A REVIEW ON HPLC METHOD DEVELOPMENT AND VALIDATION

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Abstract : High performance liquid chromatography (HPLC) serves as an important technique in assessing drug product due to its versatile features like column stability, and capacity to efficiently separate a wide range of compounds with a high detection sensitivity. This review provides information in regards to various stages involved in development and validation of HPLC method. Validation of HPLC method are described in terms of performance characteristics, like accuracy, precision, specificity, linearity, range and limit of detection, limit of quantification, robustness and system suitability testing. All these parameters are used for routine and stability analysis. HPLC method development and validation play an important role in drug discovery, development, and manufacturing of pharmaceutical drugs due to its number of advantages like rapidity, specificity, precision, accuracy and ease of automation. A sequence of events required for HPLC method development and analytical validation are been described in this article.

IndexTerms **– Accuracy, HPLC, Method development, Precision, Selectivity, Validation.**

I. INTRODUCTION:

Chromatography is used to discrete the components from a mixture by the use of mobile phase and a stationary phase. HPLC is often referred as high-pressure liquid chromatography since high pressure is used when compared to column chromatography. HPLC is considered as an accurate analytical technique with an application in qualitative and quantitative analysis of drugs [1,2]. The principle involved in HPLC technique is adsorption i.e. when a sample solution is injected into a HPLC column and the mobile phase is pumped at a high pressure the separation of components within the sample takes place based on the relative affinities towards the stationary phase. The components having lesser affinity towards the stationary phase travels faster and vice a versa. As no two components will have the same affinity towards the stationary phase separation can be achieved [1,3].

HPLC has a number of pros such as

- High resolution
- High sensitivity
- High accuracy
- Rapid Analysis
- Easy to purify the sample
- Relatively high inlet pressure and controlled flow of mobile phase
- Ease of automation
- Good repeatability [2,4]

II. CLASSIFICATION OF HPLC TECNIQUES:

1. Normal Phase HPLC:

The Stationary phase selected in this mode is polar in nature and the mobile phase is non-polar. As the non-polar compounds travels faster and are eluted first due to its lesser affinity towards the stationary phase however on the other hand the polar compounds are retained for a longer period of time in the column and eluted later due to its higher affinity towards stationary phase [1,9].

2. Reverse Phase HPLC (RP-HPLC):

Non-polar stationary phase is used in this mode and the mobile phase is polar in nature. Thus, the polar compounds are eluted first and the non-polar compounds are retained for a longer period of time in the column and eluted later. Reverse phase chromatography is more commonly used as drugs are usually hydrophilic Different columns used are ODS (Ocatyl decyl silane), C18, C8, C4 etc. [1,9].

III. HPLC SYSTEM:

a.) Solvent Reservoirs

Different solvents are used based on mode of chromatography. For normal- phase HPLC, the solvent is usually non-polar and in RP-HPLC, the solvent is normally polar in nature [6].

b.) Pump

The solvent used should be passed through the column at a high pressure of about 1000-3000 psi. which helps in providing a constant and continuous flow of the mobile phase through the system [5,6].

c.) Injector

Sample injection can be done through a manual or auto injector. Injector helps in injecting the mixture into the mobile phase before entering the column. Injector injects the mixture within the range of 0.1-100 mL of volume under high pressure (up to 4000 psi). Different devices used are septum injectors, stop flow injectors & rheo dyne injectors [5,6,7,8].

d.) Analytical Column

Column is the heart of HPLC technique as it decides the efficiency of separation. A column is in the contact of the mobile phase and stationary phase. Stainless steel column is most wide used due to its high-pressure withstanding capacity. Whereas the latest ones are Poly ether ether ketone (PEEK) column [6,7,8].

e.) Detector

The commonly used detector in pharmaceutical analysis is UV detector. Different available detectors are Refractive index detector, Flourimetric detector, Conductivity detector, Amperometric detector, Photodiode array detector [5].

f.) Records and Integrators

Recorders are used to record the response obtained from detectors after amplification. Integrators are improved version of

records used for data processing by providing a greater information on peaks [5].

IV. ANALYTICAL METHOD DEVELOPMENT:

Analytical method development & validation plays a crucial role in development and manufacturing of drug products. New methods are developed for the analysis of novel products when no definitive techniques are available. New methods are developed by optimization and validation through preliminary runs to reduce the cost along with the better precision and ruggedness. Whereas alternative methods are developed to replace the existing procedure with the comparative laboratory data [11,12].

