Chapter 1: Introduction

INTRODUCTION
1.1 INTRODUCTION: Medicinal plants and their extracts have been used by humans for different ailments since old ages. Ayurveda, the most ancient health care system, is practiced worldwide which uses medicinal plants in different forms for the treatment of various health problems. These medicinal plants parts which are used for cure of different ailments are known as herbal medicines. Herbal medicines are made up of various plant parts like leaves, fruits, seeds, aerial parts, bark, roots etc. According to World Health Organization (WHO), herbal medicines are defined as active ingredients, plant parts or plant materials in crude or processed state with certain excipients.

India has a great history of use of herbal medicines and are increasingly used worldwide during the last few decades as evidenced by rapidly growing national as well as global markets of herbal medicines. As per WHO estimates, the current requirement for medicinal plants is approximately US $14 billion a year and it would reach to ~US $5 trillion by 2050. Herbal medicines are now getting recognition in the developed countries and their use remains widespread in the developing countries. Current estimates indicate that about 80% of people in developing countries still rely on herbal medicines for their primary healthcare because they are effective, affordable and having lesser side effects as compared to allopathic medicines. Also very few effective allopathic medicines are available for age-related diseases such as memory loss, diabetic wounds, immune and liver disorders, etc. India has 2.4% of world's area with 8% of global biodiversity. Approximately 25,000 herbal formulations are used as traditional medicine and more than 1.5 million practitioners are using traditional medicinal system for health care purpose in India. More than 7800 herbal manufacturing units are running currently in India which require more than 2000 tones of a raw material from medicinal plants annually. India is one of the largest exporters of standardized herbal extracts to USA and Europe for food supplements.

Research related to pharmacognosy, chemistry, pharmacology and clinical therapeutics have been carried out on medicinal plants and many of the major pharmaceutical corporations have renewed their strategies in favor of natural products drug discovery. Numerous drugs have entered the international pharmacopoeia through the study of ethnopharmacology and traditional medicine. The R & D thrust in the pharmaceutical sector is also focused on development of new innovative/indigenous plant-based drugs through investigation of leads from the
traditional system of medicine. WHO has also recognized the importance of traditional medicine and has created strategies, guidelines and standards for botanical medicines. It has released guidelines for Good Agricultural and Collection Practices (GACP) for medicinal plants to ensure quality, safety and ecologically sound cultivation practices for future generations. WHO has also formed International Regulatory Cooperation for Herbal Medicines (IRCH) in 2006 for regulation of herbal medicines.

Government of India also has encouraged research on traditional medicines. To promote research on indigenous systems, a separate department known as AYUSH (Ayurveda, Yoga, Unani, Siddha, Homoeopathy), formerly known as Department of Indian Systems of Medicine and Homoeopathy (ISM & H), was established in March 1995. This department deals with quality control and standardization of herbal drugs, improving the educational standards, availability of medicinal plant material and awareness generation about the efficacy of the indigenous systems domestically and internationally. India has a National Medicinal Plants Board (NMPB) and 30 State Medicinal Plants Boards (SMPB) which are encouraging sustainable collection and cultivation of medicinal plants. The Central Council of Indian Medicine oversees teaching and training institutes, while Central Council for Research in Ayurveda and Siddha deals with interdisciplinary research.

1.1.1 Need of standardization: Despite of various advantages of herbal medicines over allopathic medicines, they are not globally accepted because of lack of proper standardization techniques. Herbal medicines are seen with suspect. Incidences of adverse drug reactions caused by the use of herbal medicines are also increasing because of poor quality of herbal medicines and wrong identification of plant species. Therefore cultivation, collection and classification of medicinal plants should be monitored and controlled stringently to ensure quality, efficacy and safety of herbal products. Herbal drug manufacturers are obtaining the crude materials from nontechnical persons from rural or forest area leading to increased incidences of adulteration/substitution. Another reason for adulteration/substitution is that the processed drugs are very difficult to identify. So, proper identification and standardization techniques have to be adopted before proceeding for manufacturing of herbal formulations.
Standardization means assuring that every medicine that is being sold has exact substances in the appropriate concentration and will produce desired therapeutic activity. It helps in adjusting the herbal drug formulation to a defined content of a constituent/s with therapeutic activity. Standardization of plant-based medicines becomes mandatory for their global acceptance. The technique of standardization is extensively acknowledged to set up the standard criteria for uniformity and quality control of herbal preparations.

