoric acid	2.1	1.1-3.1	<200 nm
Potassium acetate / acetic acid	4.8	3.8-5.8	210 nm (10 nm)
Potassium formate / formic acid	3.8	2.8-4.8	210 nm (10 nm)
Tri-fluoro-acetic acid	<2	1.5-2.5	210 nm (10 nm)
Borate	9.2	8.2-10.2	-
H_3BO_3 / $Na_2B_4O_7$ 10 H_2O			
Tri-K Citrate / hydrochloric acid	3.1	2.1-4.1	230 nm (10 mM)
Tri-K Citrate / hydrochloric acid	3.1	3.7-5.7	230 nm (10 mM)

Parameters of HPLC method Validation

- Accuracy
- Precision
- Repeatability
- Intermediate precision
- Linearity
- Limit of Detection
- Limit of Quantification
- Specificity
- Range
- Robustness
- System suitability determination
- Forced degradation studies
- Solution stability studies

a. Accuracy

Accuracy is the closeness of the test results obtained by the analytical method to the true value. Accuracy is usually determined in one of four ways. First, accuracy can be assessed by analyzing a sample of known concentration (reference materials) and comparing the measured value to the true value. The second approach is to compare test results from the new method with results from an existing alternate well-characterized procedure that is known to be accurate. The third approach, based on the recovery of known amounts of analyte, is performed by spiking analyte in blank matrices. The fourth approach is the technique of standard additions, which can also be used to determine recovery of spiked analyte. This approach is used if it is not possible to prepare a blank sample matrix without the presence of the analyte [11].

b. Precision

The precision of an analytical procedure express the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision of an analytical procedure is usually expressed the variance, standard deviation or coefficient of variance of a series of measurement. The first type of precision study is **instrument precision or injection repeatability**. A minimum of 10 injections of one sample solution is made to test the performance of the chromatographic instrument. The second type is **repeatability or intra-assay precision**. Intra-assay precision data are obtained on one day. From these

precision studies, the samples preparation procedure the number of replicate samples to be prepared and the number of injections required for each sample in the final method procedure will be set. An example of precision criteria for an assay method is that the instrument precision (RSD) will be 1% and the intra assay precision will be 2% [10].

Repeatability

Repeatability expresses the precision under the same operating conditions over a short of time. Repeatability is also termed intra-assay precision.

c. Intermediate Precision

Intermediate precision expresses within laboratories variations: different days, different analysts and different equipment, etc.

d. Reproducibility

Reproducibility expresses, the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

e. Linearity

The linear range of obeys Beer's Law is dependent on the compound analyzed and detector used. The working sample concentration and samples tested for accuracy should be in the linear range. [(11)] Linearity is the ability of analytical procedure to obtain a response that is directly proportional to the concentration (amount) of analyte in the sample [12]. For assay methods, this study is generally performed by preparing standard solutions at five concentration levels. Five levels are required to allow detection of curvature in the plotted data acceptability of linearity data is often judged by examine the correlation and y-intercept of the linear regression line for the response verses concentration plot. A correlation coefficient of >0.999 is generally considered as evidence of the data to the regression line. The y-intercept should be less than a few percent responses obtained for the analyte at the target level [10].

f. Limit of Detection

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantified. It is expressed as a concentration at a specified signal-to-noise ratio, usually 3:1. LOD may also be calculated based on the SD of the response and the slope (S) of the calibration curve at levels approximating the LOD according to the formula LOD = 3.3(SD/S). The SD of the response can be determined based on the SD of the blank, on the residual SD of the regression line, or the SD of *y*-intercepts of regression lines [11].

g. Limit of Quantification

The limit of quantification (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. The ICH has recommended a signal: noise ratio 10:1. LOQ may also be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve at levels approximating the LOQ according to the formulae LOQ 10*(SD/S) [12].

h. Specificity

The specificity of an analytical method is its ability to measure accurately an analyte in the presence of interferences that are known to be present in the product e.g. synthetic precursors, excipients, enantiomers, and known degradants that may be present. For separation techniques, this means that there is resolution of > 1.5 between the analyte of interest and the interferents [13-14].

i. Range

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.

j. 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. [(12)] To check the column-to-column ruggedness, the specificity (selectivity) of at least three columns from three different batches supplied by one column manufacturer should be checked.

k. System suitability testing

After a method has been validated, an overall system suitability test should be routinely run to determine if the operating system is performing properly. According to the USP, a system can be considered suitable if it meets the requirements for both precision and one of the tests listed in Table 2 [14].

