



Analytical Method Development and Validation: A Review

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Abstract This review focuses on approach to the validation of HPLC method with the compliance of restrictive needs and accepted pharmaceutical practices. The information during this review provides the explanations for performing analytical method validation. The validation parameters needed to be performed in validation for assay and organic impurities strategies. Individual validation parameters are mentioned in reference to the kind of method such assay and organic impurities method to be valid. This review was written to assist chemists/analysts to perform for method validation. This review study might facilitate to academics and pharmaceutical industry personnel to know the analytical method validation of HPLC as per USP and ICH guidelines.

Keywords Validation, HPLC, USP, ICH, Regulatory, QC Lab

Introduction

Method validation is defined as the process which proves that an implied analytical method is acceptable for its intended purpose, determined by means of well- documented experimental studies. Guidelines from the United States Pharmacopoeia (USP), International Conference on harmonization (ICH), Food and Drug Administration (FDA) etc., provide guidelines for performing such validations in pharmaceuticals. Results from method validation is used to judge the quality, reliability and consistency of analytical results [1].

Method development and validation are an iterative process. The influence of operating parameters on the performance of the method can be assessed at the validation stage which was not done during development/optimization stage of the method. The most significant point raised for validation is that the validity of a method can be demonstrated only through laboratory studies.

Reasons for validation

There are basically two important reasons for validating assays of an active pharmaceutical ingredients in the pharmaceutical industry:

- i). Validation is considered as an integral part of the quality-control system.
- ii). CGMP (Current good manufacturing practice) regulation requires assay validation. In pharmaceutical industry it would be difficult to confirm that the product being manufactured is uniform and that meet the predetermined standards set to assure fitness for use [2].

Parameters of method validation

The parameters for method validation have been defined in different working groups of national and international committees. An attempt at harmonization was made for pharmaceutical applications through the ICH. The defined validation parameters by the ICH and other regulatory bodies are summarized as under [3].





Figure 1: Parameters for method validation

Accuracy

Accuracy is defined as the measure of exactness of an analytical procedure. It expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

The ICH document on validation methodology recommends accuracy to be assessed using a minimum of nine determination over a minimum of three concentration levels covering the specified range (for example, three concentrations with three replicates each). Accuracy should be reported as percent recovery by the assay of known added amount of standard in the sample or as the difference between the mean and the accepted true value, together with the confidence intervals.

Thus, accuracy of the method was studied by recovery experiments using standard addition method at three different levels (80%, 100% and 120). Known amounts of Standard solutions containing analyte were added to pre-quantified sample solutions to get 80,100 and 120 %. These samples were analysed by injecting the sample solution and % recovery was calculated. In the present study %recovery was calculated by the following formula [4].

$$\% \text{ Recovery} = \frac{\text{Area of spiked sample} - \text{Area of unspiked sample}}{\text{Area of standard}} \times \frac{\text{Conc. of standard}}{\text{Conc. of added drug}} \times 100$$

Acceptance limit for % recovery is 98-100%.

Accuracy for analytical procedures is determined by two methods:

- i). Absolute method
- ii). Comparative method

i). Absolute method

The test for the accuracy of the method is carried out by taking varying amounts of the constituents and proceeding according to the specified instructions. The difference between the means of an adequate number of results and amount of constituent actually present, usually expressed as parts per hundred (%) i.e., % error. The constituent in question will usually have to be determined in the presence of other substances, and it will therefore be necessary to know the effect of these upon the determinations. This will require testing the influence of a large number of



probable compounds in the chosen samples each of varying amounts. In few instances, the accuracy of the compound is controlled by separation (usually solvent extraction or chromatographic technique) involved [5].

ii). **Comparative method**

In the analysis of pharmaceutical formulations or laboratory prepared samples of desired composition, the content of the constituents sought determined by two or more (proposed and official or reference) 'accurate' methods of essentially different character can usually be accepted in the absence of an appreciable formulation either in the proposed or reference methods comprising of various operations which include sampling, preparation of solution, separation of interfering ingredients if any and the method for the quantitative assay [6].