1.1.2 Marker-based standardization:
Standardization of herbal formulations is essential to ensure quality and finest levels of active principles for their bioactivity. Nowadays, marker-based standardization of herbal drugs is gaining thrust. Marker-based standardization involves identification of major and unique component/s in herbs as markers and development of appropriate analytical method for monitoring them.

In many herbal preparations, the therapeutic activity is due to synergistic effects of more than one constituent. There are very few herbs available for which the activity is assigned to single constituent or group of well-defined constituents. These are called as bioactive components. Bioactive components are chemically identified and known to contribute to the therapeutic activity of a plant material or herbal preparation. If compounds which are specifically responsible for the biological activity are not known, specific constituent or group of constituents present in the plant material are chosen and used for quality control purpose. These constituents are known as marker compounds.

1.1.3 Classification of markers: According to EMEA (European Medicines Agency) guidelines marker compounds are broadly classified into two categories:

A) Analytical markers: They are constituents or groups of constituents that serve solely for analytical purposes.

B) Active markers: Active markers are constituents or groups of constituents which are generally accepted to contribute to the therapeutic activity.

Hongxi Xu et al proposed eight new categories of chemical markers as given below:

(1) Therapeutic components: Therapeutic components are responsible for therapeutic effects of a herbal drug and are used for both qualitative and quantitative assessments.
(2) **Bioactive components**: Bioactive components are structurally different from each other, the individual components may not have direct therapeutic effects but the combination of their bioactivities contributes to the therapeutic effects.

(3) **Synergistic components**: They do not contribute to the therapeutic activity or related bioactivities directly. Synergistically they reinforce the bioactivities of other components consequently the therapeutic activity of the herbal medicine is modulated.

(4) **Characteristic components**: As the name suggests, characteristic components are specific to a herbal medicine which may contribute to the therapeutic activity.

(5) **Main components**: The concentration of main components in herbal medicine is very high. They are not specific components of herbal medicine and their bioactivities may not be known. They are mainly used for differentiation and stability purpose.

(6) **Correlative components**: Correlative components are closely related to each other. They are used to evaluate the quality of herbal medicines originated from different geographical regions and stored for different periods of time.

(7) **Toxic components**: They exhibit toxicity and their levels in herbs are utilized to control toxic components in herbal drugs.

(8) **General components**: General components are common but specific components present in a particular species, genus or family. These components may be used along with 'fingerprints' to differentiate plant from its substitutes and adulterants.

1.1.4 **Ideal characteristics of a marker compound**: Marker compound should have following characteristics:

a) It should be a pure substance with an established chemical structure.

b) Selected marker should be present in sufficient quantity.

c) It should be sufficiently stable.

d) It should be commercially available.

1.1.5 **Applications of marker based standardization**: Marker based standardization is useful for following applications to ensure quality, efficacy and stability of herbs, herbal extracts or herbal medicines:

a) To identify adulterants and substituents in crude drugs

b) Analysis of herbal formulations

c) Stability assessment of herbal formulations

d) Quality control of plant parts
1.1.6: Quality control of herbal medicines: Commonly used chromatographic techniques for quality control of herbal medicines are High Performance/Pressure Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC) and Gas Chromatography (GC). These techniques require 'reference standards' to produce accurate and reliable results. Reference standards for many plant-based formulations are difficult to obtain or very expensive to purchase.商性 unavailable reference standards are either synthesized using appropriate methods or isolated from respective medicinal plant. Chromatographic techniques such as column chromatography (CC), preparative TLC, preparative HPLC, flash chromatography, vacuum liquid chromatography (VLC), centrifugal thin layer chromatography (CTLC), countercurrent chromatography (CCC), solid phase extraction technique (SPE), etc. are used either alone or in combination for isolation of marker compounds or bioactives.

