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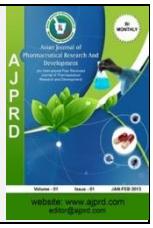

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Review Article

A Review on Validation of RP-HPLC Method for the Simultaneous Estimation

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A B S T R A C T

The development of the pharmaceuticals brought a revolution in human health. These pharmaceuticals would serve their intent only if they are free from impurities and are administered in an appropriate amount. To make drugs serve their purpose various chemical and instrumental methods were developed at regular intervals which are involved in the estimation of drugs. These pharmaceuticals may develop impurities at various stages of their development, transportation and storage which makes the pharmaceutical risky to be administered thus they must be detected and quantitated. For this analytical instrumentation and methods play an important role. This review highlights the role of the analytical instrumentation and the analytical methods in assessing the quality of the drugs. The review highlights a variety of analytical techniques such as titrimetric, chromatographic, spectroscopic, electrophoretic, and electrochemical and their corresponding methods that have been applied in the analysis of pharmaceuticals.

Key words: Chromatographic, HPLC, Analytical Techniques.

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INTRODUCTION

Pharmaceutical Analysis may be defined as the application of analytical procedures used to determine the purity, safety and quality of drugs and chemicals. The pharmaceutical analysis comprises the procedures necessary to determine the "identity, strength, quality and purity" of such compounds. It also includes the analysis of raw material and intermediates during manufacturing process of drugs.

TYPES [1]

Pharmaceutical analysis includes both qualitative and quantitative of drugs and pharmaceutical substances starts from bulk drugs to the finished products.

Qualitative analysis

Qualitative inorganic analysis seeks to establish the presence of a given element or inorganic compound in a sample.

Qualitative organic analysis seeks to establish the presence of a given functional group or organic compound in a samples

Quantitative analysis[2]

Quantitative analysis seeks to establish the amount of a given element or compound in a sample.

Importance of analytical chemistry is to gain information about the qualitative and quantitative composition of substance and chemical species, that is, to find out what a substance is composed of and exactly how much it is present For the past two decades, the pharmaceutical analyst has been a spur in development of analytical techniques for various medicinal principles both in pure and combined state and also in finished products. Some of the methods shine in modern technological and electronics and microprocessor-based developments have really shown faster and best results with more accuracy than the conventional methods.

There are various methods used for quantitative analysis of mixtures. One of them is spectrophotometry, which utilizes the measurement of intensity of electromagnetic radiation emitted or absorbed by the analytes. Another technique which has gained large popularity during last decade is high performance liquid chromatography. The technique is very much useful to pharmaceutical analyst in analyzing complex formulation containing number of ingredients as it permits simultaneous separation and determination of components of mixture.

METHODS OF ANALYSIS

Generally analytical methods are classified as follows:

1. Chemical methods
2. Instrumental methods

Chemical methods

1. Volumetric method
2. Gravimetric method

Instrumental methods:

The analytical chemist, to save time, to avoid chemical separation or to obtain increased accuracy may use instrumental methods. This can be classified into:

1. Spectrophotometric methods – UV, IR, NMR etc.
2. Fluorimetric methods
3. Polarimetric method
4. Flame photometric method
5. Turbidimetric method
6. Chromatographic method
7. Refractrometric method
8. Thermal method
9. Electro chemical method

INTRODUCTION TO CHROMATOGRAPHY[3]

The word is obtained from the Greek word *chroma* + *graphia*, literally "colour writing". Chromatography is a technique for analyzing mixtures of gases, liquids or solutes by exploiting differences in their distribution between a stationary and a mobile phase. Chromatography is a fundamental technique in the detection, identification and quantization of chemical species. It comes in two basic formats, planar and column chromatography.

Classification of chromatography based on stationary phase

Planar Chromatography

The origins of planar chromatography can be traced back to the practice amongst dyers of testing pigments by placing a drop on paper and observing the colours as the drop spreads. The scientific use of paper chromatography can be traced to the mid-19th century. The important separation of amino acids and peptides by paper chromatography was developed in 1994 by Consden, Gordon, and Martin. Thin layer chromatography (TLC) was developed in 1938 by Izmailov and Shraiber based on Mikhail Tswett's earlier description of column chromatography.

Column Chromatography

Russian botanist Mikhail Tswett's invented column chromatography in 1906 as a means of studying plant pigments, but it soon became clear that the technique provided a means for separating many complex homogeneous mixtures into their individual components. Today instrumental chromatographic techniques are essential tools in areas such as chemistry, biology, medicine, forensic science, manufacturing and the environment.