Precision

The precision determines the closeness of agreement (degree of scatter) between a series of measurements attained from multiple sampling of the standardized sample under the prescribed conditions. The precision of an analytical method is normally expressed as the percent relative standard deviation for a statistically significant number of samples. The most common statistical terms employed is the standard deviation of a population of observation. Standard deviation is the square root of the sum of squares of deviations of individual results from the mean, divided by one less than the number of results in the set [7].

According to the ICH (International conference on harmonisation), precision should be accomplished at three different levels viz; repeatability, intermediate precision and reproducibility.

- **Repeatability**

Repeatability is a measure of the precision under the same operating conditions over a short interval of time. It is sometimes denoted as intra-assay precision [8].

- **Intermediate precision or Inter-day precision**

It is defined as the variation within the same laboratory. Typical parameters that are investigated include day-to-day variation, analyst variation, and equipment variation [9].

- **Reproducibility or Ruggedness**

It measures the precision between laboratories in collaborative studies. To validate this, the characteristic similar studies need to be performed at other laboratories using the same homogenous sample lot and the same experimental design [10].

Limit of Detection

The detection limit of an individual analytical procedure is defined as the lowest concentration of an analyte in a sample that can be detected but not quantified as an exact value.

Limit of detection (LOD) is calculated by using the formula:

$$LOD = 3.3 \times \left(\frac{\sigma (\text{Standard deviation})}{S (\text{Slope of the curve})} \right)$$

The standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line or the standard deviation of y-intercepts of regression lines [11].

Limit of Quantification

It is defined as the lowest concentration of an analyte in a sample that can be quantitatively determined with acceptable precision and accuracy under the stated operational conditions of the method.

Limit of quantification (LOQ) is calculated by using the formula [12]:

$$LOQ = 10 \times \left(\frac{\sigma (\text{Standard deviation})}{S (\text{Slope of the curve})} \right)$$

Specificity

Specificity is defined as the ability to access accurately and specifically the analyte in the presence of other components that may be expected to be present in the sample matrix. It is a measure of the degree of interference



from such things like other active pharmaceutical ingredients, excipients, impurities and degradation products ensuring that a peak response is due to a single component only, that is no co-elutions exist. Specificity is measured and then documented in a separation by the resolution, plate count (efficiency) and tailing factor.

This reserves the use of “specific” for those procedures that produce a response for a single analyte only. ISO/IEC most likely has the same understanding because it requires a method to be “selective” rather than specific. Our goal is to distinguish and quantify the response of the target compounds from the responses of all other compounds. Selectivity is the ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix. Specificity for an assay ensures that the signal measured comes from the substance of interest, and that there is no interference from excipient and/or degradation products and/or impurities.

Determination of this can be carried out by assessing the peak identity and purity [13].

Linearity and Range

The linearity of an analytical procedure is defined as the ability (within a given range) to obtain test results of variable data (e.g., absorbance and area under the curve) which are directly proportional to the concentration of the analyte in the sample. This is the method's ability (within a given range) to obtain results which are either directly, or after mathematical transformation proportional to the concentration of the analyte within a given range. The range is normally expressed in the same units as the test results. For assay tests, ICH requires the minimum specified range to be 80 to 120 percent of the test concentration [14].

Robustness

Robustness is defined as the capacity of an analytical method to remain unaffected by small deliberate variations in method parameters and provides an indication of its reliability during normal usage. It is evaluated by various method parameters such as percent organic, pH, ionic strength, temperature, etc., and determining the effect on the results of the method [15].

Solution Stability

Many solutes readily decompose prior to chromatographic investigations, for example, during the preparation of the sample solutions, extraction, clean up, phase transfer or storage of prepared vials (in refrigerators or in an automatic sampler). Under these circumstances, method validation should investigate the stability of the analytes and standards in solution form (in analytical preparations) [16].

