Various Criteria in Development & Validation of HPLC Analytical Methods: -Review

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ABSTRACT
HPLC method development and validation play important role in the discovery, development and manufacture of pharmaceutical products. This article mainly focuses on the optimization of HPLC conditions and other important perspectives during method development and validation. Various critical steps related to analytical method development and validation is discussed. A sequence of events required for method development and analytical validation are described. The steps involved in developing a stability-indicating HPLC method influences the analysis of degradation products/impurities in stability study and its validation demonstrate the suitability for its intended purpose.

Key words: Method validation, Method development, International conference on Harmonization (ICH).

INTRODUCTION
Analytical chemistry\(^1\) is the science and art of determining the components of materials in terms of the elements or compound contained. Analytical Chemistry seeks ever improved means of measuring the chemical composition of natural and artificial materials.

Selection of Analytical Method\(^2\)
First step in the selection or development of method is to establish what is to be measured and how accurately it should be measured. Unless one has series of methods at hand to assess quality of the product, validation program may have limited utility. The selected method must have the following salient features:
- It should be, As simple as possible, Most specific, Most productive, economical and convenient,
- As accurate and precise as required, Should be fully optimized before transfer for validation of its characteristics such as accuracy, precision, sensitivity, ruggedness etc.

Classification of Analytical Methods \(^3\)
Various methods of analysis can be broadly classified into two categories; Classical methods and Instrumental methods:

1. Classical Methods:
   A. Volumetric Methods   B. Gravimetric Methods
2. Instrumental Methods:
   These methods are based upon the measurement of some physical properties as conductivity, electrode potential, light absorption or emission, mass-to-charge ratio and fluorescence of substance.
   (a) Spectroscopic Techniques
   Ultraviolet and visible spectrophotometry, Fluorescence and phosphorescence spectrophotometry, Atomic spectrophotometry (emission & absorption), Infrared spectrophotometry, Raman spectroscopy, X-ray spectroscopy, Radiochemical techniques including activation analysis, NMR spectroscopy, ESR spectroscopy.

(b) Electrochemical Techniques
   Potentiometry, Voltammetry, Stripping techniques, Amperometric techniques, Coulometry, Electrogravimetry, Conductance techniques.

(c) Chromatographic Techniques
   Gas chromatography (GC), High performance liquid chromatography (HPLC), High-performance thin layer chromatography (HPTLC), Supercritical fluid chromatography (SFC), Ultra pressure liquid chromatography (UPLC)

(d) Miscellaneous Techniques
   Thermal analysis, Mass spectrometric, Kinetic techniques.

(e) Hyphenated Techniques\(^4\)
   GC-MS, ICP-MS, GC-IR, MS-MS, CE-MS, LC-NMR, LC-MS, LC-MS-NMR.

UV Visible Absorption Spectroscopy\(^5\)\(^6\)
- a. Introduction to UV visible Absorption Spectroscopy
  The absorption of electromagnetic radiation of wavelengths between 200 and 800 nm by molecules which have \(\pi\) electrons or atoms possessing unshared electron pairs can be employed for both qualitative and quantitative analysis. As a wide variety of pharmaceutical substances absorb radiation in the near-UV (220-380 nm) and visible (380-800 nm) regions of the electromagnetic spectrum, the technique is widely employed in pharmaceutical analysis. On passing electromagnetic radiation in the ultra-violet and visible regions through a compound with multiple bonds, a portion of the radiation is normally absorbed by the compound. The absorption of radiation is due to the subtraction of energy from the radiation beam when electrons in orbitals of lower energy are excited into orbitals of higher energy. Energy absorbed in the ultraviolet region by complex organic molecules causes transitions of valence electrons in the molecules. These transitions are:

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n. \( \pi^\ast \) transitions: These type of transitions are shown by unsaturated molecules which contain atoms such as oxygen, nitrogen and sulphur. These transitions exhibit a weak band in their absorption spectrum. These are forbidden transitions.