Table 2: System suitability parameters

S. No.	System Suitability Parameters	Acceptance Criteria
1.	% RSD for Five Replicate Injection of analyte Peak in Standard Solution	Should be NMT 2.0%
2.	USP Tailing Factor	Should be NMT 2.0
3.	USP Plate Count	Should be NLT 2000
4.	USP Resolution	Should be NLT 2.0

5. ICH guidelines of analytical method validation

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose.

Types of Analytical Procedures to be validated:

1. Identification tests;
2. Quantitative tests for impurity content;
3. Limit tests for the control of impurities;
4. Quantitative tests of the active moiety in samples of drug substance or drug or other selected component(s) in the drug product.

Table 3: Types of Validation Parameter

Type of analytical procedure Characteristics	Identification	Testing for impurity Quantitative	Testing for impurity Limit	Assay
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Intermediate precision	+	+	-	+
Specificity	+	+	+	+
Detection limit	-	-	+	-
Quantification limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- signifies that this characteristic is not normally evaluated.

+ signifies that this characteristic is normally evaluated [15].

- **Some Important Parameters of HPLC:**

a) System Resolution

$$\text{System resolution} = \frac{2(t_2 - t_1)}{W_2 + W_1}$$

Where t_2 and t_1, are the retention times of the two components and W_2 and W_1 are the corresponding peak widths. The resolution factor should be greater than 2.00 for two HPLC peaks.

b) Determination of System Precision

After a standard solution is injected a number of times, the relative standard deviation of the peak responses is measured as either the peak height or peak area. When using an internal standard method, the response ratio is calculated. Maximum allowable system related standard deviations made at the 99% confidence level have been tabulated. For the USP monographs, five replicate chromatograms are used if the stated limit for relative standard

deviation is 2% or less. Six replicate chromatograms are used if the stated relative standard deviation is more than 2.0%.

c) Asymmetry Factor or Tailing Factor

The increase in the peak asymmetry is responsible for a decrease in chromatographic resolution, detection limits, and precision. Measurement of peaks on solvent tails should be avoided. The peak asymmetry factor or tailing factor can be calculated by following formula:

$$T = \frac{W_{0.05}}{2f}$$

Where $W_{0.05}$ is the width of the peak at 5% peak height. The system suitability test for antibiotics and antibiotic drugs recommends measurement at 10% of the peak height from the baseline.

d) Column Efficiency

Column efficiency is generally determined by calculating the number of theoretical plates for a column. It is mainly required for the assay of antibiotics and antibiotic containing drugs.

$$N = 5.545 (t / W_{h/2})^2$$

Or

$$N = 16(t / W)^2$$

Where t is the retention time of the analyte and $W_{h/2}$ is the peak width at half-height or W is the width at the base of the peak. The height equivalent to one theoretical plate is calculated by

$$h = L / n$$

Where L is the length of the column.

e) Column Capacity

The column capacity factor is calculated by

$$K = (t_r - t_m) / t_m$$

Where the retention time of the solute is t_r and the retention time of solvent or un-retained substance is t_m. Retention volumes are sometimes preferred, because tr varies with flow rate. The factor is then calculated by

$$V = (V_r - V_m) / V_m$$

Where V_r is the retention volume of the solute and V_m is the elution volume of an unretained substance [14].

- **Types of process validation**

a. Prospective Validation

It is defined as the established documented evidence that a system does what it purports to do based on a preplanned protocol. This validation usually carried out prior to distribution either of a new product or a product made under a revised manufacturing process. It was performed on at least three consecutive batches. In Prospective Validation, the validation protocol is executed before the process is put into commercial distribution of product. During the product development phase, the production process should be categorized into individual steps. Each step should be evaluated on the basis of experience or theoretical considerations to determine the critical parameters that may affect the quality of the finished product [16].