System suitability

In addition, prior to the start of laboratory studies to demonstrate method validity, System suitability is considered appropriate when the RSD, theoretical plates, tailing factor and resolution parameters calculated on the results obtained at different time intervals, does not exceed more than of specified limit of the corresponding value of the system precision [17].

Table 1: System Suitability Test Parameters and Recommendations [18]

Parameters	Recommendation
Theoretical Plates (N)	Should be > 2000
Capacity Factor (k')	The peak should be well-resolved from other peaks and the void Volume, generally $k' > 2.0$
Relative retention Resolution (R_s)	Not essential as long as the resolution is stated. R_s of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipient, degradation product, internal standard) etc.
Tailing Factor (T)	T of ≤ 2
Repeatability	RSD $\leq 1\%$ for N ≥ 5 is desirable.



Stability indicating assay method (SIAM)

According to FDA guidelines, a SIAM is defined as “a validated analytical procedure that accurately and precisely measures active ingredient free from potential interferences like degradation products, process impurities, excipients or the other potential impurities”, and the FDA recommends that all assay procedures or stability studies [19].

The stability-indicating method is used to detect the changes with time in the chemical, physical, or microbiological properties of drug substance and drug product. The quality of a drug product depends on quality of API. The guidelines explicitly require conduct of forced degradation studies under variety of conditions, like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products. The method is expected to allow analysis of individual degradation products [20].

Purpose of stability testing of the drugs

SIAM is developed routinely by stressing the API under conditions exceeding those normally used for accelerated stability testing. In addition to demonstrating specificity in SAIM, stress testing, also referred to as forced degradation, also can be used to provide information about degradation pathways and the products that could form during storage and helps to facilitate formulation development, manufacturing and packaging.

Types of SIAM

Specific stability indicating assay method:

It can be defined as “a method that able to measure unequivocally the drugs in the presence of all degradation, in presence of excipients and additives, expected to be present in the formulation” [21].

Selectively Stability indicating assay method:

It can be defined as “a method that is able to measure unequivocally the drugs and all the degradation products in the presence of excipients and additives which are expected to be present in the formulation” [22].

Objective of stability testing

The purpose of stability testing is to provide evidence on how the quality of drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light, and enables recommended storage condition, re test periods and shelf lives to be established. Specified stress condition should result in approximately 10-20% degradation of the drug substance or represent a reasonable maximum condition achievable for the drug substance. If no degradation is observed under the conditions achievable for the drug substance [23].

Forced degradation studies are carried out for following reasons

Forced degradation or stress testing is undertaken to demonstrate specificity when developing stability-indicating methods, particularly when little information is available about potential degradation products [24].

Table 2: Conditions for forced degradation [25]

Study	Conditions
Acidic pH	Using HCl at various temperatures
Neutral pH	Using water or buffer at various temperature
Basic pH	Using NaOH at various conditions
Oxidation	O ₂ atmosphere, or H ₂ O ₂
Hydrolysis	Using water
Thermal degradation	At 10°C increment of temperature than accelerated condition

Chromatography

The term ‘chromatography’ is coined by the Russian botanist M.S. Tswett from the Greek words meaning ‘colour (chroma)’ and ‘writing (graphy)’. Afterwards, a number of well-known scientists whose contributions are too



numerous to be recounted here, and their work has led to the development of modern liquid chromatography, which is often called high-pressure or (HPLC) high-performance liquid chromatography [26-27].

High-Performance Liquid Chromatography

Chromatography is based on the principal of separation and is defined as the “technique in which the substances of a mixture are separated depending on the rates at which they are distributed through two phases, one of which does not move (stationary phase) and the other which moves (mobile phase)”. HPLC is known as the most widely used analytical separation technique which provides major improvements over the old chromatography techniques [28].

Advantages of HPLC

All the below mentioned advantages make HPLC more efficient over the all-other chromatographic techniques in case of separation, speed, sensitivity, easy sample recovery, automation, integration, handling and maintenance.

