IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Review Article
June 2021 Vol.:21, Issue:3

Pharmaceutical Analysis and Validation: A Review

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

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Submitted: 25 May 2021
Accepted: 02 June 2021
Published: 30 June 2021


www.ijppr.humanjournals.com

Keywords: HPLC, Pharmaceutical validation, Method development

ABSTRACT

The development of analytical method is of supreme importance during the process of drug discovery, release to market and development, culminating in a marketing approval. HPLC is the dominant separation technique to detect, separate and quantify the drug. HPLC method development depends on chemical structure of the molecules, synthetic route, solubility, polarity, pH and pKa values, and functional groups activity etc. Validation is establishing documented evidence which provides high degree on assurance that a specific process consistently produced a product meeting its predetermined specifications and quality characteristic. Validation of HPLC method as per ICH Guidelines covers all the performance characteristics of validation, like accuracy, precision, specificity, linearity, range and limit of detection, limit of quantification, robustness and system suitability testing.

INTRODUCTION:

Analytical chemistry can be defined as the study of the separation, identification, and quantification of chemical components of natural and artificial materials composed of one or more compounds or elements. Pharmaceutical analysis is extremely important in the examination of pharmaceutical formulations and bulk drugs for quality control and assurance.

The following key points are required for modern pharmaceutical analysis.

1) Validation should focus on the analytical procedure's performance under normal operating conditions.

2) Suitability is inextricably linked to both the requirements and the design of the individual analytical procedure.

3) Consequently, the analyst has to identify relevant parameters which reflect the routine performance of the given analytical procedure, to design the experimental studies accordingly and to define acceptance criteria for the results generated.

4) Acceptance criteria should be absolute, preferably normalized parameters. These can be defined based on regulatory requirements, statistical considerations, or personal experience. Statistical significance tests should be used with caution because they do not consider practical relevance.

5) Validation must not be regarded as a singular event. The analyst is responsible for the continued maintenance of the validated status of an analytical procedure. [(1)]

Analytical Method Development:

Analytical method development is the process of demonstrating that the developed chromatography method is suitable for use in the development and manufacturing of pharmaceutical drug substances and drug products. The following are the basic separation techniques and principles involved in the development of analytical methods using HPLC and UPLC:

1. Selection of chromatography mode
2. Selection of detector
3. Selection of column (stationary phase)

4. Selection and optimization of mobile phase Buffer and its strength
   - Buffer and its strength
   - pH of buffer
   - Mobile-phase composition
5. Selection of organic modifiers
6. Selection of ion-pair reagents
7. Selection of flow rate
8. Selection of solvent delivery system (elution mode)
9. Selection of diluent
10. Methods of extraction
11. Samples to be used
12. Experimentation to finalize the method
13. Selection of test concentration and injection volume
14. Forced degradation studies (stress resting)
15. Evaluation of stress testing
16. Mass balance study
17. Finalization of wavelengths
18. Stability of solution
19. System suitability
20. Robustness of the method
21. Relative response factor
22. Quantification methods [2]

Classification of HPLC:

1. Preparative HPLC and analytical HPLC (based on scale of operation)
2. Affinity chromatography, adsorption chromatography and size exclusion chromatography
3. Ion exchange chromatography, chiral phase chromatography (based on principle of separation)
4. Gradient separation and isocratic separation (based on elution technique)
5. Normal phase chromatography and reverse phase chromatography (based on modes of operation). [3]

Steps involve in Method Development:

1. Understand the physicochemical properties of drug molecule
2. Set up HPLC conditions
3. Preparation of sample solution for method development
4. Method optimization
5. Validation of method