1.1.7 High Performance/Pressure Liquid Chromatography (HPLC): In modern pharmaceutical and herbal industry, HPLC is major and integral analytical tool useful at all stages of drug discovery and product development. HPLC is a versatile technique which offers a combination of speed, reproducibility and sensitivity. In HPLC, liquid/s containing sample is transported through fixed porous media. The fixed media is known as “stationary phase” while the moving liquid/s is known as “mobile phase”. The separation occurs based on differential affinity of sample to the stationary phase and mobile phase.\(^\text{15}\)

Based on type of the interactions of the analyte with the stationary phase and according to relative polarities of the stationary and mobile phases, HPLC is divided into four main modes\(^\text{15}\):

**Normal phase chromatography:** In this mode, stationary phase is polar and mobile phase is non polar. Here polar forces are used.

**Reversed phase chromatography:** In this mode, stationary phase is non-polar and mobile phase is polar. Here dispersive forces are used.

**Ion-exchange chromatography:** The separation is based on the difference in the affinity of the ionic analytes for the counter ions present on the stationary phase surface. Here ionic forces are used.

**Size-exclusion chromatography:** Separation is based on size of the molecules present in analyte.
Advantages of HPLC\textsuperscript{16}

a) No requirements of volatile compounds  
b) Compounds with a wide polarity range can be analysed in a single run  
c) Thermolabile compounds can be analysed  
d) Great tool for reaction monitoring  
e) Used for both qualitative and quantitative purpose

Disadvantages of HPLC\textsuperscript{16}

a) Basic instrument is very expensive as compared to other analytical tools  
b) Columns are expensive and have limited operating life

The basic instrument of HPLC consists of solvent reservoirs, pumps, an injector, a column, a detector and a data recorder.

1.2 METHOD DEVELOPMENT: Throughout the drug discovery and drug development paradigm, rugged analytical HPLC methods are developed and are adapted by each development group. At each phase of development, the analyses of a numerous of samples are performed to adequately control and monitor the quality of the prospective drug, excipients and final products. Effective and fast method development is of paramount importance throughout this drug development life cycle. This requires a thorough understanding of HPLC principles and theory.

The following factors should be considered during HPLC method development\textsuperscript{16,17}:

1.2.1 Nature of analyte: Physicochemical properties of the analyte of interest such as solubility, chemical structure, pKa, log P, molecular weight, etc. should be known as it can offer valuable clues for choice of initial conditions for HPLC method. Regular sample can be classified as neutral or ionic. If analyte is neutral, pH of mobile phase will not affect the retention. These samples generally do not require addition of buffers to the mobile phase. Ionic sample are further classified as acids, basis, amphoteric compounds and organic salts. Acidic and basic analytes require addition of buffers to mobile phase. For ionizable compounds, the pKa of the analyte should be known. The optimal pH of the mobile phase is a pH that is at least 1-2 units from pKa of the analyte. Knowledge of log P is useful in determining types of stationary phases and organic content needed to elute the components present in the sample. The solubility of analyte and selection of diluents is of prime importance. The diluent should be close to the starting mobile phase composition to avoid peak distortion. If the analyte is more soluble in the diluent than the starting mobile phase composition,
the compound will tend to reside in the “solvent plug” being injected onto the column resulting in a peak fronting or skewing.

1.2.2 Selection of type of stationary phase: Selection of stationary phase (column) depends on the nature of analyte and the goal of analysis. The columns should be stable within operating mobile phase pH and column oven temperature regions.

Selection of column is based on type of differences that will separate the analyte. For polarity based separations, solubility is a key factor. If analyte is soluble in organic solvents, normal phase columns (polar columns) such as silica, cyano, diol, amine columns are used. Analytes soluble in aqueous solvents should be run on reversed phase columns (non polar columns) such as C18, C8 and phenyl columns. Polarity based separations can be modified by addition of various additives to mobile phase such as strong solvents, buffers and ion pairing agents. For charged molecules ion exchange (cation-exchange or anion-exchange) columns are used. Anion-exchange columns are used for negatively charged analyte while cation-exchange columns are used for analyte with positive charge. For size based separations, either size-exclusion or gel permeation columns are used.

1.2.3 Column dimensions and column packing: Column is a heart of HPLC system where actual separation takes place. For desired separation it is necessary to know column chemistry and to optimize the physical dimensions of the HPLC column hardware.