- HPLC technique is more popular because it is non-destructive and may be applied to thermally liable compounds as well.
- HPLC is ideally appropriate for the separation of macromolecules and ionic species of biomedical interest, liable natural substances, and various less stable and high molecular weight compounds.
- HPLC uses both phases in the chromatographic process to increase more selective interactions with the sample molecule hence, it helps in attaining majority of difficult separations easily.
- Short, small-bore columns containing densely packed particles of stationary phase offers rapid exchange of compounds between the mobile and stationary phases. A large variety of unique column packings (stationary phase) provide a wide range of selectivity to separation through HPLC.
- HPLC also provides wide choice of detection methods as number of unique detectors are available.
- HPLC contains automatic instrumentation and calculation which is carried out by integrator itself which help saving of manual labour. In addition to receiving and reporting detector output, computers are used to control chromatographic settings and operations, thus providing for long periods of unattended operation [29].

Instrumentation of HPLC

A liquid chromatograph consisting of a reservoir having the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column (a column temperature controller may be also used) to attain retention, a detector to detect analyte response, and a data collection device such as a computer, integrator, or recorder [30, 31, 32]. Further, in some cases, degasser with vacuum pump and pre-column facility can implement in the modern HPLC, presented in figure below.

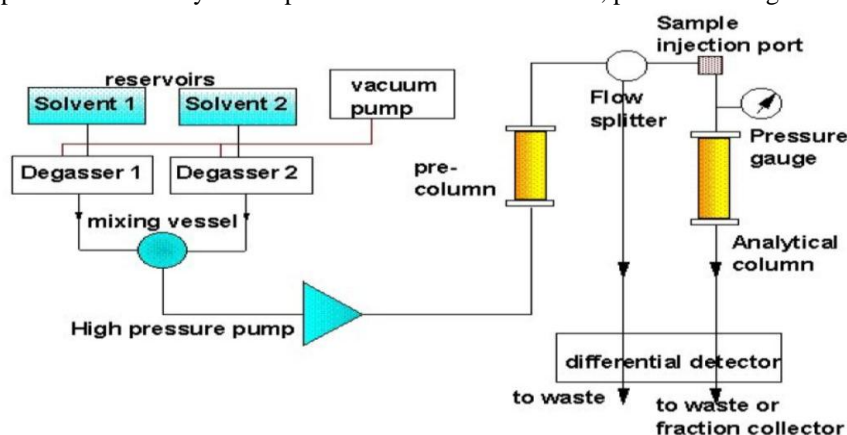


Figure 2: Designing of HPLC path



A brief introduction of HPLC components is discussed below:

➤ **Pumping systems**

HPLC pumping system distributes measured amounts of mobile phase from the solvent reservoirs to the column with the help of high-pressure tubing and fittings. Operating pressures up to 5000 psi or higher, with delivery rates up to about 10 mL min^{-1} is typical. Pumps used for quantitative analysis should be made up of constituents that are inert to corrosive mobile phase components and be capable of delivering the mobile phase at a constant rate with minimal fluctuations over extended period of time. Advanced pumping system is equipped with a degasser to remove dissolved air and other gases from the solvent through solvent delivery system.

➤ **Injectors**

After dissolution of compounds in mobile phase or in suitable diluent, they are injected into the mobile phase, either manually by syringe/loop injectors or automatically by autosampler. Autosampler is an injection device that is used to transfer sample from the vials to a loop from which it is loaded into the chromatograph.

➤ **Columns**

The column is usually made up of stainless-steel substance in order to withstand high pressure. Stationary phases for modern, reverse-phase liquid chromatography typically consist of an organic phase which are chemically bound to silica or other materials. Small particles thinly coated with organic phase provide for low mass transfer resistance and, hence, rapid transfer of compounds between the stationary and mobile phases. Unmodified silica, porous graphite or polar chemically modified silica, e.g., cyanopropyl or diol, used as the stationary phase for normal phase liquid chromatography.