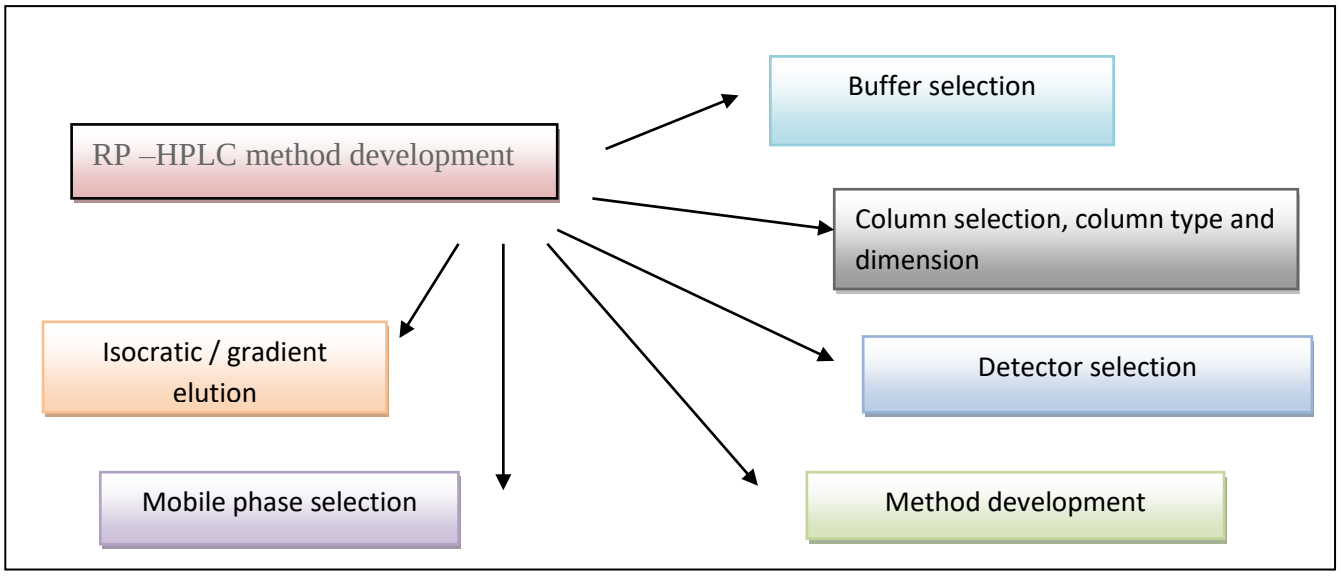

Figure No. 1: Steps involve in Method Development

1. Understand the physicochemical properties of drug molecule

The physicochemical properties of a drug molecule are critical in method development. To develop a method, one must first investigate the physical properties of the drug molecule, such as solubility, polarity, pKa, and pH. A compound's polarity is a physical property. It assists an analyst in determining the solvent and mobile phase composition. In a *nonpolar covalent* bond, the electrons are shared equally between two atoms. A *polar covalent* bond is one in which one atom has a greater attraction for the electrons than the other atom. The solubility of molecules can be explained on the basis of the polarity of molecules. Polar, e.g. water, and nonpolar. e.g. benzene, solvents do not mix.

In general, like dissolves like, which means that materials with similar polarities dissolve in each other. The solubility of the analyte is used to select diluents. The analyte must be soluble in the diluents and must not react with any of them. The pH and pKa values are important in the development of HPLC methods. The pH value is defined as the negative of the logarithm to base 10 of the concentration of the hydrogen ion.

$$pH = -\log 10[H^3O^+]$$

The acidity or basicity of a substance is defined most typically by the pH value. Selecting a proper pH for ionizable analytes often leads to symmetrical and sharp peaks in HPLC. Sharp, symmetrical peaks are necessary in quantitative analysis in order to achieve low detection limits.

2.Set up HPLC conditions

A buffer is a partially neutralized acid that resists pH changes. To partially neutralise the acid, salts such as sodium citrate or sodium lactate are commonly used. The buffer's buffering capacity is its ability to withstand pH changes.

(i) Buffering Capacity increases as the molar concentration (molarity) of the buffer salt/acid solution increases.

(ii) The closer the buffered pH is to the pKa, the greater the Buffering Capacity.

(iii) Buffering Capacity is expressed as the molarity of Sodium Hydroxide required to increase pH by 1.0.

The effect of pH on analyte retention, the type of buffer to use and its concentration, solubility in the organic modifier, and its effect on detection are all important considerations in the development of reversed-phase chromatography (RPC) methods for ionic analytes. An improper choice of buffer, in terms of buffering species, ionic strength and pH, can result in poor or irreproducible retention and tailing in reverse-phase separation of polar and ionizable compound. [4]

2.1 Buffer selection

The pH of the solution is usually the deciding factor when selecting a buffer. The pH range for reversed-phase on silica-based packing is typically 2 to 8. Because buffers control pH best at their pKa, it is critical that the buffer has a pKa close to the desired pH. A rule is to select a buffer with a pKa value of 2 units higher than the desired mobile phase pH.

2.1.1 General considerations during buffer selection

1. Phosphate is more soluble in methanol/water than in acetonitrile/water or THF/water.

2. Some salt buffers are hygroscopic. This may lead to changes in the chromatography (increased tailing of basic compounds, and possibly selectivity differences).

3. Ammonium salts are generally more soluble in organic/water mobile phases·

4. TFA can degrade with time, is volatile, absorbs at low UV wavelengths.

5. Microbial growth can quickly occur in buffered mobile phases that contain little or no organic modifier. This growth will accumulate on column inlets and can damage chromatographic performance.

6. At pH greater than 7, phosphate buffer accelerates the dissolution of silica and severely shortens the lifetime of silica-based HPLC columns. If possible, organic buffers should be used at pH greater than 7.

7. Ammonium bicarbonate buffers usually are prone to pH changes and are usually stable for only 24 to 48 hours. The pH of this mobile phase tends to become more basic due to the release of carbon dioxide.

8. After buffers are prepared, they should be filtered through a 0.2-µm filter.

9. Mobile phases should be degassed. [5]

2.1.2 Buffer Concentration

For small molecules, a buffer concentration of 10-50 mM is usually sufficient. In general, a buffer should not contain more than 50% organic material. This will be determined by the type of buffer as well as its concentration. The most common buffer systems for reversed-phase HPLC are phosphoric acid and its sodium or potassium salts. Sulfonate buffers can replace phosphonate buffers when analyzing organophosphate compounds. [6]

2.2 Mobile phase

2.2.1 Mobile Phase Reservoirs

1. Inert container with inert lines leading to the pump are required.

2. Reservoir filters (2-10 mm) at reservoir end of solvent delivery lines

3. Degassed solvent

- Vacuum filtration

- Sparge with inert gas (N or He)

- Ultrasonic under vacuum

2.2.2 Isocratic elution

A separation that employs a single solvent or solvent mixture of constant composition.