Column length and diameter: Small column diameters provide higher sensitivity than larger column diameters for the same injected mass because the concentration of the analyte in the mobile phase is higher. Smaller diameter columns also use less mobile phase per analysis because a slower flow rate is required to achieve the same linear velocity through the column. Larger diameter columns provide greater sample loading and lower back pressure. Longer columns often provide increased resolution. Column back pressure for a given flow rate increases as the column length increases and as internal diameter decreases.

Commercially available columns for analytical purpose have following column length and column internal diameter.

Column length: 30 mm to 250 mm
Column internal diameter: 1.0 mm to 4.6 mm
**Particle size and shape:** Particle size for HPLC column packing refers to the average diameter of the packing particles. Stationary phase particles range in size from 1.7 to 25 \( \mu \). The most common particle size is 5 \( \mu \). Commercially columns are available with particle size such as 1.7 \( \mu \), 3 \( \mu \) and 5 \( \mu \) etc. With larger particles, the generated column back pressure will be low. Smaller particles generally provide greater surface area and better separation but produce higher column back pressures. Smaller particles provide not only increased efficiency, but also the ability to work at increased linear velocity without loss of efficiency, providing both resolution and speed. Spherical particles offer reduced back pressure and longer column life when using viscous mobile phases.

**Pore size:** The pore size of packing material is the average size of the pores within each particle. It is generally reported in angstrom. Larger pores permit analyte to be retained longer through maximum exposure to surface area of the particles. Molecular weight of the analyte should be considered while selecting pore size of the stationary phase. For analyte having molecular weight below 3000, a pore size of 100 \( \text{Å} \) or less should be used. For samples having molecular weight in the range of 3,000 - 10,000, a pore size of 100 \( \text{Å} \) - 130 \( \text{Å} \) is used while for those with molecular weight above 10,000, packing material with a pore size of 300 \( \text{Å} \) is recommended.

**Bonding type:** Two types of bonding are available for HPLC columns, monomeric bonding (single point attachment of bonded phase molecule) and polymeric bonding (multi point attachment of bonded phase molecule). Monomeric bonding provides increased mass transfer, higher column efficiency and faster column equilibration. Polymeric bonding provides increased column stability and can accept high sample loading.

**Carbon load:** The carbon load is a measure of the amount of bonded phase bound to the surface of the packing. High carbon loads provide greater column capacities and resolution. Low carbon loads produce less retentive packing and faster analysis times.

**1.2.4 Mobile phase composition:** Commonly used mobile phases for reversed phase HPLC are hydro-organic mixtures mainly methanol and acetonitrile alone, in the form of mixtures or in combination with aqueous phase. Other modifiers used are isopropyl alchol (IPA), tetrahydrofuran (THF) and dimethyl sulfoxide (DMSO). But the problem with these modifiers is that they produce high back pressure and background noise. For normal phase chromatography solvents such as hexane, chloroform,
dichloromethane (DCM), ethyl acetate, etc. are used. The solvents used as mobile phase must be of HPLC grade to minimize contamination and background absorbance. Solvent used for mobile phase can affect sensitivity of detection. Solvent properties such as UV cutoff, refractive index and boiling point are important for UV detector, refractive index detector and evaporating light-scattering detector, respectively. Using polar solvents retention time is increased in reversed phase chromatography and reduced in normal phase chromatography. Pressure drop is proportional to solvent viscosity. Mobile phase viscosity should be minimum to maintain acceptable pressure drop. Use of buffers as mobile phase should be critically evaluated. A buffer concentration in the range of 10 to 50 mM is adequate for the majority of reversed phase applications. Mobile phase should be filtered through 0.2 μ filter before use.¹⁹ ²⁰

1.2.5 Flow rate: The flow rate is defined as volume of mobile phase passed through the column in unit time and is usually measured in ml/min. Flow rate affects HPLC system pressure, chromatographic quality (column efficiency), and analysis time. Therefore, selection of optimum flow rate for given analytical method is essential. Higher flow rates decrease the analysis time but it also increases column back pressure. Increasing flow rate above optimum will also reduce column efficiency and resolution. At higher flow rate, analyte will not get sufficient time to interact with stationary phase. At low flow rate, retention time of analyte is increased and thus analysis time is increased. If flow rate is reduced below optimum for given particle size, it can also reduce column efficiency and resolution due to increased band dispersion.²¹