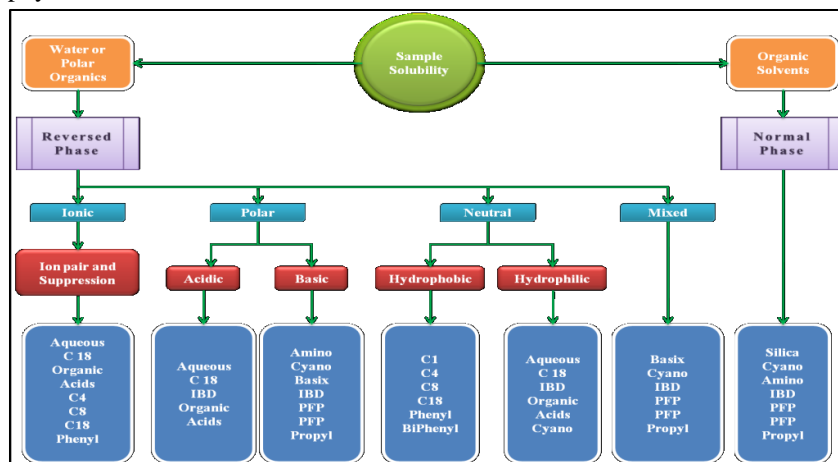


Figure 3: Stationary phase selection for HPLC

The nature of the bonded phase is an important parameter for determining the separation properties of the chromatographic system.

➤ **Detectors**

The detector measures a physical parameter of the column effluent or of the components in the column effluent and transforms it to an electrical signal. Three types of detectors are universal, selective, and specific LC detectors.

UV-Visible spectrophotometers, including diode array detectors, are the most commonly employed detectors. Fluorescence spectrophotometers, differential refractometers, electrochemical detectors, mass spectrometers, light scattering detectors, radioactivity detectors or other special detectors may also be used. In diode array multi-wavelength detectors, continuous radiation is passed through the sample cell, and then resolved into its constituent wavelengths, which are individually detected by the photodiode array.



Factors Affecting Method Development in HPLC [33, 34]**▪ Polarity**

In chemistry, polarity refers to a separation of electric charge leading to molecule or its chemical groups having an electric dipole or multiple moment. Polar molecules interact through dipole-dipole intermolecular forces and hydrogen bonds. Molecular polarity is dependent on difference in electro negativity between two atoms in a compound and asymmetry of compound nature's structure. Polarities underlies a number of physical properties including surface tension, solubility, melting and boiling points. Thus, when an acid or base undergoes ionization, it becomes more hydrophilic and less interacting with column binding sites as a result the ionized analyte is less retained on the column and eluted faster from non-polar column.

▪ Mobile Phase

The selection of mobile phase is based mainly on polarity and solubility of the compound. Usually in RP HPLC method water-organic solvent used as mobile phase. In Non-polar solvents like n- hexane is used. If the Formulation contains ionic or ionizable compounds, then buffered mobile phase is used to ensure the reproducible results.

▪ Organic Solvent

The organic solvent (modifier) is added to lower the polarity of aqueous mobile phase. The lower the polarity of the mobile phase, the greater the eluting strength in reverse phase chromatography. The two mostly used are methanol and acetonitrile, although acetonitrile is more popular choice. Both acetonitrile and methanol are less viscous than isopropanol. All three are essentially UV transparent.

▪ Ion Suppression

The retention of peptides and proteins in reversed phase chromatography can be modified by mobile phase pH since these particular solutes contain ionisable groups. The degree of ionization will depend on the pH of the mobile phase. The acids maintain a low pH environment and suppress ionization of the acidic groups in the solute molecules. Varying the concentration of strong acid components in the mobile phase can change the ionization of the solutes and therefore their retention behavior.