2.2.3 Gradient elution

In this case, two or more solvent systems with significantly different polarities are used. After elution begins, the solvent ratio is varied in a predetermined manner, sometimes continuously and sometimes in a series of steps. Gradient elution improves separation efficiency significantly.

The mobile phase has an impact on resolution, selectivity, and efficiency. The mobile phase in reverse phase chromatography is made up of an aqueous buffer and a non-UV active water miscible organic solvent. The effect of the organic and aqueous phases, as well as the proportions in which they are mixed, will have an impact on the drug molecule analysis. The ionogenic nature of the analyte and the hydrophobicity of the analytes in the mixture

influence the selection of the mobile phase and gradient conditions, respectively. The aqueous buffer has several functions.

The mobile phase protonates free silanols on the column and reduces peak tailing at low pH. Basic analytes are protonated at sufficiently low pH; when ionized, the analyte elutes more quickly but with improved peak shape. Acidic analytes will remain uncharged in buffers with sufficiently low pH, increasing retention. At higher pH, neutral basic compounds are more retained, while ionised acidic compounds elute faster. Peak splitting can occur when the pKa of a compound is similar to the pKa of the buffer and the analyte elutes as both a charged and uncharged species. The pH of a buffer will not change. (07)

2.2.4 Selection of Mobile Phase

The mobile phase has an impact on resolution, selectivity, and efficiency. The composition of the mobile phase (or solvent strength) is critical in RP-HPLC separation. Acetonitrile (ACN), methanol (MeOH), and tetrahydrofuran (THF) are common RP-HPLC solvents with low UV cut-off wavelengths of 190, 205, and 212 nm, respectively. These solvents are water miscible. The best solution is an acetonitrile-water mixture.(08)

Table No. 1: Different compositions of Mobile Phases

Mode	**Solvent type used**	**Type of compound used**
Reversed Phase	Water/Buffer, ACN, Methanol	Neutral or non-ionized compounds which can be dissolved in water/organic mixtures
Ion-pair	Water/Buffer, ACN, Methanol	Ionic or ionizable compounds
Normal Phase	Organic solvents	Mixtures of isomers and compounds not soluble in organic/water mixtures
Ion exchange	Water/Buffer	Inorganic ions, proteins, nucleic acids, organic acids
Size exclusion	Water, tetrahydrofuran, chloroform	High molecular weight compounds

2.3 Selecting an HPLC Column:

The column is the heart of an HPLC system. During method development, changing a column will have the greatest impact on analyte resolution. The best column for an application must take into account stationary phase chemistry, retention capacity, particle size, and column dimensions. The hardware (column housing), the matrix, and the stain are the three main components of an HPLC column.

Propyl (C_3), Butyl (C_4), and Pentyl (C_5) phases are useful for ion-pairing chromatography (C_4) (vide infra) and peptides with hydrophobic residues, and other large molecules. C_3–C_5 columns generally retain non-polar solutes more poorly when compared to C_8 or C_{18} phases. Examples include Zorbax SB-C_3, YMC-Pack C_4, and Luna C5. These columns are generally less stable to hydrolysis than columns with longer alkyl chains. Octyl (C_8, MOS) phases have wide applicability.[9]

2.3.1 Internal Diameter

The internal diameter (ID) of an HPLC column is a critical parameter that affects detection sensitivity and separation selectivity in gradient elution. It also determines how much analyte can be loaded into a column.[10]

2.3.2 Particle size

The stationary phase is usually attached to the outside of small spherical silica particles in traditional HPLC. These silica particles are available in a variety of sizes, with 5 m beads being the most commonly used. Smaller particles typically have more surface area and better separations, but the pressure required for optimal linear velocity increases by the inverse of the particle diameter squared. Larger particles are used in preparative HPLC where column diameters are in range of 5 cm to 30 cm and for non-HPLC application such as solid-phase extraction.[11-12]

2.3.3 Pore size

Pore size of column defines an ability of the analyte molecules to penetrate inside the particle and interact with its inner surface. [13]

2.3.4 Column Temperature

Because temperature can affect selectivity, column temperature control is critical for long-term method reproducibility. A target temperature in the 30-40°C range is usually sufficient for good reproducibility. Temperature has been an underutilized operational parameter in HPLC, despite the potential benefits of higher column temperatures, particularly enhanced kinetic and transport properties. In most instances, the objective of using elevated or high temperature is to increase the speed of separation to obtain higher efficiencies and faster results, though there are some situations where selectivity can be manipulated through change of temperature. Temperature-programmed HPLC can be used as an alternative to using solvent gradient elution for variation of solvent strength during the run, and this is expected to be of particular utility with small-bore columns which have low thermal mass. A number of papers have considered the effect of change of temperature on retention. The effect of temperature on retention factor k can be described by the Van't Hoff equation, the retention factor decreases with increase of temperature.[7]

2.4 Selection of detector

The detector is a critical component of HPLC. The chemical nature of the analytes, potential interference, detection limit required, availability and/or cost of the detector all influence detector selection. UV-Visible detector is a dual-wavelength absorbance detector for HPLC. This detector provides the high sensitivity required for routine UV-based applications such as impurity identification at low levels.