\( \sigma \) \( \sigma^\ast \) transitions: These transitions can occur in compounds in which all the electrons are involved in single bonds and there are no lone pair of electrons.

n. \( \pi^\ast \) transitions: Saturated compounds with lone pair(non-bonding) of electrons undergo these transitions in addition to \( \sigma \) \( \sigma^\ast \) transitions.

\( \pi \) \( \pi^\ast \) transitions: A \( \pi \) \( \pi^\ast \) transition corresponds to the promotion of an electron from a bonding \( \pi \) orbital to an antibonding \( \pi^\ast \) orbital. This transition can in principle occur in any molecule having a \( \pi \) electron system. The quantitative analysis by UV-Visible spectrophotometry is governed by the Beer-Lambert’s law. It states that, the intensity of a beam of monochromatic light when passed through transparent medium decreases exponentially as the thickness and concentration of absorbing media increases arithmetically.

\[ A = \log \left( \frac{I}{I_0} \right) \]

\( A \) = Absorbance of the solution at particular wavelength of the light beam
\( I_0 \) = Intensity of incident light beam
\( I \) = Intensity of transmitted light beam
\( a \) = Absorptivity of molecule at the wavelength of beam
\( b \) = Path length of cell in cm
\( c \) = Concentration of solution in gm/lit.

b. Terms Used In Absorption Spectroscopy

1. Transmittance (T): It is the ratio of intensity of transmitted light to that of incident light.

\[ T = \frac{I}{I_0} \]

2. Absorbance (A): It is the negative logarithm of transmittance to the base 10.

\[ A = - \log_{10} T = \log_{10} \left( \frac{I}{I_0} \right) \]

3. Molar Absorptivity (c): When concentration ‘c’ in equation A = \( a \)bc is expressed in mole/lit and cell length in ‘cm’ then absorptivity is called as molar absorptivity.

\[ c = \frac{A}{bc} \]

c. Steps Involved in Spectrophotometric Analysis

1. Solvent selection: The solvent selected should have the following characteristics:
   - A good solubilizing power
   - It should have stable interactions with the absorbing species.
   - The solvent should be transparent in the region of measurement.
   - It should be of consistent purity.

2. Selection of analytical wavelength: The wavelength selected should be such that the absorbing species should show maximum or appreciable absorbance at that wavelength.

3. Type of instrument: This forms the nucleus of the analytical method because more advanced the instrument; greater will be the degree of accuracy and precision of the results.

4. Preparation of working curve: The concentration of an unknown sample can be estimated by comparing with the standard solution, it is most accurately found by means of a working curve or calibration plot. The absorbance of several standard solutions are measured and plotted against their respective concentrations to obtain the standard working curve.

5. Sample measurements and calculations: The concentration of the sample should also be adjusted such that the absorbance falls within the range selected at that specific wavelength at which the standard solutions are measured. The concentrations are read from the calibration curve.

6. Evaluation of reproducibility and recognition of variations: To ensure that proper conditions have been selected and that no important variables have been overlooked, the tentative method should be critically evaluated with respect to Beer’s law conformity, reproducibility and possible effect due to aging or minor changes in the procedural details. Reproducibility tests are performed over an extended period by replicate determinations on different days using both the first set and freshly prepared set of all solutions each day.

d. Methods of Multicomponent Analysis Using a Double Beam UV-Visible Spectrophotometer

8. Simultaneous equation method: Consider a multicomponent system consisting of two components X and Y, \( \lambda_1 \) is the wavelength of maximum absorbance of X (\( \lambda_{\text{max}} \)) and \( \lambda_2 \) being the wavelength of maximum absorbance of Y. Figure 1: represents the overlain spectra of drugs X and Y.

It may be possible to determine both drugs by technique of simultaneous equation method (Vierodt’s method). The information required is –

- The absorptivities of X at \( \lambda_1 \) and \( \lambda_2 \), \( a_1 \) and \( a_2 \), respectively.
- The absorbivities of Y at \( \lambda_1 \) and \( \lambda_2 \), \( a_1 \) and \( a_2 \), respectively.
- The absorbances of the diluted sample mixture at \( \lambda_1 \) and \( \lambda_2 \), \( A_1 \) and \( A_2 \), respectively.
- \( c_x \) and \( c_y \) be the concentrations of X and Y respectively in the diluted sample.