Table: 1 Classification of column chromatographic methods [2]

General Classification	Specific Method	Stationary Phase	Type of Equilibrium
Liquid Chromatography (LC) (mobile phase: liquid)	Liquid-Liquid or partition	Liquid adsorbed on a solid	Partition between immiscible liquids
	Liquid-Bonded phase	Organic species bonded to a solid surface	Partition between liquid and bonded surface
	Liquid-Solid or adsorption	Solid	Adsorption
	Ion exchange size exclusion	Ion-exchange resin Liquid in interstices of a polymeric solid	Ion exchange Partition/sieving
Gas Chromatography (GC) (mobile phase: gas)	Gas-liquid	Liquid adsorbed on a solid	Partition between gas and liquid
	Gas-bonded phase	Organic species bonded to a solid surface	Partition between liquid and bonded surface
	Gas-Solid	Solid	Adsorption
Supercritical-fluid chromatography (SFC) (mobile phase: Super critical fluid)		Organic species bonded to a solid surface	Partition between supercritical fluid and bonded surface

Classification of chromatography based on mobile Phase

Chromatographic methods are also classified depending upon the medium of the mobile phase. There are two methods under this category:

Gas chromatography

Gas chromatography (GC) is based on a partition equilibrium of analyte between a solid stationary phase (often a liquid silicone-based material) and a mobile gas (most often Helium). The stationary phase is adhered to the inside of a small-diameter glass tube (a capillary column) or a solid matrix inside a larger metal tube (a packed column). It is widely used in analytical chemistry; though the high temperatures used in GC make it unsuitable for high molecular weight biopolymers or proteins (heat will denature them), frequently encountered in biochemistry, it is well suited for use in the petrochemical,environmental monitoring and remediation, and industrial chemicalfields. It is also used extensively in chemistry research.

Liquid chromatography

Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid. Liquid chromatography can be carried out either in a column or a plane. Present day liquid chromatography that generally utilizes very small packing particles and a relatively high pressure is referred to as high performance liquid chromatography(HPLC).

Supercritical Fluid Chromatography (SFC)

SFC is aseparation technique in which the mobile phase is a fluid above and relatively close to its critical temperature and pressure. SFC is a form of normal phase chromatography that is used for the analysis and purification of low to moderate molecular weight, thermally labile molecules. It can also be used for the separation of chiralcompounds. Principles are similar to those of high performance liquid chromatography(HPLC), however SFC typically utilizes carbon dioxideas the mobile phase; therefore the entire chromatographic flow path must be pressurized.

Classification of chromatography based on the mechanism of separation[4]

Adsorption chromatography

It involves the interaction between the sample molecule and the stationary phase. It is a competitive situation in which the molecules of the mobile phase and the solute are in competition for discrete adsorption sites on the surface of the column. Interaction between a solute molecule and the adsorbent surface is optimum when solute functional groups exactly overlap these adsorption sites. The adsorbent may be packed in a column e.g. silica, alumina.

The separation is achieved by changes in the composition of mobile phase. It is used for analysis of non-ionizing, water insoluble compounds.

Bonded phase chromatography

It is widely used column packing for liquid-liquid partition chromatography with chemically bonded, organic stationary phases. Partition occurs between the bonded phase and a mobile liquid phase. Bonded phase supports are made from silica by the covalent attachment of an organic hydro carbon moiety to the surface. A stationary phase chemically bonded to a support that is used for the separation. It is the most commonly used LC mode. The most popular support used is micro particulate silica gel. An organosilane, such as octadecyl (for reversed-phase chromatography) is the most accepted type of bonded phase.

Normal phase chromatography

Also known as Normal phase HPLC (NP-HPLC), in this method separation of analyte is based on polarity, it was the first kind of HPLC that chemists developed. NP-HPLC uses a polar stationary phase and a non-polar mobile phase and works effectively for relatively polar analyte. The polar analyte associates with and is retained by the polar stationary phase. Adsorption strength increase with increased analyte polarity and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time.

Size exclusion chromatography (SEC)

It is a chromatographic method in which particles are separated based on their size or in more technical terms their hydrodynamic volume. It is usually applied to large moleculesor macromolecular complexes such as proteins and industrial polymers. When an aqueous solution is used to transport the sample through the column the technique is known as gel filtration chromatography. The name gel permeation chromatography is used when an organic solvent is used as a mobile phase. The main application of gel filtration chromatography is the fractionation of proteins and other water-soluble polymers, while gel permeation chromatography is used to analyze the molecular weight distribution of organic soluble polymers. Polymer chemists typically use either silica or cross linked polystyrene medium under a higher pressure. This media are known as the stationary phase.

Ion-exchange chromatography

It is a process that allows the separation of ions and polar molecules based on the charge properties of the molecules. It can be used for almost any kind of charged molecule including large proteins, small nucleotides and amino acids. It is often used in protein purification, water analysis and quality control. Ion exchange chromatography retains analyte molecules based on coulomb(ionic) interactions. The stationary phase surface displays ionic functional groups (R-X) that interact with analyte ions of opposite charge. This type of chromatography is further subdivided into cation exchange chromatography and anion exchange chromatography. The ionic compound consisting of the cationic species M+ and the anionic species B- can be retained by the stationary phase.

Bio-affinity chromatography

This chromatographic process relies on the property of biologically active substances to form stable, specific and reversible complexes. The formation of these complexes involves the participation of common molecular forces such as the Waals interaction, electrostatic interaction, dipole-dipole interaction, hydrophobic interaction and the hydrogen bond. An efficient bio specific bond is formed by a simultaneous and concerted action of several of these forces.