Advanced optical detection for Waters analytical HPLC, preparative HPLC, or LC/MS system solutions. Its integrated software and optics innovations deliver high chromatographic and spectral sensitivity. Refractive Index (RI) Detector offers high sensitivity, stability and reproducibility, which make this detector the ideal solution for analysis of components with limited or no UV absorption. Multi-Wavelength Fluorescence Detector offers high sensitivity and selectivity fluorescence detection for quantitating low concentrations of target compounds.[14-15]

Table No. 2: Types of Detectors with applications

Detector	**Type of compound can be detected**
UV-Visible &Photodiode array	Compounds with chromophores, such as aromatic rings or multiple alternating double bonds.
Fluorescence detector	Fluorescent compounds, usually with fused rings or highly conjugated planer system.
Conductivity detector	Charged compounds, such as inorganic ions and organic acid.
Electrochemical detector	For easily oxidized compounds like quinines or amines
Refractive Index detector & Evaporative light scattering detector	Compounds that do not show characteristics usable by the other detectors, eg. polymers, saccharides.

3. Sample preparation

Sample preparation is an important part of HPLC analysis because it ensures that the solution is reproducible and homogeneous enough to be injected onto the column. The goal of sample preparation is to create a sample aliquot that is relatively free of interferences, will not damage the column, and is compatible with the intended HPLC method, that is, the sample solvent will dissolve in the mobile phase with the intended HPLC method.

4. Method optimization

Identify the method's "weaknesses" and optimize the method using experimental design. Understand how the method performs under different conditions, with different instrument setups, and with different samples.[16]

5. Method Validation

Validation is the examination and provision of objective evidence that the specific requirements for a specific intended use are met. A method of assessing method performance and demonstrating that it meets a specific requirement. In other words, it understands what your method is capable of delivering, especially at low concentrations.

Pharmaceutical Validations:

The validation (evaluation of suitability) of an analytical technique is a procedure aimed at obtaining experimentally justified evidence of this technique's ability to produce results with the required accuracy and precision. Validation is required for all analytical techniques used development of pharmaceuticals and the determination of their quality characteristics. Figure 1 show the general scheme of evaluation of the suitability of an analytical procedure, which takes into account specific features of HPLC. In the case of using methods stipulated and described in the State Pharmacopoeia, it is not necessary to evaluate their suitability, provided that the analyses are conducted with strict observation of the text of each particular article. In most other cases, especially in cases of modification of the drug composition, the scheme of synthesis, or the analytical procedure, it is necessary to re-evaluate the suitability of the analytical techniques.[17]