Then the absorbances of the sample mixture at \( \lambda_1 \) and \( \lambda_2 \) may be expressed as follows:

\[ A_1 = a_1 c_x + a_2 c_y \quad \text{......(1)} \]
\[ A_2 = a_1 c_x + a_2 c_y \quad \text{......(2)} \]

For measurements in 1 cm cell, \( b = 1 \).

Rearranging Equation (2),

\[ c_y = \frac{A_2 - a_2 c_x}{a_2} \quad \text{......(3)} \]

Substituting for \( c_y \) in equation (1) and rearranging gives

\[ c_x = \frac{a_2 A_1 - A_2}{a_2} \quad \text{......(4)} \]
Each solute will also have a characteristic retention volume.

The corrected retention time ($t'_R$) is the time elapsed between the dead point and the peak maximum.

The corrected retention volume ($V'_R$) is the volume of mobile phase passed through the column between the dead point and the peak maximum. It will also be the retention volume minus the dead volume. Thus, $V'_R = V_R - V_d = Q (t_R - t_d)$ where $Q$ is the flow rate in ml/min. The peak height ($h$) is the distance between the peak maximum and the baseline geometrically produced beneath the peak. The peak width ($W$) is the distance between each side of a peak measure at 0.6065 of the peak height (0.607$h$). The peak width measured at this height is equivalent to two standard deviations (2$s$) of the Gaussian curve and thus has significance when dealing with chromatography theory. The peak width at half height ($W_{0.5}$) is the distance between each side of a peak measured at half the peak height. The peak width measured at half height has no significance with respect to chromatography theory. The peak width at the base ($W_B$) is the distance between the intersections of the tangents drawn to the sides of the peak and the peak base geometrically produced. The peak width at the base is equivalent to four standard deviations of the Gaussian curve and thus also has significance when dealing with chromatography theory.

Using equations (4) and (5), the concentrations of X and Y in the sample mixture can be determined.

### 9. Multicomponent mode method

The total absorbance of a solution at a given wavelength is equal to the sum of absorbance of the individual components present. This relationship makes possible the quantitative determination of the individual constituent of a mixture even if their spectrum overlaps. This method has advantages over the other methods, as it does not involve any manual calculations. It directly gives the concentration of the components in the sample mixture.

### Chromatography

Chromatography is probably the most powerful and versatile analytical technique available to the modern chemist. Its power arises from its capacity to determine quantitatively many individual components present in a mixture in one single analytical procedure. Its versatility comes from its capacity to handle a very wide variety of sample; they may be gaseous, liquid or solid in nature.

#### a. Introduction to Chromatography

Chromatography is dealing with chromatography theory. High-Performance Liquid Chromatography (HPLC) has the ability to separate, identify, and quantify the compounds that are present in any sample. The principle is that a solution of the sample is injected into a column of a porous material (stationary phase) and a liquid (mobile phase) is pumped at high pressure through the column. The separation of sample is based on the differences in the rates of migration through the column arising from different partition of the sample between the stationary and mobile phase. Depending upon the partition behavior of different components, elution at different time takes place.

Some important terminologies:
The baseline is any part of the chromatogram where only mobile phase is emerging from the column.
The peak maximum is the highest point of the peak.
The injection point is that point in time where the sample is placed on the column.
The dead point is the position of the peak-maximum of an unretained solute.
The dead time ($t_d$) is the time elapsed between the injection point and the dead point.
The dead volume ($V_d$) is the volume of mobile phase passed through the column between the injection point and the dead point. Thus, $V_d = Q t_d$ where $Q$ is the flow rate in ml/min and $t_d$ is dead time.
The retention time ($t_R$) is the time elapsed between the injection point and the peak maximum. Thus, $t_R = t_d + W$ where $W$ is the width at half height.
The retention volume ($V_R$) is the volume of mobile phase passed through the column between the injection point and the peak maximum. Thus, $V_R = Q t_R$ where $Q$ is the flow rate in ml/min and $t_R$ is retention time.

$$c_V = \frac{A_1 aX_2 - A_2 aX_1}{aX_2 aY_1 - aX_1 aY_2}$$ (5)

#### b. Terms Used in HPLC

1) **Column resolution**

The resolution $R$, of a column provides a quantitative measure of its ability to separate two analytes. Column resolution is defined as the difference between the two retention times divided by their average peak width.

$$R = 2 \left| t_{R_B} - t_{R_A} \right| / W_{A+B}$$

Where $t_{R_B}$, $t_{R_A} =$ retention time of compound B & A respectively.