Hydrophobic Interaction Chromatography (HIC)

Hydrophobic Interaction Chromatography is a separation technique uses the properties of hydrophobic to separate proteins from one another. In this type of chromatography, hydrophobic groups such as phenyl, octyl or butyl are attached to the stationary column. Proteins that pass through the column that have hydrophobic amino acid side chains on their surfaces are able to interact with and bind to the hydrophobic groups on the column. HIC separations are often designed using the opposite conditions of those used in ion exchange chromatography. In this separation, a buffer with a high ionic strength, usually ammonium sulfate is initially applied to the column. The salt in the buffer reduces the solvation of sample solutes thus as solvation decreases, hydrophobic regions that become exposed are adsorbed by the medium. The stationary phase is designed to form hydrophobic interactions with other molecules.

Selection of suitable method for estimation of drug in dosage forms is an impending challenge for an analytical chemist. The method so selected should provide analytical data as accurate as required, technically sound, defensible with low level of uncertainty and above all amenable to routine laboratory use and capable of giving reproducible results. HPLC has become the back bone of the biotechnology and pharmaceutical industries where it is used to identify, characterize and purify molecules at all stages of a process, from R&D to quality assurance and validation.

Why HPLC having high importance compared with other techniques?

Different types of chromatographic techniques are available to analyze the samples, but one of the technique very familiar, is High Performance Liquid Chromatography. HPLC technique is not only useful for separation, but also useful for qualifying (identifying) and quantifying the small and neutral molecules also. A few microgram of sample (at the extreme, even less than a nanogram) is enough to ensure the required accuracy. Secondly, HPLC separations are usually relatively fast, precise, accurate and an analysis can be completed in short span possibly in a few seconds. Another advantage of these techniques is relative simplicity and ease of operation compared with other instrumental techniques. If the established procedure is well controlled and the apparatus is maintained under calibrated condition, good accuracy and precision can be achieved.

Analytical Methods Development

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs.

Pharmaceutical products formulated with more than one drug, typically referred to as combination products, are intended to meet previously unmet patients need by combining the therapeutic effects of two or more drugs in one product. These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods.

Basic criteria for new method development of drug analysis:

- The drug or drug combination may not be official in any pharmacopoeias,
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations,
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients,
- Analytical methods for the quantitation of the drug in biological fluids may not be available,
- Analytical methods for a drug in combination with other drugs may not be available,
- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.

HPLC Methods of Analysis for Drugs in Combination

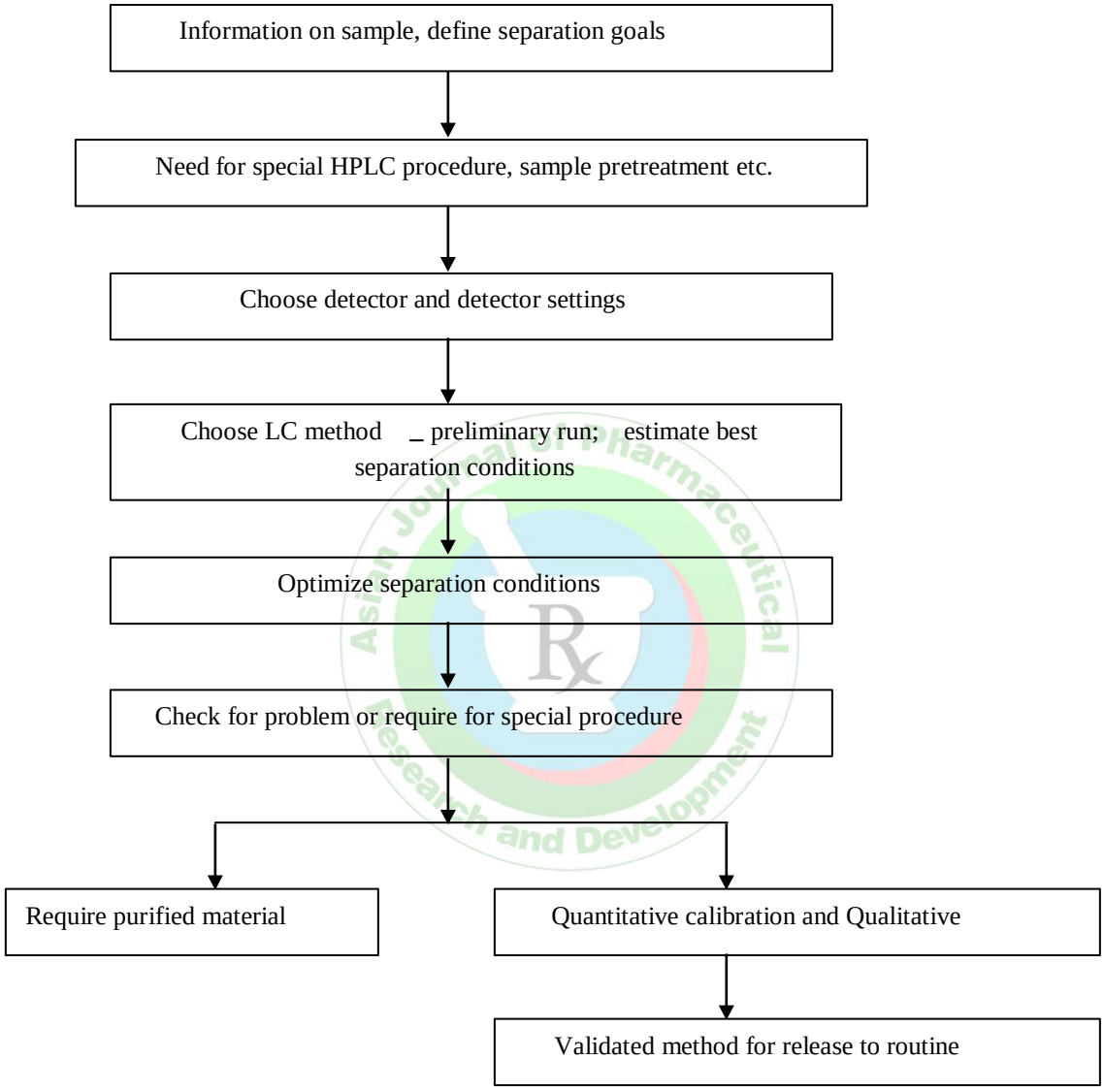
Most of the drugs in multi-component dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures. Some of the advantages are:

- Speed (analysis can be accomplished in 20 minutes or less),
- Greater sensitivity (various detectors can be employed),
- Improved resolution (wide variety of stationary phases),
- Reusable columns (expensive columns but can be used for many analysis),
- Ideal for the substances of low volatility,

- Easy sample recovery, handling and maintenance,
- Instrumentation tends itself to automation and quantitation (less time and less labor),
- Precise and reproducible,
- Calculations are done by integrator itself,
- Suitable for preparative liquid chromatography on a much larger scale.

HPLC Method Development [5]

HPLC method development seems complex. The process is influenced by the nature of the analytes and generally involves the following steps:



Instrumentation of HPLC[2,3,4]

The general instrumentation for HPLC incorporates the following components,

- Solvent reservoir.
- Pump.
- Sampling valves or loops
- Guard column
- Pressure gauge
- Analytical column
- Detector
- Data acquisition system

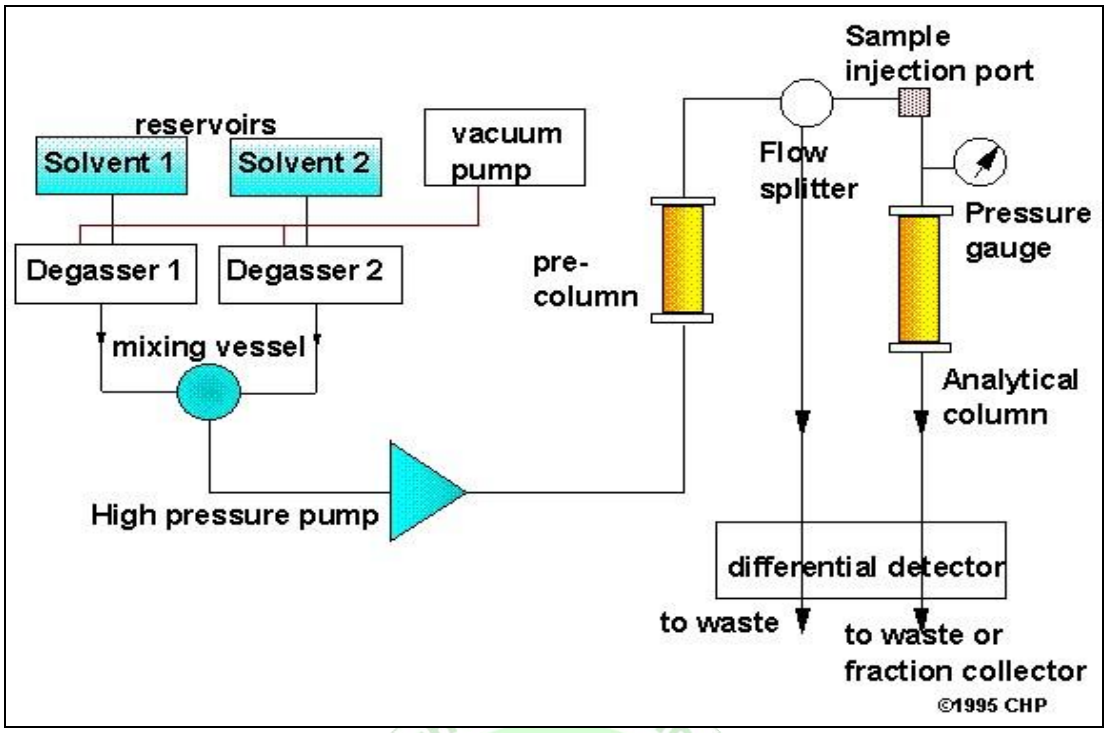


Figure: 1 Block diagram of a HPLC instrument

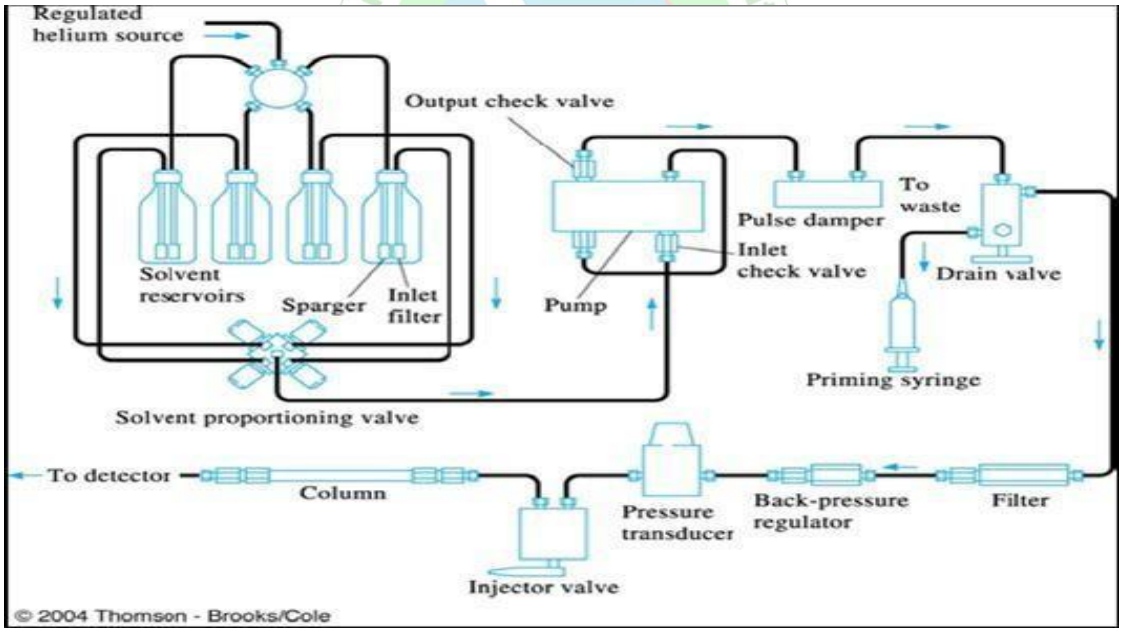


Figure: 1 Schematic diagram of a HPLC instrument

TYPES OF HPLC BASED ON MODES OF SEPARATION

- NP-HPLC
- RP-HPLC

Inthe normal phase mode, the stationary phase is a polar substance such as polyethylene glycol or the untreated silica surface itself, and the mobile phase is non polar (e.g. hexane) under these circumstances polar compounds retarded preferentially and non polar substances elute more quickly.

In reversed phase mode, the stationary phase is non polar (e.g. ODS) and the mobile phase is polar, usually a mixture of water, methanol and/or acetonitrile. Non polar compounds are retained more strongly, while polar solutes elute first. Reversed phase separations are the most frequently used methods in HPLC.

Table: 2 Comparison of NP – HPLC and RP – HPLC

Parameter	Normal	Reversed
Packing polarity	High	Low
Solvent polarity	Low	High
Elution order	Non-polar first, then polar	Polar first, then non-polar
Effect of increasing solvent polarity	Decreases retention time	Increases retention time