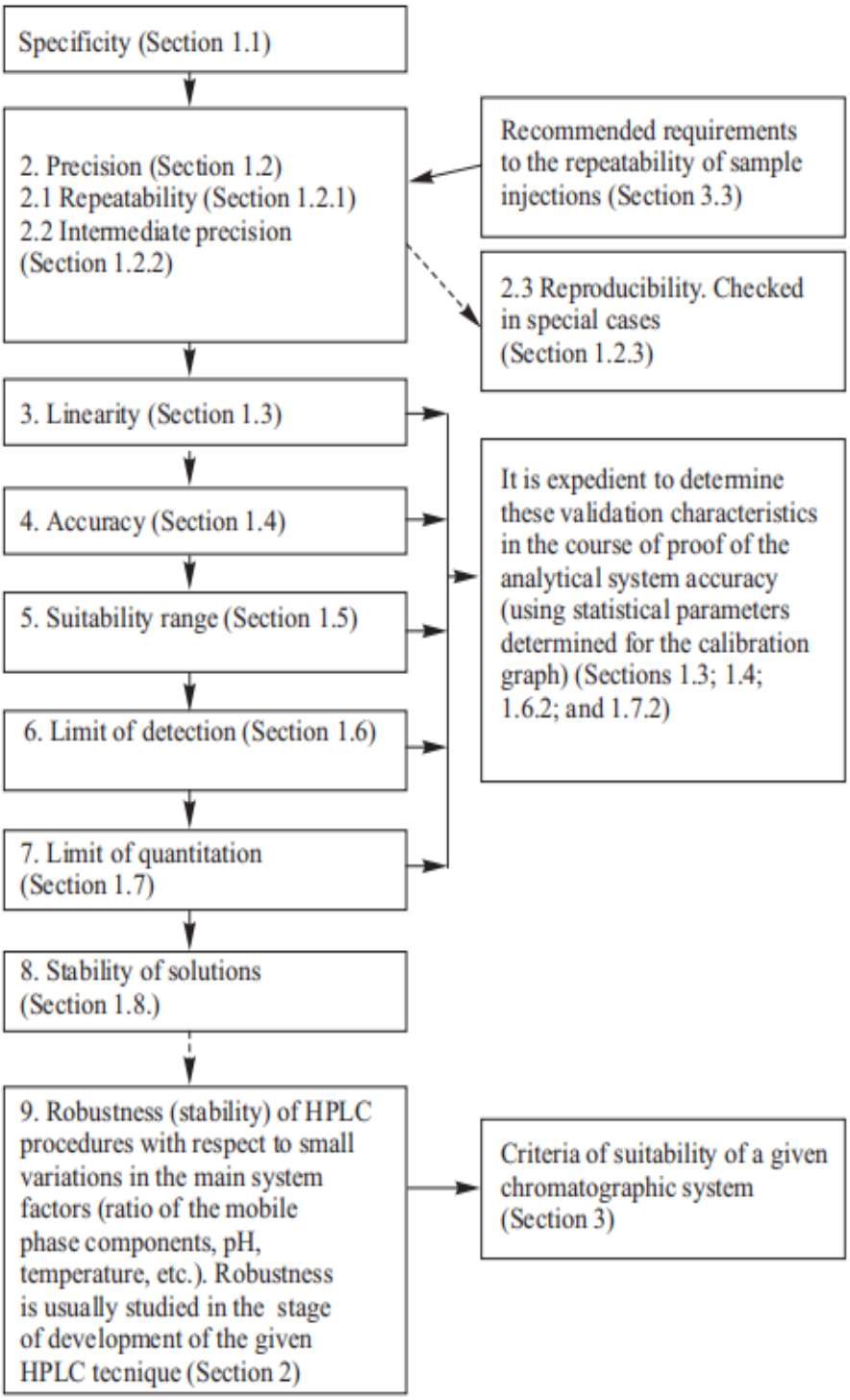

Figure No. 2: General scheme of validation of HPLC based analytical procedure.

Table no. 3: Validation characteristics according to the United States Pharmacopoeia (2003)

Characteristic	Category				
	I	II		III	IV
		Quantitative tests	Limiting tests		
Accuracy	Yes	Yes	MB	MB	No
Precision	Yes	Yes	No	Yes	No
Specificity	Yes	Yes	Yes	MB	Yes
Limit of detection	No	No	Yes	MB	No
Limit of quantitation	No	Yes	No	MB	No
Linearity	Yes	Yes	No	MB	No
Suitability range	Yes	Yes	MB	MB	No

Note: Yes = usually studied; No = usually not studied; MB= may be required;

I = Methods for determination of content of main component

II = Method for determination of impurities and decomposition products

III = Method for determination of the parameters of dissolution, drug release, etc.

IV = Includes identification tests

Importance of Validation

1. Assurance of quality

2. Time bound

3. Process optimization

4. Reduction of quality cost

5. Nominal mix-ups and bottle necks

6. Minimal batch failures, improved efficiently and productivity

7. Reduction in rejections

8. Increased output

9. Avoidance of capital expenditures

10. Fewer complaints about process related failures

11. Reduced testing in process and in finished goods

12. More rapid and reliable start-up of new equipments

13. Easier scale-up from development work

14. Easier maintenance of equipment

15. Improved employee awareness of processes

16. More rapid automation

17. Government regulation (Compliance with validation requirements is necessary for obtaining approval to manufacture and to introduce new products). [18]

Need of Pharmaceutical Validation:

Validation is an essential component of quality assurance; it entails conducting a systematic examination of systems, facilities, and processes to determine whether they perform their intended functions adequately and consistently as specified. A validated process is one that has been shown to provide a high level of assurance that uniform batches that meet the required specifications will be produced. Validation in itself does not improve processes but confirms that the processes have been properly developed and are under control.[18]

Scope of Validation:

Pharmaceutical Validation is a broad field that encompasses almost every aspect of pharmaceutical processing activities, making defining the Scope of Validation a difficult task. A systematic examination of pharmaceutical operations, on the other hand, will reveal at least the following areas for pharmaceutical validation.

1. Instrument Calibration
2. Process Utility services
3. Raw materials
4. Packaging materials

5. Equipment
6. Facilities
7. Manufacturing operations
8. Product Design
9. Cleaning
10. Operator [19]

Analytical Method Validation:

Method validation is the process of "creating documented evidence" that provides a high level of assurance that the product (equipment) will meet the requirements for the intended analytical applications. [18]

Types of analytical procedures to be validated:

Discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures:

1. Identification tests
2. Quantitative tests for impurities content
3. Limit tests for the control of impurities
4. Quantitative tests of the active moiety in samples of drug

Parameters for Method Validation

The various validation parameters are

1. Accuracy
2. Precision (repeatability and reproducibility)
3. Linearity
4. Range
5. Limit of detection (LOD)

6. Limit of Quantitation (LOQ)

7. Selectivity/ specificity

8. Robustness

9. Ruggedness

10. System Suitability Studies

1.Accuracy

The accuracy of an analytical procedure expresses the degree of agreement between the value accepted as a conventional true value or an accepted reference value and the value discovered. This is referred to as "truthfulness" at times. Accuracy should be established across the analytical procedure's specified range. Accuracy should be evaluated using a minimum of 9 determinations spread over a minimum of 3 concentration levels covering specified range.

2.Precision

An analytical procedure's precision expresses the degree of agreement (degree of scatter) between a series of measurements obtained from multiple samplings of the same homogeneous sample under the prescribed conditions. Precision is classified into three categories: repeatability, intermediate precision, and reproducibility.

a. Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time.