V. HPLC METHOD DEVELOPMENT:

Following steps are involved for method development of HPLC

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

1.) Understanding the Physicochemical properties of drug molecule:

Understanding of the physicochemical properties of drug molecule like pH, pKa, solubility etc. is a prerequisite for method development as it is required to evaluate the solvent and composition of mobile phase. Selection of diluents is done on the basis of solubility of analyte. The acidity and the basicity of a substance is defined by the pH value. Selecting a appropriate pH for ionizable analytes leads to sharp and symmetrical peak in HPLC. To achieve low limit of detection sharp symmetrical peaks are required. The pH value is defined as the negative of the logarithm to base 10 of the concentration of the hydrogen ion [13,14,15].

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

2.) Setup HPLC conditions:

Buffer Selection: It depends on desired pH. It is important that the buffer has a pKa value close to the desired pH since buffer controls pH best at their pKa.

Key points considered during buffer selection:

- Phosphate is more soluble in methanol/water than in acetonitrile/water or THF/water.
- Ammonium salts are generally more soluble in organic/water mobile phases.
- TFA can degrade with time, is volatile in nature & absorbs at low UV wavelengths.
- Microbial growth can damage chromatographic performance.
- Mobile phases should be degassed.
- At pH > 7, phosphate buffer accelerates the dissolution of silica and shortens the lifetime of silica-based HPLC

columns. Organic buffers should be used at a pH > 7.

- Ammonium bicarbonate buffers are only stable for 24 to 48 hours. Thus, become more basic due to the release of carbon dioxide.
- After preparation of buffer solution, it should be filtered through a 0.2µm filter.
- Phosphoric acid and its salts are mostly used buffer systems for RP-HPLC [6,12,15,16].

Isocratic Elution:

Elution techniques are used for pumping the mobile phase through a column. The same mobile phase composition is maintained throughout the separation process; means equilibrium conditions in the column and the actual velocity of compounds moving through the column are constant. The longer the component is retained on the column the wider is the resultant peak [12,13,17].

Gradient Elution:

The mobile phase combination of gradually increasing polarity is used. In determing whether a gradient or isocratic elution 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. If the calculated ratio is <0.25 isocratic is adequate. When the ratio is >0.25 gradient would be adequate [15, 17].

Column Selection:

It is an important parameter to verify the system suitability criteria as it influences the sensitivity and selectivity in gradient elution. Column selection also evaluates the quantity of analyte that can be loaded into a column [15, 17].

Particle size:

As smaller particles provide more surface area, better separation is achieved. Larger particles are used in preparative HPLC where column diameters are in range of 5 - >30 cm [15].

Pore size:

Pore size of column defines an ability of the analyte molecules to penetrate inside the particle [15].

Selection of Mobile Phase:

Composition of mobile phase serves an important role in RP-HPLC separation as it affects resolution, selectivity and efficiency. Most widely used solvents in RP-HPLC is acetonitrile, methanol & tetrahydrofuran. Mixture of acetonitrile and water is the best choice for the mobile phase during method development. At low pH, basic analytes are protonated giving an improved peak shape & acidic analyte at low pH will remain unchanged and increases the retention [15].

Selection of detectors:

Detector is a very important part of HPLC. Selection of detector depends on the chemical nature of analysis, possible interference, desired limit of detection, availability and cost of detector [15, 17]. A number of detectors are used in HPLC method development like UV-Visible Detectors due to their versatile, dual wavelength absorbance and higher sensitivity for compounds with chromophoric groups or double bonds. Photodiode Array Detector (PDA) are also used due to its advanced optical detection for Waters analytical HPLC, preparative HPLC, or LC/MS system solutions and higher sensitivity usually

for compounds with double bonds. Refractive Index Detector (RI) are generally used for Polymers and saccharides. Fluorescence Detectors can also be used for quantitating low concentrations of target compounds generally with fused rings or highly conjugated planer system [15, 17].

3.) Developing the approach of analysis:

During the development of analytical method on RP-HPLC the first step is the selections of various chromatographic parameters like selection of mobile phase, column selection, mobile phase flow rate, pH. Typical parameters of system suitability are e.g.

