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HPLC method development and validation – A review

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ABSTRACT

High Performance liquid chromatography analytical tool used to qualitative and quantitative the drug product and drug stability, impurities. The separation is done by column, detection wavelength and other composition (organic & PH) for analysis the drug. The analytical method development and validation play a important role for drug discovery and development. The validations are done by [Accuracy, Precision, Repeatability, Specific limit etc….] the review articles are discussed about by the High-Performance Liquid Chromatography Method Development and Validation of drug.

Keywords— *HPLC, Instrumentation, Method Development, Method validation*

1. INTRODUCTION

Chromatography is a technique used for separation of the components of mixture by continuous distribution of the component between two phases. One phase moves (mobile phase) over the other phase (stationary phase) in a continuous manner[1].High Performance Liquid Chromatography which is also known as High Pressure Liquid Chromatography[2].HPLC has gained its popularity mainly due to its reliability (use of pressure driven liquid support) and versatility (possibility of adjusting the composition of both mobile and stationary phases)[3].HPLC is the most accurate analytical methods widely used for the quantitative as well as qualitative analysis of drug product and used for determining drug product stability. It has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid[4]. Therefore, analytical methods developed using sophisticated instruments such as spectrophotometer, HPLC, GC and HPTLC have wide applications is assuring the quality and quantity of raw materials and finished products[1].

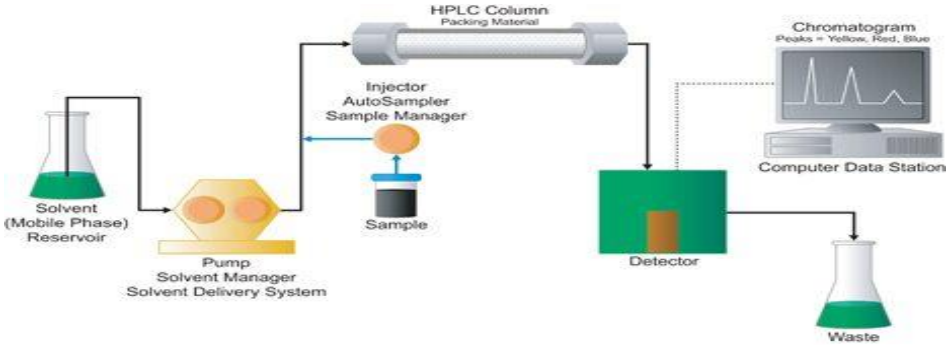
2. INSTRUMENTATION

HPLC instrumentation involve the principles is separation of solution is injected into a column of porous material (stationary phase) and liquid phase (Mobile phase) is pumped at higher pressure through the column[5].

3. PRINCIPLE

The Principle of high-performance liquid chromatography is to separate by absorption, ion exchange, Partition of solute on stationary phase based on affinity towards stationary phase[4]. The solvent usually flows through column with the help of gravity but in the HPLC techniques the solvent will be forced under high pressure up to 400 atmospheres[2]. HPLC, pumps will be used to pass pressurized liquid solvent including the sample mixture which is allowed to enter into a column filled with solid adsorbent material. E.g.- water, acetonitrile, methanol and is considered as mobile phase[2].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 phase[4].



(a) Solvent reservoir: The contents of mobile phase are present in glass container. In HPLC the mobile phase or solvent is a mixture of polar and non-polar liquid components. Depending on the composition of sample, the polar and non-polar solvents will be varied[6].

(b) Pump: A high-pressure pump (solvent delivery system or solvent manager) is used to generate and meter a specified flow rate of mobile phase, typically milli litters per minute. The pump suctions the mobile phase from solvent reservoir and forces it to column and then passes to detector. The operating pressure depends on column dimensions, particle size, flow rate and composition of mobile phase. Normal flow rates in HPLC are in the 1 to 2ml/min range. Typical pumps can reach pressures in the range of 6000-9000 psi (400-to 600-bar)[7]. Pump pressure depends on column dimension, particle size, flow rate and composition of mobile phase[8].

(c) Sample injector: The injector serves to introduce the liquid sample into the flow stream of the mobile phase. Typical sample volumes are 5- to 20-microliters (µL). The injector must also be able to withstand the high pressures of the liquid system. An auto sampler is the automatic version for when the user has many samples to analyse or when manual injections are not Practical[9].

Two means for analyte introduction on the column are injection in to a flowing stream and a stop flow injection. Several devices are available either for manual or auto injection.