Introduction to RP – HPLC[6]

Separation by RP – HPLC is similar to the extraction of different compounds from water into an organic solvent, where more hydrophobic (non - polar) compounds extract into the non - polar phase. The column (typically C8and C18 bonded phase) is less polar than the water - organic phase. Sample molecules partition between the polar mobile phase and non – polar C8and C18stationary phase and more hydrophobic (non - polar) compounds are retained more strongly. Polar compounds are less strongly held and elute from the column first and vice versa. In RP – HPLC the retention of a compound is determined by its polarity, experimental conditions, mobile phase, column and temperature. RP – HPLC columns are efficient, stable and reproducible.

Mobile phase effects

Retention is preferable adjusted by changing mobile phase composition or solvent strength. In this the retention is less for stronger, less polar mobile phase. Solvent strength depends on the choice of organic solvent and its concentration in the mobile phase.

Mobile phase strength

1. In RP-HPLC solvent strength varies as
2. Water < Methanol < Acetonitrile < Ethanol < Tetrahydrofuran < Propanol < Methylene chloride.
3. Acetonitrile is best initial choice of solvent and Acetonitrile and Water mixture used for UV detection.

Various Methods of Quantitative Analysis in HPLC

The sample or solute is analyzed quantitatively in HPLC by either peak height or peak area measurements. Peak areas are proportional to the amount of constant rate. Peak heights are proportional to the amount of material only when peak width are constant and are strongly affected by the sample injection techniques. Once the peak height or the peak areas are measured, there are five principle evaluation methods for quantifying the solute[3,7].

Calibration by Standards

Calibration curves for each component are prepared from pure standards, using identical injection volumes of operating conditions for standards and samples. The concentration of solute is read from its curve if the curve is linear.

$$X = K \times Area.$$

Where, X = Concentration of solute.