b. Concurrent Validation

This validation involves in-process monitoring of critical points in process and product testing. It gives a documented evidence to show that the production process is in a state of control [17]. A process where current production batches are used to monitor processing parameters. It gives of the present batch being studied, and offers limited assurance regarding consistency of quality from batch to batch [18]. Concurrent Validation may be the practical approach under certain circumstances. Example

- A previous validated process is being transferred to a third party contract manufacturer or to another site.
- The product is of different strength of a previously validated product with the same ratio of active/inactive ingredients.
- The number of lots evaluated under the Retrospective Validation was not sufficient to obtain a high degree of assurance demonstrating that the process is fully under control [19].

c. Retrospective Validation

It is defined as the established documented evidence that a system does what it purports to do on the review and analysis of historical information. This is achieved by the review of the historical manufacturing testing data to prove that the process always remained in control. This type of validation used for a product already in distribution. Retrospective validation is only acceptable for well-established processes and will be inappropriate where there have been recent changes in the composition of the product, operating procedures or equipment. Batches selected for retrospective validation should be representative of all batches made during the review period, including any batches that failed to meet the specifications, and should be sufficient in number to demonstrate the process consistency [20].

d. Process Re-Validation

Re-validation required when there is a change in any of the critical process parameters: formulation, primary packaging components, raw material fabricator, major equipment or premises. Failure to meet product and process specifications in batches would also require process re-validation.

Re-Validation becomes necessary in certain situations. The following are examples

- Changes in raw materials (physical properties such as density, viscosity, particle size distribution, and moisture, etc., that may affect the process or product).
- Changes in the source of active raw material manufacturer.
- Changes in packaging material (primary container/closure system).
- Changes in the process (e.g., mixing time, drying temperatures and batch size).
- Changes in the equipment (e.g. addition of automatic detection system).
- Changes of equipment which involve the replacement of equipment on a "like for like" basis would not normally require a revalidation except that this new equipment.
- Must be qualified.
- Changes in the plant/facility.
- Variations revealed by trend analysis (e.g. process drifts) [19].

Elements of validation

The validation of a process requires the qualification of each important elements of the process. The relative importance of an element may vary from process to process. Some of the elements commonly considered in a process validation study are presented below.

a. Design qualification (DQ)

The first element of validation of new facilities, systems or equipment could be design qualification (DQ). The compliance of the design with GMP should be demonstrated and documented [20].

b. Installation Qualification (IQ)

Establishing by objective evidence that all key aspects of the process equipment and ancillary system installation adhere to the manufacturer's approved specification and that the recommendation of the supplier of the equipment are suitably considered [16].

IQ consideration is

- Equipment design features
- Installation conditions (wiring, utility, functionality, etc.)
- Calibration, preventative maintenance, cleaning schedules.
- Safety features.
- Supplier documentation, prints, drawings and manuals.
- Software documented.
- Spare parts list.
- Environmental conditions (such as clean room requirements, temperature, and humidity) [19].

a. Operational Qualification (OQ)

Establishing by objective evidence that process control limits and action levels which result in product quality meets all predetermined requirements.

OQ consideration is

- Process control limits (time, temperature, pressure, line speed, setup conditions, etc.)
- Software parameters.
- Raw material specifications
- Process operating procedures.
- Material handling requirements.
- Process change control.
- Training.
- Short term stability and capability of the process, (latitude studies or control charts).
- Potential failure modes, action levels and worst-case conditions.
- The use of statistically valid techniques such as screening experiments to optimize the process can be used during this phase.

b. Performance Qualification (PQ)

Establishing by objective evidence that the process under anticipated condition consistently produce a product which meets all its predetermined requirements.

PQ consideration is

- Actual product and process parameters and procedures established in OQ.
- Acceptability of the product.
- Assurance of process capability as established in OQ.
- Process repeatability, long term process stability [18].

Conclusion

This review article describes about High performance Liquid Chromatography and the general technique of HPLC method development and validation of optimized method. A general and simple approach for the method development for the separation of compounds was discussed. Knowledge of the pH, pKa and solubility of the primary compound is of most importance prior to the HPLC method development. The advantages of HPLC technique were high selectivity, sensitivity, economic, less time consuming and low limit of detection. Optimized method is validated with various parameters (e.g. accuracy, precision, specificity, selectivity, linearity, robustness etc.). It also involves some important parameters required for determining the efficiency of the developed method.

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