HPLC METHOD DEVELOPMENT AND VALIDATION: A REVIEW

Azim Md. Sabir*, Mitra Moloy, Bhasin Parminder S.

Analytical Research, R&D-V, Ranbaxy Laboratories Limited, Gurgaon, India

Email: sabirazim@gmail.com

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ABSTRACT

High performance liquid chromatography (HPLC) is an essential analytical tool in assessing drug product. HPLC methods should be able to separate, detect, and quantify the various drugs and drug related degradants that can form on storage or manufacturing, detect and quantify any drugs and drug-related impurities that may be introduced during synthesis. Validation is the process of establishing the performance characteristics and limitations of a method and identification of the influences which may change these characteristics and to what extent. This article discusses the strategies and the issues pertinent to designing HPLC method development and validation.

Key word: HPLC, degradant, Impurities, method development, validation.

INTRODUCTION

Analytical chemistry is used to determining the qualitative and quantitative composition of material under study. Both these aspects are necessary to understand the sample material. Analytical chemistry is divided into two branches quantitative and qualitative. A qualitative analysis gives us the information about the nature of sample by knowing about the presence or absence of certain components. A quantitative analysis provides numerical information as to the relative amount of one or more of this component. For analyzing the drug samples in bulk, pharmaceutical formulations and biological fluids, different analytical methods are routinely being used.

In non-instrumental, the conventional and physicochemical property are use to analyze the sample. The instrumental methods of analysis are based upon the measurements of some physical property of substance using instrument to determine its chemical composition. The instrumental methods are simple, precise and reproducible as compared to classical methods. Therefore, analytical methods developed using sophisticated instruments such as spectrophotometer, HPLC, GC and HPTLC have wide applications in assuring the quality and quantity of raw materials and finished products¹.

Chromatography

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.

Chromatography according to USP can be defined as a procedure by which solute are separated by a differential migration process in a system consisting of two or more phases, one of which move continuously in a given direction.

Principle of chromatography

Adsorption Chromatography: When the stationary phase is a solid and mobile phase is liquid or gaseous phase, it is called Adsorption Chromatography.

Examples: Thin layer chromatography, Column Chromatography, Gas-solid chromatography.

Partition Chromatography: When the stationary phase and mobile phase are liquid, it is called Partition Chromatography.

Example: Paper partition chromatography, Gas-liquid chromatography.

Theory of Chromatography

Two theoretical approaches have been developed to describe the processes involved in the passage of solutes through a chromatographic system.

The Plate Theory

According to Martin and Synge, a chromatographic system consists of discrete layers of theoretical plates. At each of these, equilibration of the solute between the mobile and stationary phases occurs. The movement of solute is considered as a series of stepwise transfers from plate to plate.

The Rate Theory

This theory considers the dynamics of the solute particles as it passes through the void space between the stationary phase particles in the system as well its kinetic as it is transferred to and from the stationary phase.

Phases of Chromatography

Normal Phase Chromatography: In Normal Phase mode the stationary phase is polar and the mobile phase is non polar in nature. In this technique, non polar compounds travel faster and are eluted first. This is because of the lower affinity between the non polar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore take more times to elute. Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute.

Reversed Phase Chromatography: It is the most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode,

the stationary phase is non polar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. The polar compound gets eluted first in this mode and non polar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are Octa Decyl Silane (ODS) or C₁₈, C₈, C₄, (in the order of increasing polarity of the stationary phase). An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity.

Ion Exchange Chromatography: The stationary phase contains ionic groups like NR₃⁺, SO₃⁻ which interact with the ionic groups of the sample molecules. This is suitable for the separation of charged molecules only. Changing the pH and salt concentration can modulate the retention.

Ion Pair Chromatography: This technique is also referred to as Reversed Phase Ion Pair Chromatography or Soap Chromatography. It may be used for the separation of ionic compounds and this method can also substitute for Ion Exchange Chromatography. Strong acidic and basic compounds may be separated by reversed phase mode by forming ion pairs (coulombic association species formed between two ions of opposite electric charge) with suitable counter ions.

Affinity Chromatography: This technique uses highly specific biochemical interactions for separation. The stationary phase contains specific groups of molecules which can absorb the sample if certain steric and charge related conditions are satisfied. This technique can be used to isolate proteins, enzymes as well as antibodies from complex mixtures.

Size Exclusion Chromatography: It separates molecules according to their molecular mass. Largest molecules are eluted first and the smallest molecules last. This method is generally used when a mixture contains compounds with a molecular mass difference of at least 10%. This mode can be further subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solvents).