- Theoretical plates should be >2000,
- Retention time should be >5 min
- Tailing factor should be <2,
- Resolution between 2 peaks should be more than 5 [15]

4.) Sample preparation:

Sample preparation is a crucial part of method development. The following points must be considered while preparing the sample

- Provide a reproducible and homogenous solution that is suitable for injection onto the column
- Sample must be free of interferences
- Should not damage the column
- Is compatible with the intended HPLC method.

Filters of 0.22 or 0.45 µm are generally employed for filtration to remove the particulate matter [12, 15].

5.) Method optimization:

HPLC conditions like different components of the mobile phase, solvent, gradient, flow rate, temperature, sample amounts, injection volume, and diluents solvent type, column dimensions, column-packing & particle size should be optimized to get the desired separation and sensitivity. Thus, a desired balance between resolution and analysis time after satisfactory selectivity has been achieved can be determined [12, 13].

6.) Method validation:

Method validation is the process used to ensure that the analytical procedure employed for a specific test meets the pre-determined quality attributes and is suitable for its intended use. Results from method validation can be used to determine the quality, reliability and consistency of obtained analytical results; it is an integral part of any good analytical practice. Use of equipment that is within specification, working correctly and adequately calibrated is essential for method validation process. Analytical methods need to be validated or revalidated.

- Before routine use;
- When conditions changes for a validated method
- When the method itself is changes [12,19].

Typical parameters recommended by FDA, USP, and ICH are as follow [12, 20]:

1. Accuracy (Recovery)
2. Specificity
3. Linearity & Range
4. Precision
   i. Method precision (Repeatability)
   ii. Intermediate precision (Reproducibility)
5. Solution stability
6. Limit of Detection (LOD)
7. Limit of Quantification (LOQ)
8. Robustness
9. System suitability

1. Accuracy (Recovery):

Accuracy expresses the closeness of agreement between the accepted reference value and the observed value. It is determined by applying the method to samples to which known amounts of analyte have been added which are analyzed against standard and blank solutions and then the accuracy is calculated from the test results as a percentage of the analyte recovered by the assay. Accuracy can be determined by using atleast 9 determination over a minimum of 3 concentration over the specified range [13,20,21].

2. Specificity:

Specificity of an analytical method as its ability to measure accurately an analyte in the presence of interference [13, 21].

3. Linearity and range:

The linearity of an analytical method is defined as the ability to obtain test results within a given range, which is directly proportional to the analyte concentration in the sample. Linearity is usually expressed as the confidence limit around the slope of the regression line [13,19,21]. To establish linearity, minimum of five concentrations are recommended by ICH guideline. The range of an analytical method is the interval between the upper and lower concentration of analyte in the sample that have been demonstrated to be determined with precision, accuracy and linearity using the method [21].

4. Precision:

The precision of an analytical method is expressed as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the recommended conditions. Precision is determined at 3 different levels: repeatability, intermediate precision and reproducibility. Precision is the measure of reproducibility of the whole analytical method. Repeatability is the variation observed on a single instrument by a single analyst. Intermediate precision is expressed within laboratory variations, as on different days, or with different analysts or equipment within same laboratory. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (RSD) of series of measurements [13, 21].

5. Solution stability:

During validation the stability of standards and samples is determined at normal storage conditions or in the instrument to determine if special storage conditions are required [20, 21].

6. Limit of Detection (LOD):

LOD is defined as the lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact value. The signal-to-noise ratio is determined by

s = H/h

Where H = Peak height of the component.

h = absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution [20, 21, 23].

7. Limit of Quantification (LOQ):

LOQ of an individual analytical procedure is the least quantity of analyte in a sample that can be quantify with precision and accuracy [20].

8. Robustness:

It is defined as the measure of the ability of an analytical method to remain unaffected by small variations in method parameters and provides an indication of its reliability during normal usage (e.g. pH, mobile phase composition, temperature and instrumental settings) [9, 20].

9. System Suitability:

They are used to determine that the sensitivity, resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. Factors, such as the number of theoretical plates, capacity factor, relative retention, peak tailing, resolution and relative standard deviation are measured to determine the suitability of the method [9, 20, 21].

VI. CONCLUSION

This article gives an insight on a simple approach for the HPLC method development for the separation of compounds and validation in accordance with FDA, USP, and ICH guidelines. Method development and validation are interrelated processes used to ensure that the quality is worked into the procedures for drug production. HPLC conditions such as selection of column, buffer, detector etc. can be optimized to get the desired separation and sensitivity and the optimized method is further validated with various parameters (e.g. precision, accuracy, LOQ, LOD etc.).

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