- **Septum injectors-** for injecting the sample through a rubber septum.
- **Stop flow-** in which the flow of the mobile phase is stopped for a while and the sample is injected through a valve device.
- **Rheodyne injector (loop valve type)-** it is the most popular type; this has a fixed volume loop like 20-50 µl or more. The injector has two modes, i.e., load position when the sample is loaded in the loop and the inject mode, when the sample is injected. The injector can be a solitary infusion or a computerized infusion framework. An injector for a HPLC framework should give infusion of the fluid specimen inside the scope of 0.1 mL to 100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).
- **Automatic injector-** is a microprocessor-controlled version of the manual universal injector. Usually, up to 100 samples is capable of being loaded on to the auto injector tray. The system parameters such as flow rates, volume to be injected, gradient, run time, etc. are selected, stored in memory and sequentially executed on consecutive injections[10].

(d) Column: Considered the "heart of the chromatograph" the column's stationary phase separates the sample components of interest using various physical and chemical parameters. The small particles inside the column are what cause the high backpressure at normal flow rates. The pump must push hard to move the mobile phase through the column and this resistance causes a high pressure within the chromatograph[9].Provides separation through high pressure created by the small particles. Analytical columns are stainless steel (most often) or glass tubes of inner diameter between 1 to 10 mm and length of 5 to 50cm[11].Different types of HPLC columns are used in analysis of different pharmaceutical compounds according to their nature and column separation capacity. Columns are the main component in HPLC because the column is responsible for the separation of the sample components. The sample passes through the column with the mobile phase and separates in its components when it comes out from the column.

Generally, silica gel is filled in the high-performance liquid chromatography columns because of its particle size and porosity that helps in separation of components and silica gel is also an inert material that does not react with mobile phases. Therefore, silica columns can be used to analyse the compounds of different chemical natures. The material filled in the HPLC columns is known as a stationary phase. There are different types of chromatography columns on the basis of their composition and method of separation. Some of them are described here.

- **Normal Phase HPLC Columns:** This type of columns has more polar stationary phase than the mobile phase. The packing material of the column should be more polar than the mobile phase and this condition is fulfilled by the silica that is polar material. But water is more polar than the silica, therefore, water is not used and methylene chloride, hexane and chloroform or a mixture of these with diethyl ether is used as mobile phase. Separation of the sample components occurs on the basis of the polarity of the sample components. Sample components having more polarity interact more with polar stationary phase resulting in separation from the less polar component that interacts with less polar mobile phase. Silica columns are widely used in the pharmaceutical analysis. The chromatography column packing in which normal phase columns are used is known as Normal Phase Chromatography.
- **Reverse Phase HPLC Columns:** In reverse phase columns as its name states, it is reverse of the normal phase columns. It has a non-polar or less polar stationary phase than the more polar mobile phase. Bonded hydrocarbons like C8 and C18 and other non-polar hydrocarbons are used as stationary phase in reverse phase columns while aqueous organic solution

like water-methanol or water-acetonitrile mixture is used as mobile phase. Separation of sample components in reverse phase columns also occurs on the basis on the polarity of the sample components but it happens just opposite of the normal phase HPLC columns, therefore, this type of chromatography is known as Reverse Phase Chromatography.

- **Ion Exchange HPLC Columns:** The compounds those can easily ionize are analysed using these columns. Stationary phase in these columns remains acidic or basic having negative or positive charge while mobile phase a polar liquid as the salt solution in water. Separation of molecules occurs on the basis of the attractive ionic force between molecules and the charged stationary phase. Due to the exchange of ions during the separation of sample components, it is known as Ion Exchange Chromatography.
- **Size Exclusion HPLC Columns:** Porous stationary phase in these columns allows the separation of the components according to their size. Combination of polymers like polysaccharides and silica is used as stationary phase in these columns. Small sample molecules penetrate in the pores of stationary phase while the large molecules penetrate partially into the pores. Therefore, the large molecules of the sample elute first than the small molecules and this chromatography is called Size Exclusion Chromatography. These columns are generally not used in the analysis of pharmaceutical compounds.

HPLC columns have a different length varying from 30 mm to 250 mm and their particle size or porosity from 3µ to 5µ. These factors affect the analysis of sample; therefore, these are considered important during the HPLC analytical method development. Columns are selected according to the nature of the compound to be analysed and the mobile phase. Column performance should also be evaluated time to time generally after 1000 runs or as required[12].