Repeatability should be assessed using:

a) A minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each); or

b) A minimum of 6 determinations at 100% of the test concentration.

b. Intermediate precision

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

c. Reproducibility:

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology). Reproducibility is assessed by means of an inter-laboratory trial.[18]

Accuracy and precision are not the same, as the diagram below indicates a method can have good precision and yet not be accurate. [22]

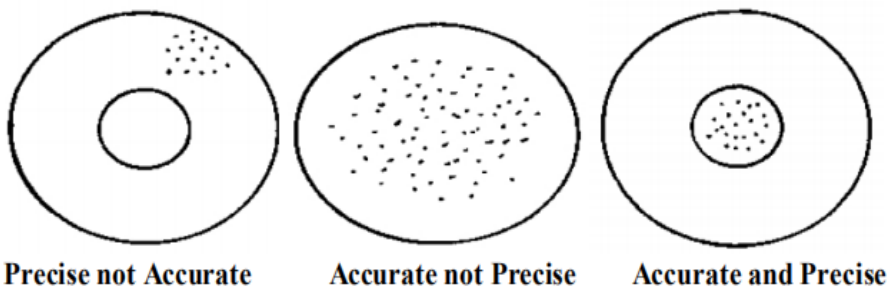

Figure No. 3: Example for Accuracy and precision

3. Specificity (Selectivity):

Specificity is the ability to assess the analyte unequivocally in the presence of components that are expected to be present. Specificity is defined by an ICH guideline as the ability to assess the analyte unequivocally in the presence of other compounds that are likely to be present. Typically, these are impurities, degradants, matrix, and so on. The following are the implications of the definition:

1. Identification test: Identification tests should be able to differentiate compounds of closely related structure which are expected to be present i.e., to assure identity of an analyte.

2. Purity test: To ensure that the analytical procedure performed allows an accurate statement of content of the impurity of an analyte i.e. related substances, residual solvents content, heavy metals, etc.

3. Assay: To arrive at an accurate result, this permits a correct report on the potency or content of analyte in a sample. [20]

4. Linearity

The linearity of an analytical procedure refers to its ability (within a given range) to produce test results that are directly proportional to the concentration (amount) of analyte in the sample. Linearity should be assessed visually by inspecting a plot of signals as a function of analyte concentration or content.

5. Range

The range of an analytical procedure is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which the analytical procedure has been demonstrated to have a suitable level of precision, accuracy, and linearity. [21]

It is based on a linear or nonlinear response curve (i.e., when more than one range is involved). [22]

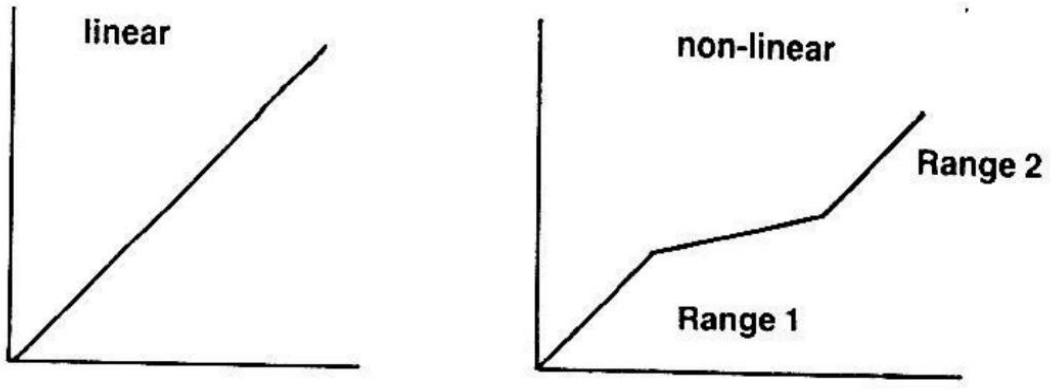

Figure No. 4: Linearity graphs

The range is normally expressed in the same units as the test results (for example percentage, parts per million) obtained by the analytical method.

a. For Assay - 80 to 120% of test concentration

b. Content uniformity - 70 to 130% of test concentration

c. Dissolution - Q-20% to 120%

d. Impurities - reporting level - 120% of impurity specification limit

e. Assay & Impurities - Reporting level to 120% of assay specific. [21]

6. Detection Limit (LOD)

An individual analytical procedure's detection limit is the smallest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. Depending on whether the procedure is non-instrumental or instrumental, there are several approaches to determining the detection limit. Alternative approaches to those listed below may be acceptable.

a. Based on Visual Evaluation

b. Based on Signal-to-Noise

c. Based on the Standard Deviation of the Response and the Slope

The detection limit (DL) may be expressed as:

$$DL = 3.3\ \sigma/S$$

Where σ = the standard deviation of the response,

S = the slope of the calibration curve.

7. Quantitation Limit (LOQ)

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

Approaches other than those listed below may be acceptable.

a. Based on Visual Evaluation

b. Based on Signal-to-Noise Approach

c. Based on the Standard Deviation of the Response and the Slope

The quantitation limit (QL) may be expressed as:

$$QL=10\ \sigma/S$$

Where σ = the standard deviation of the response,

S = the slope of the calibration curve.

8. Robustness

The robustness of an analytical procedure is a measure of its ability to remain unaffected by small but deliberate variations in method parameters, and it provides an indication of its dependability under normal conditions.

9. Ruggedness

Ruggedness is a measure of the reproducibility of test results under conditions that vary from laboratory to laboratory and from analyst to analyst. The robustness of an analytical method is the degree of reproducibility of test results obtained by analyzing the same samples under different conditions, such as different laboratories, analysts, instruments, reagents, and temperatures.[18]

10. System Suitability

System suitability testing was originally thought by the pharmaceutical industry to determine whether a chromatographic system is being used in pharmaceutical laboratories today in a routine manner where quality of results is most important which is suitable for a definite analysis.