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 analyte's physicochemical properties (pKa, log P, solubility) and determining which mode of detection would be suitable for analysis 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-mediate and any degradants²⁻⁴. There are many steps involve in method development which are:

- Physicochemical properties of drug.
- Set up HPLC conditions.
- Sample preparation.
- Method optimization.
- Validation of developed method.

Physicochemical properties of drug:

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 non-polar 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 non-polar, 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, $\text{pH} = -\log_{10} [\text{H}_3\text{O}^+]$. 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. 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 $[\text{H}_3\text{O}^+]$ 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 $[\text{H}^+]$ or its solvated form in as $[\text{H}_3\text{O}^+]$ 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⁻,

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

$$K_{\text{eq}} = \frac{[\text{H}_3\text{O}^+][\text{A}^-]}{[\text{H}_2\text{O}][\text{HA}]}$$

Now in dilute solutions of acid, $[\text{H}_2\text{O}]$ stays roughly constant. Therefore define a new equilibrium constant- the acidity constant K_a.

$$K_a = \frac{[\text{H}_3\text{O}^+][\text{A}^-]}{[\text{HA}]}$$

This is also in logarithmic form are follows:

$$\text{pK}_a = -\log_{10} K_a.$$

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:

$$\text{pH} = \text{pK}_a - \log\left(\frac{[\text{AH}]}{[\text{A}^-]}\right)$$

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 $pKa \pm 2$, though buffering is weak outside the range $pKa \pm 1$. At $pKa \pm 1$, $[A^-]/[HA] = 10$ 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 monoprotic acid can be easily calculated⁵⁻⁹.

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:

- Buffering Capacity increases as the molar concentration (molarity) of the buffer salt/acid solution increases.
- The closer the buffered pH is to the pKa, the greater the Buffering Capacity.
- 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 compounds¹⁰⁻¹².

Buffer selection

Choice of buffer is typically governed by the desired pH. The typical pH range for reversed-phase 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 (Table-1).

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

Buffer	pKa	Useful pH Range
Ammonium acetate	4.8	3.8-5.8
	9.2	8.2-10.2
Ammonium formate	3.8	2.8-4.8
	9.2	8.2-10.2
KH ₂ PO ₄ /phosphoric acid	2.1	1.1-3.1
KH ₂ PO ₄ /K ₂ PO ₄	7.2	6.2-8.2
Potassium Acetate/ acetic acid	4.8	3.8-5.8
Borate (H ₃ BO ₃ /Na ₂ B ₄ O ₇ 10H ₂ O)	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

General considerations for 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 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. Phosphate buffers can be replaced with sulfate buffers when analyzing organophosphate compounds¹³.

Selection of detector

Detector is a very important part of HPLC. Selection of detector depends on the chemical nature of analytes, potential interference, limit of detection required, availability and/or cost of detector. UV-Visible detector is versatile, dual-wavelength absorbance detector for HPLC. This detector offers the high sensitivity required for routine UV-based applications to low-level impurity identification and quantitative analysis. Photodiode Array (PDA) Detector offers advanced optical detection for Waters analytical HPLC, preparative HPLC, or LC/MS system solutions. 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 compounds¹⁴⁻¹⁵.

Column selection

The heart of a HPLC system is the column. Changing a column will have the greatest effect on the resolution of analytes during method development. Generally, modern reverse phase HPLC columns are made by packing the column housing with spherical silica gel beads which are coated with the hydrophobic stationary phase. The stationary phase is introduced to the matrix by reacted a chlorosilane with the hydroxyl groups present on the silica gel surface. In general, the nature of stationary phase has the greatest effect on capacity factor, selectivity, efficiency and elution. There are several types of matrices for support of the stationary phase, including silica, polymers, and alumina. Silica is the most common matrix for HPLC columns. Silica matrices are robust, easily derivatized, manufactured to consistent sphere size, and does not tend to compress under pressure. Silica is chemically stable to most organic solvents and to low pH systems. One shortcoming of a silica solid support is that it

will dissolve above pH 7. In recent years, silica supported columns have been developed for use at high pH. The nature, shape and particle size of the silica support effects separation. Smaller particle results in a greater number of theoretical plates, or increased separation efficiency. However, the use of smaller particles also results in increased backpressure during chromatography and the column more easily becomes plugged.