$W_A$, $W_B =$ width at the base of the peak in time units of peak A & B.

Baseline resolution is achieved when $R = 1.5$.

To obtain high resolution, the three terms must be maximized.

1. **An increase in $N$, the number of theoretical plates**.

Increasing the length of column leads to an increase in retention time and increased band broadening. Instead, to increase the number of plates, the HETP can be reduced by reducing the size of the stationary phase particles.

2. **Selectivity Factor**

It is a measure of the difference in retention times between two given peaks.

The selectivity factor $\alpha$ of a column for the two species A & B is defined as

$$\alpha = \frac{K_B}{K_A}$$

Where, $K_B =$ distribution constant for more strongly retained species B.

$K_A =$ distribution constant for less strongly or more rapidly eluted species A.

$\alpha$ is always greater than unity, so small changes in $\alpha$ have large effects on the resulting resolution. Selectivity can be improved by altering the composition of the mobile phase, stationary phase, pH and temperature.

3. **Capacity Factor or Retention Factor ($K'$)**
A column must have the capacity to retain sample & the ability to separate sample components efficiently.

$$K' = \frac{t_r - t_0}{t_0}$$

Where \( t_r \) = retention time of analyte, \( t_0 \) = elution time of the void volume or non-retained components

\( t_0 \) and \( t_0 \) are easily obtained from a chromatogram. When capacity factor is less than one, elution is so fast that accurate determination of the retention time is very difficult. High retention factors (greater than 20) mean that elution takes a very long time. Ideally, the retention factor for an analyte is between 1 and 5. \( K' \) can be improved by changing the eluent strength

(4) Tailing Factor

Band shape is characterized by an asymmetry factor or tailing factor

$$T = \frac{W_2}{2f}$$

Where, \( W_2 \) = width of peak determined at either 5% or 10% from the baseline of the peak height \( f = \) distance between peak maximum and peak front

The accuracy of quantitation decreases with increase in peak tailing \((0.5 \leq T \leq 2)\)

(5) Theoretical plate number or Efficiency Factor

\( N \) defines the ability of the column to produce sharp, narrow peaks for achieving good resolution of band pairs.

$$N = 16\left(\frac{t_0}{\sigma_v}\right)^2 = \frac{L}{H}$$

Since resolution is a function of the square root of \( N \), large changes in \( N \) are required to make small changes in resolution. They also serve as a way of measuring column efficiency, either by stating the number of theoretical plates in a column, \( N \) (the more plates the better), or by stating the plate height, the Height Equivalent to a Theoretical Plate [HETP] (the smaller the better) \( N \) can be improved by increasing column length, decreasing particle size of column packing, or decreasing flow rate, Minimizing extra-column dead volume.

c. Pneumatic Pumps

Because of the relatively high pressures necessary to perform this type of chromatography, a more elaborate experimental setup is required. Figure 3 shows the block diagram of a complete HPLC apparatus

A. Solvent Reservoir

A modern HPLC consists of one or more glass or stainless steel reservoirs each of which contains 200-1000 ml of solvent. The reservoirs are equipped with degassers which removes gases usually oxygen and nitrogen because the entrapped bubbles in column and in detector system result in band broadening and errors in detection system. Degassers may consist of vacuum pumping system in which dissolved gases are swept out by fine bubbles of inert gas of low solubility. Filtration of this mobile phase is employed to remove any particulate matter, as particulate matter may cause damage to pumping and injection system as well as may result in clogging of column.

B. Pumping system

a. Reciprocating pump : It consists of small chamber in which solvent is pumped by back and forth motion by motor driven piston. Two ball check walls open and close alternately which controls flow of solvent into and out of cylinder. A solvent is in direct contact with piston.