(e) **Detector:** The detector can see (detect) the individual molecules that come out (elute) from the column. A detector serves to measure the amount of those molecules so that the chemist can quantitatively analyse the sample components. The detector provides an output to a recorder or computer that results in the liquid chromatogram (i.e., the graph of the detector response)[9]. The detectors used in HPLC are of majorly two types:

- **Selective detectors:** These detectors respond to a particular physical or chemical property of the solute, being ideally independent of the mobile phase. They are as follow:
  - Absorbance detectors
  - Fluorescence detectors
  - Electrochemical detectors
  - Mass spectrometric detectors[13]
- **Universal detectors (bulk property):** Measure the difference in some physical property of the solute in the mobile phase compared to the mobile phase alone. They are generally universal in application but tend to have poor sensitivity and limited range. Such detectors are usually affected by even small changes in the mobile-phase composition which precludes the use of techniques such as gradient elution. They are as follow:
  - Refractive index detectors
  - Evaporating light scattering detectors

(f) **Data collection devices or Integrated:**[6] Signals from the detector might be gathered on graph recorders or electronic integrators that fluctuate in many-sided quality and in their capacity to process, store and reprocess chromatographic information. It also determines the time of elution of the analysts. The PC coordinates the reaction of the indicator to every part and places it into a chromatograph that is anything but difficult to interpret[14]. The data that collected by the detector is transmitted to a printer or computer software to draw the chromatogram. Then this chromatogram can be analysed manually or by specialized software used in the procedures that aim to purify a certain compound from a mixture. i.e. the output of this system is data only[15].

2. METHOD DEVELOPMENT

Analytical method development and validation play important roles in the discovery development and manufacture of pharmaceuticals. These methods used to ensure the identity, purity, potency, & performance of drug products. There are many factors to consider when developing methods. The initially collect the information about the analyst's physicochemical properties (pKa, log P, solubility) and determining which mode of detection would be suitable for analysis (i.e., suitable wavelength in case of UV detection).The majority of the analytical development effort goes into validating a stability indicating HPLC–method. The goal of the HPLC-method is to try & separate quantify the main active drug, any reaction impurities, all available synthetic inter-mediates and any degrades[16].

A step involved in method development of HPLC is as follows:

1. Understanding the Physicochemical properties of drug molecule.
2. Selection of chromatographic conditions.
3. Developing the approach of analysis.
4. Sample preparations
5. Method optimization
6. Method validation[17].

2.1 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. In a nonpolar covalent bond, the electrons are shared equally between two atoms. A polar covalent bond is one in which one atom has a greater attraction for the

electrons than the other atom. 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 analyte must be soluble in the diluents and must not react with any of the diluent components. The diluent should match to the starting eluent composition of the assay to ensure that no peak distortion will occur, especially for early eluting components.

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.

$$\mathbf{pH = - log10[H3O^{+}]}$$

The acidity or basicity of a substance is defined most typically by the pH value. Selecting a proper pH for ionisable analysts 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.

The acidity of an aqueous solution is determined by the concentration of **$[H3O^{+}]$** ions. Thus, the pH of a solution indicates the concentration of hydrogen ions in the solution. The concentration of hydrogen ions can be indicated as [H+] or its solvated form in as **$[H3O^{+}]$** whose value normally lies between 0 and 14.

The lower the pH, the more acidic is the solution. The pH of a solution can be changed simply by adding acid or base to the solution. The pKa is characteristic of a particular compound, and it tells how readily the compound gives up a proton.

An acid dissociation constant is a particular example of equilibrium constant. For the specific equilibrium between a monoprotic acid, HA and its conjugate base A

$$\mathbf{HA + H2O = A^{-} + H3O}$$

The position of equilibrium is measured by the equilibrium constant, K_{eq}.

$$\mathbf{K_{eq} = \frac{[H3O^{+}]\,[A^{-}]}{[H2O]\,[HA]}}$$

Now in dilute solutions of acid, [H2O] stays roughly constant. Therefore, define a new equilibrium constant- the acidity constant K

$$\mathbf{K_a = \frac{[H3O+]\,[A-]}{[HA]}}$$

This is also in logarithmic form are follows:

$$\mathbf{p_{Ka} = -log10k_a}$$

It turns that the pKa of an acid is the pH at which it is exactly half dissociated. This can be shown by rearranging the expression for K_a.

$$\mathbf{pH = pKa - log([AH]/[A-])}$$

At half-neutralization **$[A^{-}]$ / [HA] = 1**; since **log(1)= 0**, the pH at half-neutralization is numerically equal to pKa. Conversely, when **pH = pKa,** the concentration of HA is equal to the concentration of A^{-}.

The buffer region extends over the approximate range **pKa ± 2,** though buffering is weak outside the range **pKa ± 1. At pKa ± 1, $[A^{-}]$/[HA] = 10 or 1/10.**

If the pH is known, the ratio may be calculated. This ratio is independent of the analytical concentration of the acid. When the pKa and analytical concentration of the acid are known, the extent of dissociation and pH of a solution of a monocratic acid can be easily calculated[18].