In reverse phase chromatography the stationary phase is non-polar and the mobile phase is polar, causing polar peaks to generally elute earlier than non-polar peaks. To create a stationary phase for reverse phase chromatography on silica support, the free silanols are reacted with a chlorosilane with hydrophobic functionality to introduce the non-polar surface. Due to steric constraints, only about 1/3 of the surface silanols are derivatized. The remaining free silanols can interact with analytes, causing peak tailing. Typically, after the derivatization of a column with the desired stationary phase, the column is further reacted with chlorotrimethylsilane to end cap the remaining free silanols and improve the column efficiency. Common stationary phases are C₄ (butyl), C₈ (octyl), C₁₈ (octadecyl), nitrile (cyanopropyl), and phenyl (phenyl propyl) columns. In general, longer alkyl chains, higher phase loading, and higher carbon loads provide greater retention of non-polar analytes. Commonly used reverse phase columns and their uses are listed below. Propyl (C₃), Butyl (C₄), and Pentyl (C₅) columns are useful for ion-pairing chromatography. Examples: include Zorbax SB-C₃, YMC-Pack C₄ and Luna C₅. These columns are generally less stable to hydrolysis than columns with longer alkyl chain. Octyl (C₈) columns have wide applicability. This phase is less retentive than the C₁₈ phases, but is still quite useful for pharmaceuticals. Example: include (Zorbax SB-C₈, Luna C₈ and YMC-Pack-MOS). Octadecyl (C₁₈, ODS) columns are the most widely used and tend to be the most retentive for non-polar analytes. Examples include Zorbax SB-C₁₈, YMC-Pack ODS and Luna C₁₈. Xterra RP-C₁₈ and Zorbax Extend-C₁₈ columns have been formulated to tolerate high pH systems (pH >7, normally up to pH 11). Varying the pH can affect selectivity and resolution of polar analytes, especially for ionizable compounds. Phenyl (Ph) columns offer unique selectivity from the alkyl phases and are generally less retentive than C₈ or C₁₈ phases. Phenyl columns are commonly used to resolve aromatic compounds. Examples include Zorbax SB-Phenyl, YMC-Pack Phenyl and Luna Phenyl-Hexyl. Nitrile (CN or cyano) columns are polar and can be used for both reverse and normal phase applications. This phase is often used to increase retention of polar analytes. Examples include Zorbax SB-CN, Luna-CN, and YMC-Pack CN.

The type of column chosen for a particular separation depends on the compound and the aim of analysis¹⁶⁻¹⁹.

Column temperature

Column temperature control is important for long-term method reproducibility as temperature can affect selectivity. A target temperature in the range of 30–40 °C is normally sufficient for good reproducibility. Use of elevated temperature can be advantageous for several reasons. First, operating at a temperature higher than ambient reduces the viscosity of the mobile phase and thus the overall backpressure on the column. Lower system pressures allow for faster flow rates and thus faster analyses. The temperature may also affect selectivity patterns because analytes will respond dissimilarly to different temperatures.

Finally, use of a column oven eliminates variability due to normal fluctuations in the air temperature surrounding the column.

While temperature is a variable that can affect selectivity ' α ' its effect is relatively small. Also, the k' generally decreases with an increase in temperature for neutral compounds but less dramatically for partially ionized analytes. Some effect when there is a significant difference in shape and size. Overall, it is better to use solvent strength to control selectivity than to use temperature; its effect is much more dramatic. An increase of 1°C will decrease the k' by 1 to 2%, a both ionic and neutral samples are reported to show significant changes in α with temperature changes. Possible temperature fluctuations during method development and validation, it is recommended that the column be thermo stated to control the temperature.

Mobile phase

Solvent type

Solvent type (methanol, acetonitrile, and tetrahydrofuran) will affect selectivity. The choice between methanol and acetonitrile may be dependent on the solubility of the analyte as well as the buffer used. Tetrahydrofuran is least polar among these three solvent, often responsible for large changes in selectivity and is also incompatible with the low-wavelength detection required for most pharmaceutical compounds.

The mobile phase effects resolution, selectivity and efficiency. In reverse phase chromatography, the mobile phase consists of an aqueous buffer and a non-UV active water miscible organic solvent. The effect of the organic and aqueous phase and the proportions in which they are mixed will affect the analysis of the drug molecule. Selection of the mobile-phase and gradient conditions is dependent on the ionogenic nature of the analyte and the hydrophobicity of the analytes in the mixture respectively. The aqueous buffer serves several purposes. At low pH, the mobile phase protonates free silanols on the column and reduces peak tailing. At sufficiently low pH basic analytes are protonated when ionized the analyte will elute more quickly but with improved peak shape. Acidic analytes in buffers of sufficiently low pH will remain unchanged, increasing retention. Conversely, at higher pH neutral basic compounds will be more retained, and ionized acidic compounds will elute earlier. Peak splitting may be observed if the pK_a of a compound is similar to the pK_a of the buffer, and the analyte elutes as both a charged and uncharged species. The pH of a buffer will not greatly affect the retention of non-ionizable sample components.