▪ Isocratic and Gradient Elution

In isocratic type constant eluent composition is pumped through the column during the whole analysis while in the gradient type, eluent composition (and strength) is steadily changed during run. In isocratic elution, the selectivity does not change if column dimension change that peak elute in same order. In gradient elution, the elution order may change as the dimensions or flow rate changes. Gradient elution decreases the retention of later eluting components so that they elute faster, giving narrower peaks for most components. This also improves the peak shape for tailed peaks, as the increasing concentration of the organic eluent pushes the tailing part of a peak forward.

▪ pH

pH plays a role in achieving the chromatographic separations as it controls the elution properties by controlling the ionization characteristics. The low pH results in good solubility of the sample components and ion suppression, not only acidic groups on the sample molecules, but also of residual silanol groups of silica matrix. Acids such as trifluoroacetic acid, heptafluorobutyric acid and ortho- phosphoric acid in concentration range of 0.05- 0.1% which are outside this range. This is due to the fact that siloxane linkage area cleaved below pH 2, while pH value is above 8 silica may dissolve.

▪ pKa

The Pka or (dissociation constant) is a measure of the strength of an acid or base, and allows us to determine the degree of ionization at a given pH. When pH is equal to pKa for the analyte, it is ionized and unionized species are equal. Generally, at low pH tailing is minimized and method ruggedness is maximized. It is normal to quote the pKa at 25° C.

▪ Buffer as Mobile Phase

Buffers improve peak shape of basic compounds and can help modify the band spacing and retention of acidic or basic compounds. The buffer maintains the pH when small amount of acid or base is added. A buffer is most



effective when used within ± 1 pH units from the pKa. The most popular buffer for HPLC with UV detection are phosphate and acetate as they can be used at wavelengths below 220nm.

▪ **Buffering Capacity**

Maximum amount of either strong acid or strong base that can be added before a significant change in the pH will occur. The closer the buffered pH is to pKa, the greater the buffering capacity. Buffering capacity is expressed as the molarity of sodium hydroxide required to increase pH by 1.0.

▪ **Buffer Selection**

Buffer are solution of weak acid and conjugate base, or weak base its conjugate acid. Choice of buffer is typically governed by the desired pH. It is mandatory that the buffer has pka near to the desired ph. Since buffer control pH best at their pKa. A rule of thumb is to choose a buffer with pKa value < 2 units of the desired mobile phase pH.

▪ **Peak Shape**

The peak shape in chromatography is the name given to the type of curve resulting from plotting the concentration of solute in mobile phase (or the mass of solute per unit time) eluted from chromatographic column, against time. If support in a packed column has adsorptive properties, the peak will exhibit a long tail and, again for obvious reason, is called tailing peak.

▪ **Sensitivity**

Sensitivity is a measure of the smallest detectable level of component in a chromatographic separation and is dependent on the signal-to-noise ratio in a given detector. Sensitivity can be increased by derivatization of the compound of interest, optimization of chromatographic system or miniaturization of the system.

▪ **Flow Rate**

Flow rate, more for isocratic than gradient separation, can sometime be useful and readily utilized to increase the resolution, although its effect is very modest. The slower flow rate will also decrease the column back pressure. As the flow rate increases, the retention time of the drug decreases and compound elutes faster.

Modes of HPLC [35, 36, 37]

Various modes of HPLC utilized to separate compounds are classified as follows:

- Adsorption chromatography
- Normal-phase chromatography
- Reverse-phase chromatography
- Ion-pair chromatography
- Ion-exchange chromatography
- Size exclusion chromatography

✚ **Adsorption chromatography**

This chromatography uses polar substances as stationary phases with relatively non-polar mobile phases. Separations in adsorption chromatography led to a great extent from the interaction of sample polar functional groups with discrete adsorption sites on the stationary phase. Adsorption chromatography is usually considered appropriate for the separation of non-ionic molecules that are soluble in organic solvents.

✚ **Normal-phase chromatography**

In HPLC, if stationary phase is more polar than the mobile phase, then it is known as normal-phase liquid chromatography. Due to lower affinity of non-polar compounds to the stationary phases used, non-polar compounds are eluted first while polar compounds are retained for longer time. This chromatography is widely applied for chiral separations.