K = Proportionality constant (slope of the curve).

In this evaluation method only the area of the peaks of interest is measured. Relative response factors must be considered when converting areas to volume and when the response of a given detector differs for each molecular type of compounds.

Internal Standard Method

In this technique a known quantity of the internal standard is chromatographed and area versus concentration is ascertained. Then a quantity of the internal standard is added to the raw sample prior to any sample pretreatment or separation operations.

The peak area of the standard in the sample run is compared with the peak area when the standard is run separately. This ratio serves as a correction factor for variation in sample size for losses in any preliminary pretreatment operations or for incomplete elution of the sample. The material selected for the internal standard must be completely resolved from adjacent sample components, must not interfere with the sample components and must never be present in samples.

$$\text{Area ratio} = \frac{\text{Area of sample}}{\text{Area of internal standard}}$$

$$\text{Sample concentration} = \frac{\text{Area of sample}}{\text{Area of internal standard}} \times \text{Concentration of standard}$$

Area Normalization

The technique is often used for the sample having identical components. It is used to evaluate the absolute purity of the sample. The procedure is to total up the areas under all peaks and then calculate the percentage of the total area that is contributed by the compound of interest. For this method the entire sample must be eluted, all components must be separated and each peak must be completely resolved.

Standard Addition Method

If only few samples are to be chromatographed, it is possible to employ the method of standard addition(s). The chromatogram of the unknown analyte is recorded, then a known amount of analyte(s) is added and the chromatogram is repeated using same reagents, instruments and same conditions. From the increase in the peak area (or) peak height, the original concentration can be computed by interpolation.

The detector response must be a linear function of analyte concentration and yield no signal at zero concentration of the analyte. Sufficient time must elapse between addition of the standard and actual analysis to allow equilibrium of added standard with any matrix interferon.

If an instrumental reading (area/height) 'Rx' is obtained, from a sample of unknown 'x' and a reading 'Rt' is obtained from the sample to which a known concentration 'a' of analyte has been added, then 'x' can be calculated from.

$$\frac{x}{x+a} = \frac{R_x}{R_t}$$

A correction for dilution must be made if the amount of standard added changes the total sample volume significantly. It is always advisable to check the result by adding at least one other standard.

External Standard Method

It employs a separate injection of a fixed volume of sample and standard solution. The peaks are integrated and concentration is calculated.

$$\text{Sample concentration} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{Concentration of standard}$$

The selection of suitable chromatographic (HPLC) system for a given mixtures of solutes cannot be made with certainty and must be confirmed by experiment. If the chemical nature of the sample components is known, then the phase system can be selected from the literature references. If nothing is known about the chemical nature of sample, then the sample solubility will give some indication as to which chromatographic method to employ. The essential parts of high performance liquid chromatographic system are solvent reservoir, pump, injection port, column, detector and recorders.

METHOD VALIDATION

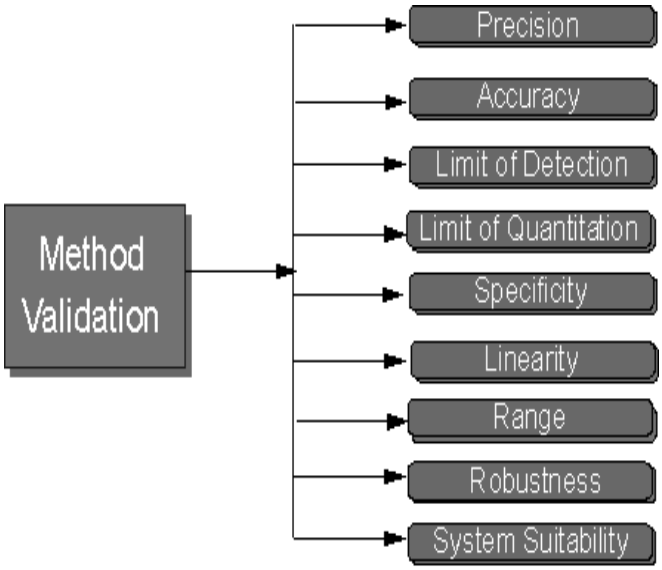
Method validation can be defined as establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics. Method Validation, however, is generally a one-time process performed after the method has been developed to demonstrate that the method is scientifically sound and that it serves the intended analytical purpose.