1.2.6 Column oven temperature: It is necessary to control column oven temperature to produce reproducible data. Retention time of analyte and viscosity of the mobile phase are temperature dependent. Higher temperature decreases retention time and viscosity of the mobile phase. Biological samples such as enzymes or proteins, may not be stable at room temperature or higher temperature. For such samples, column oven temperature should be kept lower.²¹

1.2.7 Injection volume: Injection volume refers to volume of sample injected in HPLC system. For analytical scale HPLC methods, the injection volume should be in the range of microliters. Selection of injection volume depends on the length and internal diameter of the column. Injection volume can also affect the peak width.
1.2.8 Selection of detector: Detectors of HPLC should be sensitive and should produce stable, fast, reproducible and linear response. The commonly used HPLC detectors include UV/visible detector, refractive index detector, fluorescence detector, conductivity detector, IR detector, NMR detector and mass detector.\textsuperscript{22}

**UV/visible detector:** For compounds which absorb UV and visible range. UV/visible detector include three types of detectors.

**Fixed wavelength detector (254 nm or 280 nm):** The analyte must have UV absorbance and mobile phase must have negligible absorbance at the fixed wavelength used. The disadvantage of this detector is that the analyte must have significant absorbance at chosen wavelength. Measurement at other wavelength is not possible.

**Variable wavelength detector:** One can choose wavelength based on absorption maximum of the analyte. Analysis at more than one wavelength is possible.

**PDA detector:** It gives absorbance value at all wavelengths. More useful for sample with unknown absorption spectrum.

**Refractive index detector:** This detector can be very useful for detecting those compounds that are nonionic, do not absorb in the UV, and do not fluoresce. These detectors are ideal for analysis of compounds like sugars, certain grades of polymeric material and aliphatic compounds.

**Fluorescence detector:** For analysis of only those compounds which exhibit fluorescence or have converted to fluorescent derivatives.

**Conductivity detector:** They are used for ionic compounds. For example detector of ion chromatography is conductivity detector.

**Other detectors:** LC-IR, LC-NMR and LC-MS are hyphenated techniques where IR, NMR and MS detectors are used for simultaneous separation and characterization of compounds.

1.2.9 Gradient elution: Many samples which cannot be successfully separated by the use of isocratic elution are separated by using gradient elution. Gradient elution which is also known as solvent programming is defined as change in mobile phase composition with time during chromatographic run. Using gradient elution, improved resolution can be achieved in samples with wide range of retention. It is also useful for separation of high molecular weight compounds. Gradient elution suppresses tailing of basic compounds.\textsuperscript{23,24}
1.2.10 Sample preparation: The sample preparation in HPLC analysis is as significant as the chromatographic separation itself. Sample preparation involves various techniques such as extraction, removal of solids by filtration, centrifugation, concentration procedures, derivatisation, etc. Removal of contaminants is necessary through sample preparation step as contaminants may damage sample valve, column or some other part of chromatograph. Use of devices like pre-column or guard column is useful but this can increase extra column volume leading to peak dispersion and fade resolution. In the absence of guard column, the only way to protect the analytical column is to use proper sample preparation/cleaning procedure before loading the analytical column with sample. 25

1.3 VALIDATION: Validation of analytical method may be viewed as establishment of experimental database which certifies that the developed method performs in suitable manner as per intended application. It is a process of performing several tests designed to assure that an analytical test system is suitable for its intended purpose and is capable of providing useful and valid analytical data. 26

Validation which is an integral part of any good analytical practice is a basic requirement to ensure quality and reliability of the results for all analytical applications. Method validation is required to assure quality of products. Validation helps in achieving acceptance of products by the international agencies. It is mandatory requirement for the purposes of accreditation as per ISO 17025 guidelines and registration of any pharmaceutical product or pesticide formulation. Validated methods are only acceptable for undertaking proficiency testing. 27

Validation Parameters 28

Typical validation parameters which should be considered are:

- Specificity
- Linearity
- Range
- Accuracy
- Precision (Repeatability, Intermediate precision and Reproducibility)
- Limit of detection (LOD)
- Limit of quantitation (LOQ)
- Robustness
- System suitability testing

Development and Validation of HPLC Methods for Marker compounds and Bioactive as per ICH Guidelines
1.3.1 Specificity: Specificity of analytical procedure is the ability to measure accurately and specifically analyte of interest in the presence of other component such as impurities, degradation products, and matrix components which may be expected to be present in the sample. Specificity studies should be conducted during the validation of identification tests (to ensure the identity of the analyte), the determination of impurities (for accurate statement of the content of impurities of an analyte such as related substances, heavy metals, residual solvents, etc.) and the assay (for exact determination of content or potency of the analyte in a sample). Specificity should be one of the first validation parameters. When criteria for specificity are not met that indicates the method is poorly developed.