2.2. Selection of chromatographic 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 neutralize the acid.

2.2.1 Buffering Capacity: Is the ability of the buffer to resist changes in pH:

- Buffering capacity increases as the molar concentration (molarity) of the buffer salt\acid solution increases.
- The closer the buffered pH is to the pK_a greater the buffering capacity.
- Buffering capacity is expressed as the molarity of sodium hydroxide required to increases pH by 1.0.

Consideration of the affect of pH on analyte retention, type of buffer to use, and its affect on detection are important in reversed-phase chromatography (RPC) method development of ionic analysts. 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 ionisable compounds[1].

2.2.2 Buffer selection: Choice of buffer is governed by the pH that is desired. The typical pH range for reversed phase on silica-based packing is pH 2 to 8. It is important that the buffer has a pK_a close to the desired pH since buffer controls pH best at their pK_a. A rule is to choose a buffer with a pK_a value <2 units of the desired mobile phase pH. (Table-1)

Table-1: HPLC Buffers, pKa Values and Useful pH Range

Buffer	pKa	Useful pH Range
Ammonium acetate	4.8 9.2	3.8-5.8 8.2-10.2
Ammonium formate	3.8 9.2	2.8-4.8 8.2-10.2
KH_2PO_4/ phosphoric acid	2.1	1.1-3.1
KH_2PO_4/K_2PO_4	7.2	6.2-8.2
Potassium Acetate/ acetic acid	4.8	3.8-5.8
Borate ($H_3BO_3/Na_2B_4O_7$ $10H_2O$)	9.2	8.2-10.2
Ammonium hydroxide/ ammonia	9.2	8.2-10.2
Trifluoroacetic acid	<2	1.5-2.5
Potassium formate / formic acid	3.8	2.8-4.8

2.2.3 Buffer concentration: Generally, a buffer concentration of 10-50mm 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. Phosphate buffers can be replaced with sulphate buffers when analysing organophosphate compounds[1].

2.2.4 Selection of detector

Detector is a very important part of HPLC. Selection of detector depends on the chemical nature of analysts, 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. Its integrated software and optics innovations deliver high chromatographic and spectral sensitivity. 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[19].

Detector	Type of compound can be detected
UV-Visible &Photodiode array	Compounds with chromophores, such as aromatic rings or multiple alternating double bonds.
Fluorescence detector	Fluorescent compounds, usually with fused rings or highly conjugated planer system.
Conductivity detector	Charged compounds, such as inorganic ions and organic acid.
Electrochemical detector	For easily oxidized compounds like quinines or amines
Refractive Index detector & Evaporative light scattering detector	Compounds that do not show characteristics usable by the other detectors, eg.polymers, saccharides.

2.2.5 Isocratic and Gradient Separations: 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 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.

2.2.6 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.

2.2.7 Particle size: Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles. These silica particles come in many sizes with 5 μm beads being the most commonly used. The smaller particles usually provide more surface area and better separations but the pressure required for the optimum linear velocity increases by the inverse of the particle diameter squared. Larger particles are used in preparative HPLC where column diameters are in range of 5 cm to >30 cm and for non-HPLC applications such as solid-phase extraction.

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

2.2.9 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 (Me OH) and tetra hydro furan

(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[19].

Mode	Solvent type used	Type of compound used
Reversed Phase	Water/Buffer, ACN, Methanol	Neutral or non-ionized compounds which can be dissolved in water/ organic mixtures.
Ion-pair	Water/Buffer, ACN, Methanol	Ionic or Ionizable compounds
Normal Phase	Organic solvents	Mixtures of isomers and compounds not soluble in Organic/ Water mixtures.
Ion exchange	Water/Buffer	Inorganic ions, proteins, nucleic acids, organic acids.
Size exclusion	Water, Tetrahydrofuran, chloroform	High molecular weight compounds.

2.3 Developing the approach for analysis

While developing the analytical method on RP-HPLC the first step which is followed, the selections 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 isopiestic point in the case of simultaneous estimation of 2 components[4].

2.4 Sample preparation

Sample preparation is a critical step of method development that the analyst must investigate. For example, the analyst should investigate if centrifugation (determining the optimal rpm and time) shaking and/or filtration of the sample are needed, especially if there are insoluble components in the sample. The objective is to demonstrate that the sample filtration does not affect the analytical result due to adsorption and/or extraction of leachable. The effectiveness of the syringe filters is largely determined by their ability to remove contaminants/insoluble components without leaching undesirable artefacts (i.e., extractable) into the filtrate. The sample preparation procedure should be adequately described in the respective analytical method that is applied to a real in-process sample or a dosage form for subsequent HPLC analysis. The analytical procedure must specify the manufacturer, type of filter, and pore size of the filter media. The purpose of sample preparation is to create a processed sample that leads to better analytical results compared with the initial sample. The prepared sample should be an aliquot relatively free of interferences that is compatible with the HPLC method and that will not damage the column[4].