✚ **Reversed-phase chromatography**

In HPLC, if stationary phase is less polar than the mobile phase, then it is known as reversed-phase liquid chromatography. Retention in this chromatography occurs by non-specific hydrophobic interactions of the solute with stationary phase. The global application of this chromatography arises from the fact that practically all organic



molecules have hydrophobic regions in their structures and effectively interact with the stationary phase. The rationale for this includes the simplicity, versatility, and scope of the reversed-phase method.

✚ Ion-pair chromatography

Ionic or partially ionic compounds can be chromatographed on reversed-phase columns by using ion-pairing reagents. These reagents are typically long-chain alkyl anions or cations that, when used in dilute concentrations, can increase the retention of analyte ions. C-5 to C-10 alkylsulfonates are commonly used for cationic compounds while C-5 to C-8 alkyl ammonium salts are generally used in the cases of anionic solutes.

✚ Ion-exchange chromatography

It is an adaptable technique used primarily for the separation of ionic or easily ionizable species. The stationary phase is characterized by the presence of charged centres having exchangeable counter-ions. Both anions and cations can be separated easily by choosing the suitable ion-exchange medium.

✚ Size exclusion chromatography

This chromatography separates molecules according to their molecular mass. In this chromatography, column is filled with material having precisely controlled pore sizes and the sample is simply screened or filtered according to its solvated molecular size. Largest molecules are eluted first and the smallest molecules last. This mode can be further subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solvents).

HPLC method development [38]

A variety of patterns have been used to develop analytical separations. The choice of the mode of HPLC method should be made principally from the properties of the sample that has been determined about molecular weight (MW).

The term polarity concerns the ability of a sample or solvent molecule to interact by combination of dispersion, dipole, hydrogen bonding, and dielectric interactions. The combination of these four intermolecular attractive forces constitutes the solvent polarity. Polarity is measure strength of solvent that affected selectivity. The changes in selectivity may be affected by making use of the following solvent properties:

- Proton acceptors: amines, ethers, sulfoxides, amides, esters, and alcohols.
- Proton donors: alcohols, carboxylic acids, phenols, and chloroform.
- Large dipole solvents: methylene chloride, nitrites, sulfoxides, and ketones.

Buffers are mainly used to regulate the pH and the acid-base equilibrium of the solute in the mobile phase. They may also be utilized to affect the retention times of ionizable compounds. The buffer capacity should be at maximum and should not vary in the pH range between 2 to 8 commonly used in HPLC. The buffers should be soluble, stable, inert to analytes and compatible to the detector.

The parameters that are considered for good chromatographic conditions and being optimized are:

- ❖ Resolution (R)
- ❖ Theoretical plates (N)
- ❖ Tailing factor (T)
- ❖ Capacity factor (k')
- ❖ Selectivity (α)
- ❖ Resolution (R) [39]

The resolution R is a function of column efficiency, N and is specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the drug.



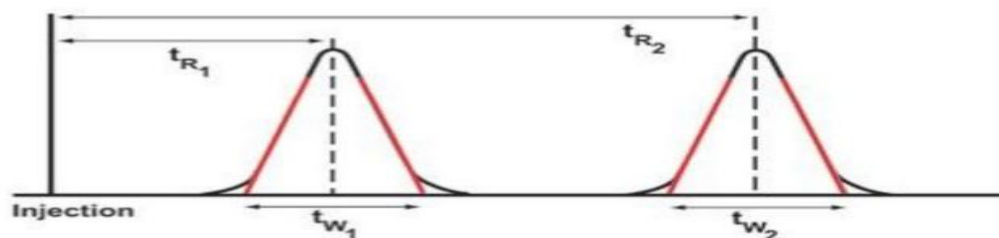


Figure 4: Expression of Resolution

R is determined by the equation:

$$R = \frac{2(t_2 - t_1)}{(W_2 + W_1)}$$

Or

$$R = \frac{2(t_2 - t_1)}{1.70(W_1, \frac{h}{2} + W_2, \frac{h}{2})}$$

Where,

t_2 and t_1 are the retention times of the two components,

W_2 and W_1 are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline,

$W_1, h/2 + W_2, h/2$ are the corresponding peak width at half-height.