Methodology: For identification tests, specificity is evaluated by ability of the method to differentiate structurally related compounds or by comparison with known reference standard.

For impurity determination, specificity is evaluated by spiking the analyte with known amount of impurities and determining them with appropriate accuracy and precision. If impurities are not known, results are compared with another well established procedure. For the assay, specificity is evaluated by analysis of spiked samples to ensure that the results are not affected by presence of impurities or excipients.

Documentation: Representative chromatograms with peak labels should be presented along with resolution, number of theoretical plates and tailing factor. The purity of the analyte of interest should be checked with photodiode array or mass detector to ensure that the response is due to only one component.

1.3.2 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 (amount) of analyte in the sample. Linearity is evaluated for assay, quantitative impurity tests and dissolution testing. Linearity is not evaluated for identification test and limit test.

Methodology: Linearity should be demonstrated over the entire range. Minimum five concentrations are recommended.

Documentation: The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included.

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

Methodology: The following range should be considered:

- For the assay of an active substance or a finished product: 80-120% of the test concentration,
- For content uniformity: 70-130% of the test concentration
- For dissolution testing: +/-20% over the specified range
- For the determination of an impurity: from the reporting level of an impurity to 120% of the specification

1.3.4 Limit of Detection: The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated.

Methodology: Based on whether the procedure is a non-instrumental or instrumental, the following procedures are used:

Non-instrumental methods: Eg. Titrations, TLC.

Based on visual evaluation: The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.
Instrumental methods:

Based on signal to noise: detection limit can be made at which signal-to-noise ratio is 3:1 or 2:1

\[ \text{LOD} = \frac{S}{N} = 3 \]

Based on the standard deviation of the response and the slope:

LOD can be calculated using formula: \( \text{LOD} = \frac{3.3 \sigma}{S} \)

Where \( \sigma \) = the standard deviation of the response based on either the standard deviation of blank, the residual standard deviation of regression line or standard deviation of y-intercept of regression line, \( S \) = slop of calibration curve

![Figure 1.2: LOD and LOQ based on standard deviation of blank](image)

![Figure 1.3: LOD and LOQ based on signal to noise ratio](image)
Documentation: LOD should be expressed as concentration of analyte. An appropriate number of samples should be analysed at the limit to validate the level.

1.3.5 Limit of Quantitation: The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitated with acceptable accuracy and precision.

Methodology: Based on whether the procedure is a non-instrumental or instrumental, the following procedures are used:

Non-instrumental methods:
Based on visual evaluation: The quantitation limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably quantitated.

Instrumental methods:
Based on signal to noise: Quantitation limit can be made at which signal-to-noise ratio is 10:1
LOQ=S/N=10

Based on the standard deviation of the response and the slope:
LOQ can be calculated using formula: \( LOQ = 10 \sigma /S \)

Where \( \sigma \) = the standard deviation of the response based on either the standard deviation of blank, the residual standard deviation of regression line or standard deviation of y-intercept of regression line, \( S \) = slop of calibration curve

Documentation: LOQ should be expressed as concentration of analyte. An appropriate number of samples should be analysed at the limit to validate the level.

1.3.6 Accuracy: The accuracy of an analytical procedure expresses the closeness of measured value to true value. Accuracy should be established across the specified range of the analytical procedure.

Methodology: Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g. 3 concentrations/ 3 replicates each of the total analytical procedure).

Drug substance: Accuracy is assessed either by comparing the results with the analysis of reference standard or by comparison to second well established method.

Drug product: Accuracy is assessed either by analyzing synthetic mixture of known amount/ spiked sample or by comparison to second well established method.
Quantitation of impurities: Accuracy should be assessed on samples (substance/product) spiked with known amounts of impurities.