Alternatively, pressure may be transmitted to solvent via flexible diaphragm. Disadvantage associated with reciprocating pump is that they produce pulsed flow. Advantages associated with reciprocating pump are that they have small internal volume (30-400µl), they give pressure upto 10,000 psi, and they are readily adaptable to gradient elution and gives constant flow rate.

b. Displacement pump: This usually consists of large syringe like chamber equipped with plunger which is moved by screw driven mechanism to move plunger forward by stepping motor. These pumps also produce a flow that tends to be independent of viscosity and atmospheric pressure. Disadvantage of is that it is having limited solvent capacity

c. Pneumatic Pumps: It is used to direct pressure from highly compressed gas to force solvent out of tube or it may be pneumatic amplifier type in which compressed gas at a lower pressure impinges on the large end of a piston to force the smaller end to deliver the liquid. Advantage of this pump is that it produces pulse less operation with high flow rate.

C. Sampling valves

Sample must be introduced in to pressurized column in the form of a narrow plug which avoids peak broadening. Usually sample ranging from 0.5 to 500µl can be introduced in to column. For this micro syringes were designed which can withstand pressure upto 1500 psi. Samples were introduced by stop flow method when flow of solvent is stopped momentarily and sample is injected directly in to the head of the column. These devices are integral part of HPLC and have capacity of holding 5-500 µl of sample. Here by this method samples are introduced at high pressure with high precision. A Loop system consists of stainless steel or Teflon block which are drilled in such a way that it provides alternate path for solvent flow which is selected by rotating valve. When valve is in fill position, the solvent flows in to the column. In the other path sample can be introduced in loop with the help of syringe. When valve is moved to inject position the mobile phase path is diverted through loop and it takes sample along with it on to column.

D. Guard column

Guard column is used to protect more expensive analytical column. These are inserted ahead of analytical column, where it acts as physical and chemical filter for analytical column. Guard column consists of larger particle size and short length (2-10cm).

E. Analytical column

The analytical column in which actual separation takes place is stainless steel tube, usually have 5-25cm in length and internal diameter of 2.0-4.6 mm. Coiled columns may be used but it leads to loss of efficiency.

F. Detector

The detector generates electrical signal which is proportional to concentration of solute. It is signal either for mobile phase or solute component. Refractive index detector measures properties of both mobile phase and solute while UV detector measures the property of solute only. The different types of detectors
used in HPLC are ultraviolet, fluorescence, refractive index, electrochemical, mass and diode.

d. HPLC Method Development

HPLC method development involves several essential steps: sample pretreatment, detection of sample bands, choosing separation conditions, quantitation and method validation. The wide variety of equipment, columns, eluents and operational parameters involved makes HPLC method development seem complex.

Step 1 - Selection of the HPLC method and initial system

When developing a HPLC method, the first step is to find the chemical composition of the sample which can provide valuable clues for the best choice of initial conditions for an HPLC separation.

Sample preparation is an essential part of HPLC analysis. The aim of sample preparation is to get a sample aliquot that

(1) is relatively free of interferences,

(2) will not damage the column, and

(3) is compatible with the intended mobile phase; that is, the sample solution will be miscible with the mobile phase without affecting sample retention or resolution. It may also be desirable to concentrate the analytes and/or derivatize them for improved detection or better separation. Types of HPLC: 

(1) Normal phase HPLC: Normal phase HPLC (NP-HPLC) was the first kind of HPLC chemistry used, and separates analytes based on polarity. This method uses a polar stationary phase and a non-polar mobile phase, and is used when the analyte of interest is fairly polar in nature. The polar analyte associates with and is retained by the polar stationary phase. (2) Reversed phase HPLC (RP-HPLC) is the choice for the majority of samples. It consists of a non polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been treated with alkyl dimethyl silylchloride (RMe₂SiCl), where R is a straight chain alkyl group such as octadecyl (C₁₈H₃₇) or octyl (C₈H₁₇). The retention time is therefore longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily. Retention time (RT) is decreased by the addition of polar solvent to the mobile phase and increased by the addition of more hydrophobic solvent.