Typically a 10 – 50 mM solution of an aqueous buffer is used. The most commonly used aqueous phase is H₃PO₄ in water i.e. phosphate buffer. The pH of a phosphate buffer is easily adjusted by using mono-, di-, or tribasic phosphate salts. However, when phosphate salts are used the solution should be filtered to remove insoluble particles with 0.22µm filter paper. Other non-UV active acids and bases may also be used to effect differences in peak shape and retention.

pH of Mobile phase

When the sample is eluted with a mobile phase with 100% organic there is no separation, as the sample is eluted in the void volume. This is because the sample is not retained; but retention is observed when the mobile phase solvent strength is decreased to allow equilibrium competition of the solute molecules between the bonded phase and the mobile

phase. When the separation is complex, that is, many components are to be separated, and when the solvent strength is decreased and there is still no resolution between two close peaks, another organic solvent of a different polarity or even a mixture of two organics may need to be tried to effect separation. Additionally, mobile phase optimization can be enhanced in combination with bonded phase optimization (i.e., substituting C18 /C8 with cyano or phenyl). A goal for the band spacing of a solute (K') should be in the range of 4 to 9 and a run time of about 15 minutes or 20 minutes at most for most routine product release or stability runs.

Separation Techniques

Isocratic separations: Isocratic, constant eluent composition means equilibrium conditions in the column and the actual velocity of compounds moving through the column are constant; analyte-eluent and analyte-stationary-phase interactions are also constant throughout the whole run. This makes isocratic separations more predictable, although the separation power (the number of compounds which could be resolved) is not very high. The peak capacity is low; and the longer the component is retained on the column, the wider is the resultant peak.

Gradient separation: Gradient separation significantly increases the separation power of a system mainly because of the dramatic increase of the apparent efficiency (decrease of the peak width). The condition where the tail of a chromatographic zone is always under the influence of a stronger eluent composition leads to the decrease of the peak width. Peak width varies depending on the rate of the eluent composition variation (gradient slope). Changing Gradient: Gradient elution is employed for complex multicomponent samples since it may not be possible to get all components eluted between k (retention factor) 1 and 10 using a single solvent strength under isocratic conditions. This leads to the general elution problem where no one set of conditions is effective in eluting all components from a column in a reasonable time period while still attaining resolution of each component. This necessitates the implementation of a gradient. Employing gradients shallow or steep allows for obtaining differences in the chromatographic selectivity. This would be attributed to the different slopes of the retention versus organic composition for each analyte in the mixture. When a gradient method is used, the column must be allowed to equilibrate at the starting mobile-phase conditions prior to the next sample injection and the start of the next gradient run.

Selection of isocratic or gradient mode depends on the number of active components to be resolved or separated. In deciding whether a gradient would be required or whether isocratic mode would be adequate, an initial gradient run is performed, and the ratio between the total gradient time and the difference in gradient time between the first and last components are calculate. The calculated ratio is <0.25 , isocratic is adequate; when the ratio is >0.25 , gradient would be beneficial^{16,20,21}.

Sample preparation for method development

The drug substance being analyzed should be stable in solution (diluent). During initial method development, preparations of the solutions in amber flasks should be performed until it is determined that the active component is stable at room temperature and does not degrade under

normal laboratory conditions. The sample solution should be filtered; the use of a 0.22 or 0.45 μm pore-size filter is generally recommended for removal of particulates. Filtration is a preventive maintenance tool for HPLC analysis.

Sample preparation is a critical step of method development that the analyst must investigate. The effectiveness of the syringe filters is largely determined by their ability to remove contaminants/insoluble components without leaching undesirable artifacts (i.e., extractable) into the filtrate. If any additional peaks are observed in the filtered samples, then the diluents must be filtered to determine if a leachable component is coming from the syringe filter housing/filter²².

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/systemic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, temperature, sample amounts, Injection volume and diluents solvent type.

Method Validation

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. 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 guidelines²³⁻²⁸.

Validation parameters

The following are typical analytical performance characteristics which may be tested during methods validation:

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

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

Precision of an analytical method is the degree of agreement

among individual test results obtained when the method is applied to multiple sampling of a homogenous sample. Precision is a measure of the reproducibility of the whole analytical method. It consists of two components: repeatability and intermediate precision.