**Documentation:** Accuracy should be reported as percent recovery of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

**1.3.7 Precision:** The precision of an analytical procedure expresses the degree of scatter among individual test results obtained from analysis of multiple samples of the same homogeneous sample. Precision should be evaluated across the specified quantitation range of the method. Precision study is carried out at three levels: repeatability, intermediate precision, and reproducibility.

**Repeatability:** Repeatability which is also known as intra-assay precision, expresses the precision under the same operating conditions over a short interval of time.

**Intermediate precision:** Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

**Reproducibility:** Reproducibility expresses the precision between laboratories.

**Methodology:** Precision is evaluated by assaying individual samples of a homogeneous preparation and calculating relative standard deviation. A minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each) or a minimum of 6 determinations at 100 % of the test concentration should be evaluated.

**Documentation:** The standard deviation, relative standard deviation, and confidence interval should be reported.

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**Figure 1.4: Diagrammatic representation of accuracy and precision**

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Development and Validation of HPLC Methods for Marker compounds and Bioactive as per ICH Guidelines
1.3.8 **Robustness**: The robustness of an analytical method is defined as a measure of its capacity to remain unaffected when small, but deliberate variations in method parameters such as change in flow rate, column temperature, mobile phase composition or pH of mobile phase are done. It should be considered in early stage of method development. If the results are susceptible to parameter variations, these parameters should be adequately controlled and a precautionary statement should be included in the method.

*Methodology:* Robustness is evaluated by purposefully changing method parameters and determining its effect on method result. One parameter is changed at a time.

*Documentation:* The relative standard deviation should be reported.

1.3.9 **System suitability tests**\(^{29}\): These are an integral part of liquid chromatographic methods which ensure both methodology and instrumentation are performing within expectation prior to the analysis of the test samples. This test also ensures that the proposed method provide adequate resolution and reproducibility for the analysis to be done. The main parameters which are evaluated during system suitability test are injection precision, tailing factor, column efficiency and resolution of the critical pair.

*Injection Precision:* Replicate injections of a standard preparation used in the assay. Data from five replicate injections of the analyte are used to calculate the relative standard deviation, (RSD), if the requirement is 2.0 % or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0 %.

*Resolution:* The resolution, \( R \), is a function of column efficiency, \( N \), and is specified to ensure that closely eluting compounds are resolved from each other. It is defined as ratio of difference in retention times of two adjacent peaks to the average peak width.

\[
R_t = \frac{\Delta t_R}{W_{avg}}
\]

\[
W_{avg} = \frac{W_1 + W_2}{2}
\]

*Figure 1.5: Diagrammatic representation of calculation of resolution*
For precise and rugged quantitative analysis requires that resolution should be greater than 1.5. **Column efficiency (N):** Column efficiency is a measure of peak sharpness, which is important for the detection of trace components.

![Diagram of No. Theoretical Plates, N](image)

\[ N = 16\left(\frac{t_R}{W_f}\right)^2 \]

\( t_n \) = retention time, analyte  
\( t_m \) = retention time, non-retained component

**Figure 1.6: Diagrammatic representation of calculation of number of theoretical plates**

**Tailing factor (T):** It is a measure of peak symmetry. For perfectly symmetrical peak the value is unity. Its value increases as tailing becomes more pronounced. The value of tailing factor decreases in case of fronting.

![Diagram of Tailing factor T](image)

\[ T = W_{0.05}/2f \]

Where, \( W_{0.05} \) = peak width at 5% peak height

**Figure 1.7: Diagrammatic representation of calculation of tailing factor**
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f= distance between leading edge of the peak and peak maxima

Acceptable limits: There are numerous guidelines which detail the expected limits for typical chromatographic methods.

According to USP and FDA guidelines the acceptable limits are given below:

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection precision</td>
<td>RSD &lt; 2.0 % for n =6</td>
</tr>
<tr>
<td>Resolution</td>
<td>Rs &gt;2 (Minimum 1.5)</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>T &lt; 2</td>
</tr>
<tr>
<td>Theoretical plate</td>
<td>N &gt; 2000</td>
</tr>
</tbody>
</table>

1.4 REVALIDATION: Revalidation may be necessary in the following circumstances:

a) Changes in the synthesis of the drug substance  
b) Changes in the composition of the finished product  
c) Changes in the analytical process