(3) Isocratic and Gradient HPLC: With regard to the mobile phase, a composition of the mobile phase that remains constant throughout the procedure is termed isocratic.

Column dimensions: For most samples (unless they are very complex), short columns (10–15 cm) are recommended to reduce method development time. Such columns afford shorter retention and equilibration times. A flow rate of 1-1.5 ml/min should be used initially. Packing particle size of 3 or 5 μm are available for faster separation. Particle size increases efficiency and lifetime of column and decreases the backpressure.
**Detectors:** In most cases HPLC method development is carried out with ultraviolet (UV) detection using either a variable wavelength (spectrophotometer) or a diode array detector (DAD). We must be reasonably sure that the detector selected will sense all sample components of interest. Variable wavelength (UV) detectors normally are the first choice, because of their convenience and applicability for most samples. Fluorescence or electrochemical detectors should be used for trace analysis. For preparative HPLC, refractive index is preferred because it can handle high concentrations without overloading the detector.

**Step 2 - Selection of initial conditions**
This step determines the optimum conditions to adequately retain all analytes, i.e., ensures no analyte has a capacity factor of less than 0.5 (poor retention could result in peak overlapping) and no analyte has a capacity factor greater than 10–15 (excessive retention leads to long analysis time and broad peaks with poor detectability). Selection of the following is then required.

**Mobile phase solvent strength:** The mobile phase in HPLC refers to the solvent being continuously applied to the column, or stationary phase. The mobile phase acts as a carrier for the sample solution. The chemical interactions of the mobile phase and sample, with the column, determine the degree of migration and separation of components contained in the sample. Another is the influence of the pH since this can change the hydrophobicity of the analyte. For this reason most methods use a buffering agent, such as sodium phosphate, to control the pH. A volatile organic acid such as formic acid or most commonly trifluoroacetic acid is often added to the mobile phase, if mass spectrometry is applied to the eluent fractions. The buffers serve multiple purposes: they control pH, neutralize the charge on any residual exposed silica on the stationary phase and act as ion pairing agents to neutralize charge on the analyte.

**Step 3 – Selectivity optimization**
The aim of this step is to achieve adequate selectivity (peak spacing). The mobile phase and stationary phase compositions need to be taken into account. To minimize the number of trial chromatograms involved, only the parameters that are likely to have a significant effect on selectivity in the optimization must be examined.

**Step 4 – System suitability parameters optimization:** This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved. The parameters involved include column dimensions, column-packing particle size and flow rate. These parameters may be changed without affecting capacity factors or selectivity.

**Validation of an Analytical Method**
Validation of a method is the process by which a method is tested by the user for reliability, accuracy and preciseness of its intended purpose. The parameters for
method validation as defined by ICH (International Conference on Harmonization) guidelines are summarized below:

(A) Specificity
The analyte should have no interference from other extraneous components and be well resolved from them. A representative HPLC chromatogram or profile should be generated and submitted to show that the extraneous peaks either by addition of known compounds or samples from stress testing are baseline resolved from the parent analyte. Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically these might include impurities, degradants, matrix etc. specificity is measured by resolution, plate count and tailing factor.

(B) Accuracy
Accuracy is the measure of how close the experimental value is to the true value. Accuracy studies for drug substance and drug product are recommended to be performed at the 80, 100 and 120% levels of label claim as stated in the Guideline for Submitting Samples and Analytical Data for Methods Validation. For the drug product, this is performed frequently by the addition of known amounts of drug by weight or volume (dissolved in diluent) to the placebo formulation working in the linear range of detection of the analyte. This would be a true recovery for liquid formulations. For formulations such as tablet, suppository, transdermal patch, this could mean evaluating potential interaction of the active drug with the excipients. This test evaluates the specificity of the method in the presence of the excipients under the chromatographic conditions used for the analysis of the drug product. It will pick up recovery problems that could be encountered during the sample preparation and the chromatographic procedures. The mean of the replicates, expressed as % label claim, indicates how accurate the test method is.