The parameters used in the system suitability tests (SST) report are as follows:

a. Number of theoretical plates or Efficiency (N)

b. Capacity factor (K)

c. Separation or Relative retention (α)

d. Resolution (Rs)

e. Tailing factor (T)

f. Relative Standard Deviation (RSD)

a. Number of theoretical plates/Efficiency (N)

Efficiency in a specified column is defined as the measurement of the degree of peak dispersion, and it must have column characteristics. The efficiency is expressed as the number of theoretical plates. The formula for calculating N is shown in the diagram below.

Half height method

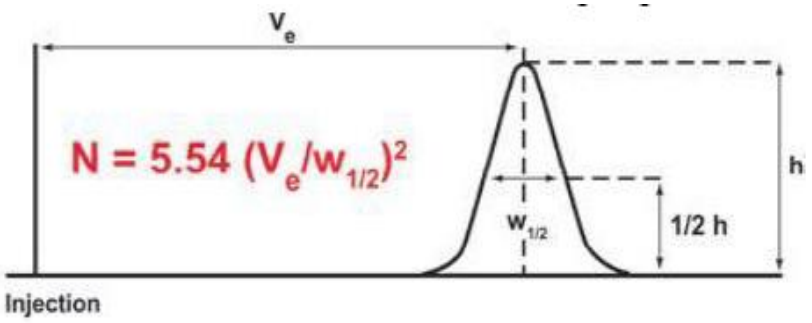

Figure No. 5: Half height method

N = Efficiency / Number of theoretical plates

Ve = Retention time of analyte

h = Height of the peak

w 1/2 = Gaussian function of the peak width at the half- height

- **Sigma/tangential method (USP method)**

With the help of sigma/tangential method N is calculated which is shown in the following figure 1.2 duly noting the formula for calculation of N.

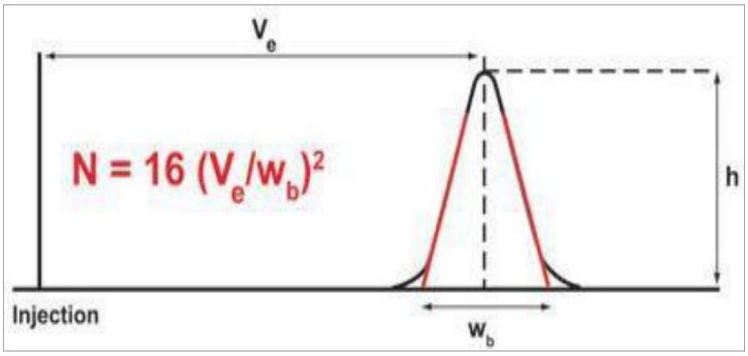

Figure No. 6: Sigma/tangential method relating to determination of N

N = Number of theoretical plates

Ve = elution volume, retention time or retention distance (mL, sec, or cm)

h = peak height

wb = width of the peak at the base line (mL, sec, or cm)

The plate number is determined by the length of the column. The theoretical plate number is a metric for column efficiency. According to plate theory, the analyte will be in instant equilibrium with the stationary phase, and the column must be divided into a number of hypothetical plates, each of which has a fixed height and where the analyte spends a finite amount of time. The height equivalent to the theoretical plate (HETP) is granted.

$$HETP = L/N,$$

Where, L = length of column

N = plate number

b. Capacity ratio or Capacity factor

$$k' = t_R - t_M / t_M$$

The above-mentioned capacity factor is also known as a retention factor because it has no dimension and is independent of mobile phase flow rate as well as column dimensions, and it is a measure of the extent of retention relating to an analyte relative to an un-retained peak. Where tR is the retention time of the sample peak and tM is the retention time of an unretained peak. k' = 0 indicates that no compound is left in the sample.

c. Relative retention or separation factor

$$\alpha = t_2 - t_a / t_1 - t_a$$

α= Relative retention

t_2= Retention time calculated from point of injection

t_a= Unretained peak time (Retention time (tR) of an inert component not retained by the column)

t_1= the retention time from the point of injection of reference peak defined. (Suppose no reference peak is found, value would be zero).

d. Resolution (Rs)

Resolution is the ability of a column to separate two drugs in two separate peaks or chromatographic zones, and it can be improved by increasing column length, decreasing particle size, increasing temperature, and changing the eluent or stationary phase. It can be expressed in terms of the tangential width average of two peaks divided by the distance between their apexes. By using the following formula resolution is calculated.