❖ Theoretical plates (N) [40]

Theoretical plate number (N) is an index that indicates column efficiency. Column efficiency is based on calculation in which the larger the theoretical plate number the sharper the peaks. Assuming a Gaussian distribution (normal distribution), the theoretical plate number is represented by equation:

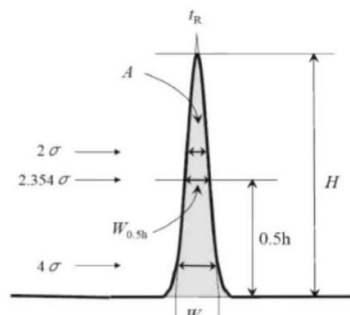
$$N = \frac{t_R}{\sigma}$$

Where,

t_R is retention time and

σ is standard deviation.

As shown in Fig. below, assuming a Gaussian distribution, the peak width W is 4σ and peak FWHM $W_{0.5h}$ is 2.354σ .



$$N = 16 \times \left(\frac{t_R}{W} \right)^2$$

$$N = 5.54 \times \left(\frac{t_R}{W_{0.5h}} \right)^2$$

Figure 5: Representation of Theoretical plates

❖ Tailing factor (T) [41]

The tailing factor (T) is a measure of peak symmetry. It is unity for perfectly symmetrical peak to value of 1.0 and its value increases as tailing becomes more pronounced. It is determined by following formula. $T = f t_{0.05} / W_{0.05}$ Where $W_{0.05}$ is width of the peak at 5% height and f is distance from the peak maximum to the leading edge of the peak which being measured at a point of 5% of the peak height from the baseline.

In general, value of tailing factor should be less than 2.0.



❖ Capacity Factor (k') [42]

Capacity factor of a sample component is a measure of the degree which that component is retained by the column relative to an unretained component.

It is determined by using following formula:

$$\text{Capacity factor } (k) = \frac{(t_r - t_0)}{t_0}$$

Where,

t_r - is the elution time of retained component and

t_0 - is the elution time of the unretained sample.

❖ Selectivity (α) [43]

The selectivity (or separation factor) is a measure of relative retention of two components in a mixture. Selectivity represents the separation power of particular adsorbent to the mixture of these particular components. In general, if the selectivity of two components is equal to 1, then there is no way to separate them by improving the column efficiency.

The ideal value of α is 2.

$$\text{Selectivity factor } (\alpha) = \frac{k'(2)}{k'(1)}$$

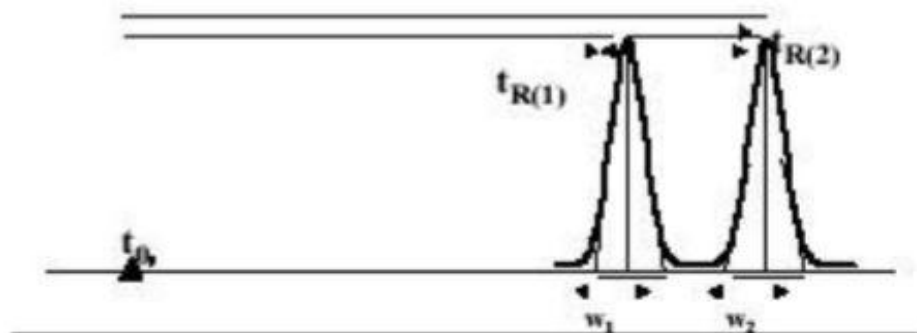


Figure 6: Representation of Selectivity

It can also be calculated by using formula,

$$\alpha = \frac{V_2 - V_1}{V_1 - V_0}$$

Where,

V_0 = The void volume of the column,

V_1 and V_2 = The retention volumes of the second and the first peak respectively.

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