(C) Precision
Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. ICH has defined precision to contain three components: repeatability, intermediate precision and reproducibility. It is normally expressed as % relative standard deviation. Precision may be considered at three levels:
(a) Repeatability: Repeatability expresses the precision under the same operating conditions over a short interval of time. It is also termed as intra-assay precision.
(b) Intermediate precision
Intermediate precision expresses within laboratories variations, different days, different analysts, different equipments, etc.
(c) Reproducibility
Reproducibility expresses the precision between laboratories (collaborative studies, usually applied for standardization of methodology).

(D) Detection limit (LOD)
These limits are normally applied to related substances in the drug substance or drug product. The detection limit of an individual analytical procedure is the lowest amount of analyte in sample, which can be detected but not necessarily quantitated as an exact value. LOD is expressed as a concentration at a specified signal to noise ratio. In chromatography detection limit is the injected amount that results in a peak with a height at least twice or three times as high as baseline noise level.

\[ \text{LOD} = 3.3(S)/s \]
where, \( S \) = standard deviation, \( s = \text{slope of the curve} \)

(E) Quantitation limit (LOQ)
The quantitation limit of an individual analytical procedure is the lowest amount of analyte in sample, which can be quantitatively determined with suitable precision and accuracy. LOQ is expressed as a concentration at a specified signal to noise ratio.

In chromatography quantitation limit is the injected amount that results in a peak with a height ten times as high as baseline noise level.

\[ \text{LOQ} = 10(S)/s \]
where, \( S = \text{standard deviation}, s = \text{slope of the curve} \)

(F) Linearity
The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of analyte in the sample.

(G) Range
The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

(H) Robustness
The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

(I) Ruggedness (As per USP)
It is the degree of reproducibility of test results obtained by analyzing the same sample under variety of normal test conditions such as different analysts, instruments, days, reagents, columns and TLC plates i.e. lack of influence of environmental variables on the method.

(J) Recovery
Recovery is expressed as the amount/weight of the compound of interest analyzed as a percentage to the theoretical amount present in the medium.

Statistical Validation\textsuperscript{22-23}

(1) Mean
It is a measure of center of distribution if the data are symmetrically distributed below and above the average. It simply involves the summing of the individual results and division by the number of results. It is denoted by \( X_m \)

\[ X_m = \frac{\sum X_i}{N} \text{ Where, } \sum X_i = \text{sum of all observations, } N = \text{number of observations} \]

(2) Standard Deviation
It is a measure of the spread of data about the mean.

\[ S = \sqrt{\left(\sum X^2 - (\sum X)^2\right)/N(N-1)} \text{ Where, } \sum X^2 = \text{sum of each value squared, } (\sum X)^2 = \text{square of sum of all values, } (\sum X)^2/N = \text{correction term} (\sum X)^2/N = \text{correction term}, N = \text{number of observations} \]

(3) Standard Deviation of Mean
It is a measure of variability of mean. 
\[ S_{xm} = \frac{S}{\sqrt{N}} \]
Where, \( S_{xm} = \) standard deviation of mean
\( S = \) standard deviation
\( N = \) number of observations

(4) Linear Regression
A common application of linear regression in analytical chemistry is to determine best linear equation for calibration data to generate a calibration curve. The concentration of analyte in a sample can then be determined by comparing a measurement of the unknown to the calibration curve.
For the line equation:
\[ Y = mX + b \]
Where, \( Y = \) estimated response / deponent variable
\( m = \) slope of the regression line
\( b = \) intercept (Y value when X=0)

(5) Correlation
Correlation is a measure of the relation between two or more variables. Correlation coefficients can range from -1.00 to +1.00. The value of -1.00 represents a perfect negative correlation while a value of +1.00 represents a positive correlation. A value of 0.00 represents a lack of correlation. The most widely used type of correlation coefficient is Pearson r, also called linear or product-moment correlation.

(6) Coefficient of Variation
It is a measure of relative variability.
\[ CV = \frac{S}{Xm} \]
Where, \( S = \) Standard deviation
\( Xm = \) Mean of observation
CV of 0.1 or 10 % means that standard deviation is one tenth of the mean.

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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