Repeatability is the variation experienced by a single analyst on a single instrument. It does not distinguish between variations from the instrument or system alone and from the sample preparation process. During validation, repeatability is performed by analyzing multiple replicates of an assay composite sample by using the analytical method. The recovery value is calculated. Intermediate precision is the variation within a laboratory such as different days, with different instruments, and by different analysts. The precision is then expressed as the relative standard deviation.

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

Accuracy and precision are not the same, as the diagram below indicates. A method can have good precision and yet not be accurate.

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

Detection Limit

The detection limit (DL) or limit of detection (LOD) of an individual procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as a n exact value. In analytical procedures that exhibit baseline noise, the LOD can be based on a signal-to-noise (S/N) ratio (3:1), which is usually expressed as the concentration of analyte in the sample.

The signal-to-noise ratio is determined by:

$s = H/h$ Where H = height of the peak corresponding to the component. h = absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.

Quantitation Limit

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

Specificity is the ability to assess unequivocally the analyte

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

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

Robustness

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

System Suitability Parameters

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

Resolution (Rs)

Resolution is the parameters describing the separation power of the complete chromatographic system relative to the particular components of the mixture. The resolution R_s two neighboring peak is defined as the ratio of the distance between two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation the ideal value of R_s is 1.5. It is calculated by using the formula (Figure-1).

Figure-1: Resolution between two Peaks

Where, $t_{R(1)}$ and $t_{R(2)}$ are the retention times of components 1 and 2 and W_1 and W_2 are peak width of components 1 and 2.

Capacity factor (K')

Capacity factor, k' , is defined as the ratio of the number of molecules of solute in the stationary phase to the number of

molecules of the same in the mobile phase. Capacity factor is a measure of how well the sample molecule is retained by a column or TLC plate during an isocratic separation. The ideal value of k' ranges from 2-10. Capacity factor can be determined by using the formula,

$$k' = \frac{V_1 - V_0}{V_0}$$

Where, V_1 = retention volume at the apex of the peak (solute) and V_0 = void volume of the system.

Column efficiency (N)

It is a measure of band spreading of a peak. Smaller the band spread, higher is the number of theoretical plates, indicating good column and system performance. Columns with N ranging from 5,000 to 100,000 Plates/meter are ideal for a good system. Efficiency is calculated by using the formula: (Figure-2)

Figure-2: Number of Theoretical plates

Where, t_R is the retention time and W is the peak width.

Peak asymmetry factor (As) and tailing factor

Peak asymmetry factor (A_s) can be used as criteria of column performance. The peak asymmetry is measured at 10 % of full peak height, divided by corresponding front half width. (Figure-3)

Figure-3: Peak asymmetry and peak tailing factor

Asymmetry factor is calculated by,

$$\text{Asymmetry factor} = B/A$$

B = Peak half width, A = Front half width

Good columns produce peaks with A_s values of 0.95 to 1.0. (Exactly symmetrical peaks have an A_s value of 1.0)

Peak Purity

Peak purity (or peak homogeneity) analysis of the main peak, to assess for the presence of impurities under the main

peak. It is an essential part of the method validation.

Quantitation Limit

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

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

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

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

Determination of robustness is a systematic process of varying a parameter and measuring the effect on the method by monitoring system suitability and/or the analysis of samples.

System Suitability Determination is the evaluation of the components of an analytical system to show that the performance of a system meets the standards required by a method²⁵. These parameters can be calculated experimentally to provide a quantitative system suitability test report: number of theoretical plates (efficiency), capacity factor, separation (relative retention), resolution, tailing factor, relative standard deviation (precision). These are measured on a peak or peaks of known retention time and peak width.

Forced Degradation Studies

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

Advantages of Analytical Method Validation

- It builds a degree of confidence, not only for the developer but also to the user.
- Although the validation exercise may appear costly and time consuming, it proves to be inexpensive by eliminating frustrating repetitions and leads to better time management in the end.
- The method validation absorbs the shock of variations of analytical conditions and pays for more than invested on the process.

Stability Studies

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 light²⁹⁻³⁶.

CONCLUSION

This review describes the general technique of HPLC method development and validation of optimized method. The general approach for the method development for the separation of pharmaceutical compounds was discussed. The knowledge of the pKa, pH and solubility of the primary compound is of utmost importance prior to the HPLC method development. 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. Optimized method is validated with various parameters (e.g. accuracy, precision, specificity, linearity, detection limit etc.) as per ICH guidelines.

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