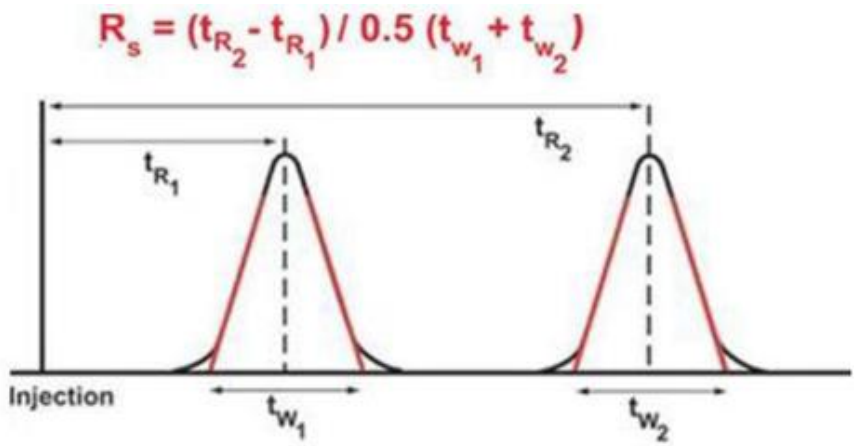

Figure No. 7: Determination of resolution between two peaks.

tR1 and tR2 are the retention times for the two peaks of components. tw1 and tw2 = At the baseline lies between tangents drawn to the sides of the peaks. (Tangents are drawn at 0.6 times the peak height). If the peaks are correctly symmetric, provided the valley between the two peaks should touch the baseline Rs is 1.5. Generally, good value of resolution is Rs ≥2 should be adequate and preferred normally.

e. Resolution factor (R)

Resolution is a function of capacity factor, selectivity, and efficiency (or number of theoretical plates) (N). To separate any two peaks, you must have the appropriate capacity factor, ideally between 2 and 10, as well as adequate selectivity, ideally 1.2, and sufficient efficiency, i.e., the number of theoretical plates (more than 2000 theoretical plates). Resolution must be made. Resolution should be ≥ 1.5. 1.5 defines baseline resolution. [20]

$$R = \frac{k'}{1+k'} \times \frac{\alpha - 1}{\alpha} \times \sqrt{\frac{N}{4}}$$

Figure no. 8: Resolution Factor.

f. Tailing factor or Asymmetry factor

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

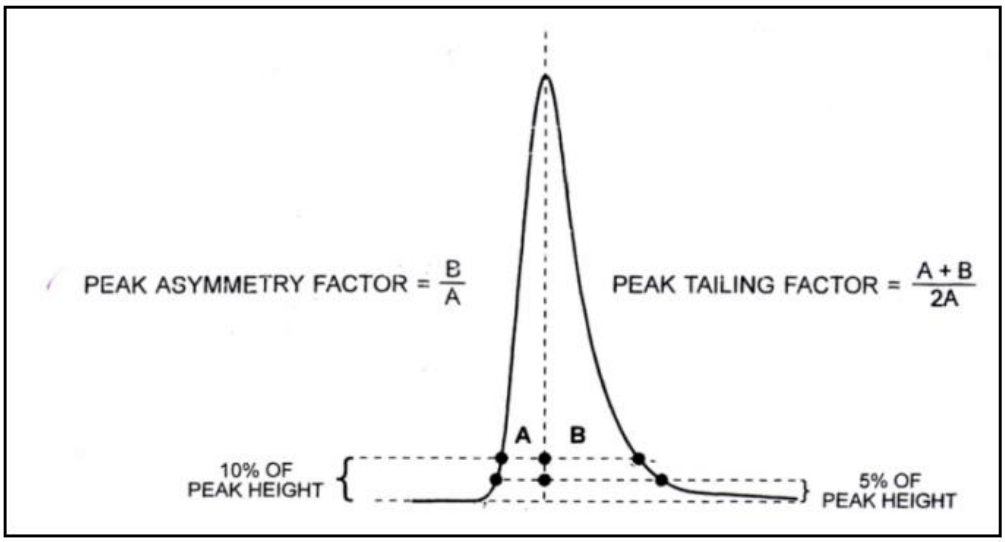

Figure no. 9: Peak asymmetry and peak tailing factor

Asymmetry factor is calculated by,

Asymmetry factor = B/A

Where B = Peak half width

A = Front half width

Good column produce peak with As values of 0.95 to 1%

(Exactly symmetrical peaks have an As value of 1.0%)[22]

Acceptance criteria:

Acceptance criteria (limits) of system suitability parameters are shown in the following Table: [20]

Table No. 4: Acceptance criteria (limits) of system suitability parameters.

S. No.	Parametric name	Acceptance criteria
1	Number of theoretical plate or Efficiency (N)	> 2000
2	Capacity factor (K)	< 1
3	Separation or Relative retention (α)	> 1
4	Resolution (Rs)	> 1.5
5	Tailing factor or Asymmetry (T)	< 2
6	Relative Standard Deviation (RSD)	< 2

CONCLUSION:

Analytical method development and validation playing a fundamental role in pharmaceutical industry for releasing the commercial batch and long term stability data therefore, the data must be produced to acceptable scientific standards. The aim of this article is to provide simple to use approaches with a correct scientific background to improve the quality of the analytical method development and validation process. The selection of Column, buffer, detector and wavelength and another conditions composition (organic and pH) plays a dramatic role on the separation selectivity The advantages of HPLC technique were high selectivity, sensitivity, economic, less time consuming and low limit of detection. Final optimization can be performed by changing the gradient slope, temperature and flow rate as well as the type and concentration of mobile-phase modifiers. Optimized method is validated with various parameters (e.g. specificity, precision, accuracy, detection limit, linearity, etc.) as per ICH guidelines.

ACKNOWLEDGEMENT:

The authors of this review article are thankful to the Principal, Government College of Pharmacy, Karad for allowing us to present this paper. We are also thankful to AICTE for providing research fellowship to complete our research work and convert our research work into publications.

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