Purpose of Validation

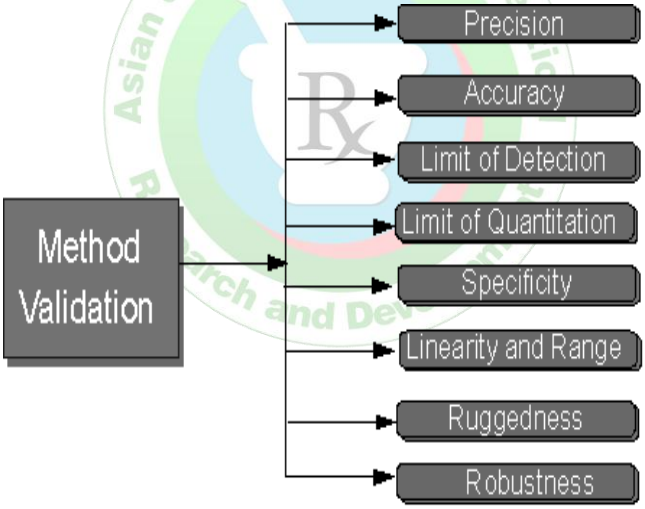
- Enable the scientists to communicate scientifically and effectively on technical matter.
- Setting the standards of evaluation procedures for checking compliance and taking remedial action.
- Economic: Reduction in cost associated with process sampling and testing.
- As quality of the product cannot always be assured by routine quality control because of testing of statistically insignificant number of samples.
- Retrospective validation is useful for trend comparison of results compliance to CGMP/CGLP.
- Closure interaction with Pharmacopoeial forum to address analytical problems.
- International Pharmacopoeial harmonization particularly in respect of impurities determination and their limits.

Guidelines for method validation [7,8,9.]

ICH Method Validation



USP Method Validation



Typical validation characteristics which should be considered and their definitions are given below.

Accuracy

The closeness of agreement between the value, which are accepted either as a conventional true value or an accepted reference value and the value found. Accuracy is represented and determined by recovery studies.

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the homogenous sample under the prescribed conditions. A more comprehensive definition proposed by the ICH divides precision into three types

1. Repeatability
2. Intermediate Precision
3. Reproducibility

Repeatability

It is the precision of a method under the same operating conditions over a short period of time. One aspect of this is instrumental precision. A second aspect is sometimes termed intra-assay precision and involves multiple measurements of the same sample by the same analyst under the same conditions.

Intermediate precision

It is the agreement of complete measurements when the same method is applied many times within the same

laboratory. This can include full analysis on different days, instruments or analysts, but would involve multiple preparations of samples and standards.

Reproducibility

It examines the precision between laboratories and is often determined in collaborative studies or method transfer experiments. Precision often is expressed by the standard deviation or relative standard deviation of the data set.

Range

The range of a method can be defined as the upper and lower concentrations for which the analytical method has adequate accuracy, precision and linearity. The range of concentrations examined will depend on the type of method and its use.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Assuring specificity is the first step in developing and validating a good method. If specificity is not assured, method accuracy, precision and linearity all are seriously compromised. Method specificity should be reassessed continually during validation and subsequent use of the method.

Linearity

The linearity of an analytical procedure is its ability to obtain test results, which are directly proportional to the concentration of analyte in the sample. Linearity can be assessed by performing single measurements at several analyte concentrations. A linearity correlation coefficient above 0.999 is acceptable for most methods, especially for major components in assay methods. The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample.

Detection Limit

Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

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.

Based on Signal-to-Noise

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

Based on the Standard Deviation of the Response and the Slope

The detection limit (LOD) may be expressed as

$$LOD = 3.3\ \sigma / S$$

Where, σ = the standard deviation of the response.

S = the slope of the calibration curve (of the analyte).

Based on the Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the Calibration Curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Quantitation Limit

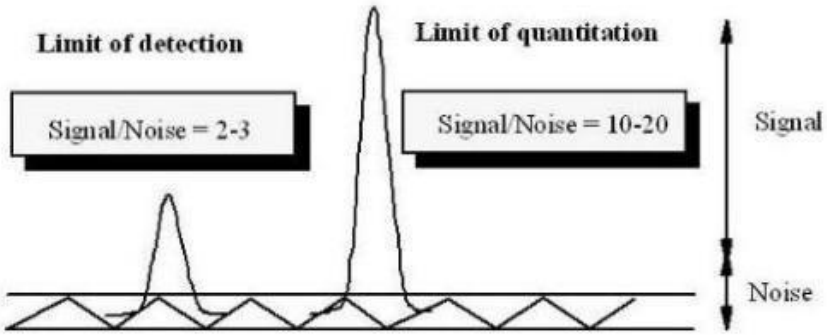
Several approaches for determining the quantitation limit are possible; depending on either the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision. b) Based on Signal-to-Noise Approach

This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.



Based on the Standard Deviation of the Response and the Slope

Quantitation Limit (LOQ) may be expressed as

LOQ = 10 σ/S

Where, σ = the standard deviation of the response.

S = the slope of the calibration curve (of the analyte). d) Based on Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the Calibration Curve

A specific calibration curve should be studied using samples, containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Ruggedness

Method ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. This includes different analysts, laboratories, columns, instruments, sources of reagents, chemicals, solvents and so on. Method ruggedness may not be known when a method is first developed, but insight is obtained during subsequent use of that method.

Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as

"a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters". The most important aspect of robustness is to develop methods that allow for expected variations in the separation parameters.