2.5 Method optimization

The experimental conditions should be optimized to get desired separations and sensitivity after getting appropriate separations. Stability-indicating assay experimental conditions will be achieved through planned/systematic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, temperature, sample amounts, injection volume, and diluents solvent type[20].

2.6. Method Validation

Validation of an analytical method is the process by which it is established by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application. Validation is required for any new or amended method to ensure that it is capable of giving reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories. The type of validation program required depends entirely on the particular method and its proposed applications[21].

3. VALIDATION

Validation is the process of establishing documentary evidence demonstrating that, a procedure, process, or activity carried out in testing and then production maintains the desired level of compliance at all stages.

3.1 Types of Analytical Procedures to be validated

The discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures:

- Identification tests
- Quantitative tests for impurities' content
- Limit tests for the control of impurities

Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product[19].

3.2 Following are typical analytical performance characteristics which may be tested during methods validation:

(a) **Accuracy**
(b) Precision
(c) Repeatability
(d) Intermediate precision
(e) Linearity
(f) Detection limit

(g) **Quantitation limit**[19]

3.2.1 Accuracy: The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose ―true value‖ is known) is analysed and the measured value is identical to the true value. Typically, accuracy is represented and determined by recovery studies. There are three ways to determine accuracy:

- Comparison to a reference standard
- Recovery of the analyte spiked into blank matrix
- Standard addition of the analyte.

It should be clear how the individual or total impurities are to be determined. E.g. Weight /weight or area per cent in all cases with respect to the major analyte[22].

3.2.2. Precision:

The precision of an analytical procedure represents the nearness of agreement between a series of measurements got from multiple sampling of the same homogenous sample under the similar analytical conditions and it is divided into 3 categories.

- Repeatability
- Intermediate precision
- Reproducibility

3.2.3 Repeatability: Precision under same operating conditions, same analyst over a short period of time.

3.2.4 Intermediate precision: Method is tested on multiple days, instruments, analysts etc.
Reproducibility: Inter-laboratory studies. The ICH guidelines suggest that repeatability should be conformed duly utilizing at least 9 determinations with specified range for the procedure (e.g., three concentrations / three replicates each) or a minimum of 6 determinations at 100 % of the test concentration[23].

3.2.5 Linearity: Linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration (amount) of the analyte in the sample. 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 examining 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 per cent responses obtained for the analyte at the target level[24].

3.2.6 Detection limit: The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value[28]. Several approaches for determining the LOD are possible, depending on whether the procedure is a non-instrumental or instrumental.

- Based on visual evaluation
- Based on signal-to-noise
- Based on the standard deviation of the response and the slope

The LOD may be expressed as: LOD =3.3 σ/S
Where, σ= Standard deviation of Intercepts of calibration curves, S = Mean of slopes of the calibration Curves

The slope S may be estimated from the calibration curve of the analyte[26].

3.2.7 Quantitation limit: The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products[24].Upper limit of quantification (ULOQ) is the maximum analyte concentration of a sample that can be quantified, with acceptable precision and accuracy. The ULOQ is identical with the concentration of the highest calibration standards[27].

4. ADVANTAGES OF HPLC

- It is extremely quick and efficient.
- It is accurate and highly reproducible.
- Repetitive reproducible analysis can be done by using the same column.
- It provides a high degree of selectivity for specific analyses
- It delivers higher resolution.
- It is versatile and extremely precise when it comes to identifying and quantifying chemical components.
- It is largely automated and hence basic HPLC runs can be performed with minimal training[28].

5. CONCLUSION

This review describes about RP-HPLC Technique. The method development and validation are continuous and interrelated processes that measure a parameter as intended and establish the performance limits of the measurement. The selection of Column, buffer, detector and wavelength and another conditions composition (organic and pH) plays a dramatic role on the

separation selectivity, the advantages of HPLC technique were high selectivity, sensitivity, economic, less time consuming and low limit of detection. Final optimization can be performed by changing the gradient slope, temperature and flow rate as well as the type and concentration of mobile-phase modifiers. Optimized method is validated with various parameters (e.g. specificity, precision, accuracy, detection limit, linearity, etc.) as per ICH guidelines.

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