Table: 3 Acceptance criteria of validation for HPLC

S.No	Characteristics	Acceptance Criteria
1	Accuracy	Recovery 98-102% with 80, 100, 120% spiked sample.
2	Precision	
2a	Repeatability	RSD < 2%
2b	Intermediate precision	RSD < 2%
3	Specificity/ selectivity	No interference
4	Detection Limit	S/N > 2 or 3
5	Quantitation Limit	S/N > 10
6	Linearity	r > 0.999
7	Range	80-120%
8	Stability	>24h or > 12h

STATISTICAL PARAMETERS[10]

Regression equation

The linear relationship is characterized by at tendency of the points of the scattered diagram to cluster along a straight line known as the regression line.

Y=a + bX

It is used to describe the dependence of one characteristic (Y) up on the other characteristic (X), both X,Y represent values of two characters a,b are two constants it will be evident that two regression lines can be computed for every set of data-one each to describe the dependence of one character to another. b is known as regressive coefficients which shows change expected in Y for unit change in X, it is dependence of Y & X; b is the regressive coefficient of Y& X. The regressive coefficient of b is estimated,

$$b = \frac{\Sigma\left(x - \bar{x}\right)\left(y - \bar{y}\right)}{\Sigma\left(x - \bar{x}\right)^2}$$

b = the slope of the regression line and is calculated by this formula x = an arbitrarily chosen value of the predictor

variable for which the corresponding value of the criterion variable is desired.

Correlation coefficient

A measure of the strength of the relationship between two variables is provided by the coefficient of correlation denoted by r, if the relationship between the two variables is of the linear form. It is also called the coefficient of linear correlation.

Pearson's correlation

The correlation coefficient calculation for data values should be +1 or -1where the values of

Correlation coefficient is +1 – positive

Correlation coefficient is -1 – negative.

$$r = \frac{\sum XY - \frac{\sum X \sum Y}{N}}{\sqrt{(\sum X^2 - \frac{(\sum X)^2}{N})(\sum Y^2 - \frac{(\sum Y)^2}{N})}}$$

Where, X – value of one character

Y – Value of another character

Stability Indicating Method[11]

A stability indicating method is a validated qualitative analytical procedure that can detect the cha nges with time in the properties of drug substance and drug product under defined storage conditions. A stability indicating assay method accurately measures the active ingredient without interference from other peaks and is sensitive enough to detect and qualify the degradation products/impurities. To develop a stability indicating method, stress testing in the form of forced degradation should be carried out at an early stage so that impurities and degradation products can be identified and characterized.

The stability-indicating assay is a method that is employed for the analysis of stability samples in pharmaceutical industry. With the advent of International Conference on Harmonization (ICH) guidelines, the requirement of establishment of Stability-Indicating Assay Method (SIAM) has become more clearly mandated. The guidelines explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products.

Stability-indicating methods according to 1987 guideline were defined as the 'quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured.'

This definition in the draft guideline of 1998 reads as: 'validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interference.'

According to FDA guidance document, a stability-indicating method is "a validated quantitative analytical procedure that can detect the changes with time in the pertinent properties of the drug substance and drug product. A stability indicating method accurately measures the active ingredients, without interference from degradation products, process impurities, excipients, or other potential impurities."

HPLC has been very widely employed in stability studies due to its high-resolution capacity, sensitivity and specificity. Non-volatile, thermally unstable or polar/ionic compounds can also be analyzed by this technique. Therefore, most of the SIAMs have been established using HPLC.

Steps in the Development of Validated SIAMS

The practical steps involved in the development of SIAMs are discussed below:

Step I : Critical study of the drug structure to assess the likely decomposition route(s)

Step II : Collection of information on physicochemical properties

Step III : Stress (forced decomposition) studies

Step IV : Preliminary separation studies on stressed samples

Step V : Final method development and optimization

Step VI: Identification and characterization of degradation products, and preparation of standards

Step VII: Validation of SIAMs

ICH guideline Q1AR and the ICH's Common Technical Document suggest the drug substance only subjected to stress conditions for the development of a SIAM.

Forced Degradation Studies[11-13]

Forced degradation or stress testing is undertaken to demonstrate specificity when developing stability-indicating methods. These studies also provide information about the degradation pathways and degradation products that could form during storage. Forced degradation studies may help facilitate pharmaceutical development as well in areas such as formulation development, manufacturing, and packaging, in which knowledge of chemical behavior can be used to improve a drug product.

Forced degradation studies (or stress testing) are discussed and differentiated from accelerated testing, which is done during formal stability testing, in ICH Q1A (R2). The guidance states,

"Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stabilityindicating power of the analytical procedures used.

The nature of the stress testing will depend on the individual drug substance and the type of drug product involved."

As stated in ICH Q1A, forced degradation studies can be used as a predictive tool . The initial purpose of these studies is to investigate stability-related properties of an API and understand the degradation products and pathways of the compound. They are also used to provide samples for the development of stability-indicating analytical methods for the API. The information gleaned from a forced degradation investigation can also be utilized in several other areas of development, including analytical development (methods development), formulation development (formulation choice and storage conditions), manufacturing/processing parameters (synthesis/salt selection of API and manufacture of formulations), safety/toxicological concerns (possible genotoxic degradation products), metabolism (identification of possible metabolites) and discovery (design of better or more stable APIs).

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