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A REVIEW ON METHOD VALIDATION FOR HPLC

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Abstract:

High performance liquid chromatography (HPLC) is an important qualitative and quantitative method. It is used to identify and quantify potency in drug substances and drug products. Chromatographic methods plays a vital role in the drug discovery, development, formulations and quality control. In this review, it explains that validation process and validation parameters. The parameters described here are according to ICH guidelines and include accuracy, precision, specificity, and limit of detection, limit of quantitation, linearity, range, and robustness.

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INTRODUCTION:

A form of column chromatography to separate, identify, and quantify the compounds. Developed in 1970s.The widely used analytical separation technique.

CHROMATOGRAPHY

Chromatography is a technique which separates components in a mixture due to the differing time taken for each component to travel through a stationary phase and carried through it by a mobile phase. It is technique used for **separation, purification, identification and extraction of compound**. It can consist of two phases **stationary phase and mobile phase.**

Stationary phase is constant phase or column packaging material. **Mobile phase** is movable phase. The basic principle of chromatography is based on **adsorption partition chromatography.**

Adsorption chromatography-The affinity of molecules towards stationary phase is known as adsorption chromatography.

Partition chromatography –The molecule can move in two phases of liquid is known as partition chromatography.

It is important for **qualitative and quantitative analysis.**

TYPES OF CHROMATOGRAPHY

- Based on modes of chromatography:
  1.Normal phase mode
  2. Reverse phase mode
- Based on principle of separation:
  1.Adsorption chromatography
  2. Ion exchange chromatography
  3. Partition chromatography
  4.Size exclusion
- Based on elution technique
  1.Isocractic separation
  2.Gradient separation
- Based on the scale of operation
  1.Analytical HPLC
  2.Preparative HPLC
- Based on the type of analysis
  1.Qualitative analysis
  2.Quantitative analysis

DIFFERENT TYPES OF CHROMATOGRAPHIC METHODS

- Paper chromatography
- Liquid chromatography
- Gas chromatography
- High performance liquid chromatography

HPLC – HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

DEFINITION:

It is a chromatographic technique used to separate components of mixture for the purpose to identity, quantify or the individual components of the mixture.

Analytical chemistry is widely used in determining the qualitative and quantitative composition of typical mixtures. These two methods are the main aspects to understand the sample materials. Generally, analytical chemistry divides into two branches i.e., qualitative and quantitative analysis. High performance liquid chromatography also is known as High Pressure Liquid Chromatography. HPLC is an analysis technique used for separation, identification, and quantification of typical mixtures such as organic, inorganic, biological, ionic and polymeric materials. HPLC is a type of column chromatography in which solvent flows with high pressure, so that sample can be separated into different constituents divided into different constituents divided into different types on modes of separation, the principle of separation, elution technique, scale of separation and based on the type of analysis. The Detector is the heart of this instrument and the efficiency of this system depends upon detecting techniques. Many types of HPLC detectors such as UV-Vs, refractive index detector, photodiode detector, fluorescence, electrical conductivity detector.

High performance liquid chromatography or high-pressure liquid chromatography (HPLC) is a specific form of column chromatography generally used in biochemistry and analysis to separate, identify, and quantify the active compounds. HPLC is a conventional extension of liquid chromatography.

HPLC is used in various fields like in pharmaceuticals , biochemicals, food products, industrial chemicals, forensic chemistry, environmental field, clinical medicine for analysis for typical mixtures like antibiotics, amino acids, fatty acids, poisons, inorganic ions, urine extracts, oestrogens.

The use of HPLC is increased day by day across the world due to its unique properties like high resolutions, high sensitivity, good repeatability, small sample size, moderate analysis condition, no need to vaporise the sample as in the gas chromatography, easy to fractionate the sample and purify.

Fig no1: High Performance Liquid Chromatography

PRINCIPLE

High performance liquid chromatography principle based on adsorption as well as partition chromatography that depending on the nature of stationary phase, if stationary phase is a solid principle that based on adsorption chromatography and if stationary phase is liquid, then the principle is based on the partition chromatography. It is important for determination of volatile and non-volatile compounds and also for qualitative and quantitative analysis. HPLC mainly utilises a column that holds packing material (stationary phase), a pump that moves the mobile phase through the column, and a detector that shows the retention times of the molecules.

The separation principle of HPLC is based on the distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase (packing material of the column). Depending on the chemical structure of the analyte, the molecules are retarded while passing the stationary phase. The specific intermolecular interactions between the molecules of a sample and the packing material define their time "on column". Hence, different constituents of a sample are eluted at different times. Thereby, the separation of the sample ingredients is achieved. A detection unit (e.g., UV detector) recognises the analytes after leaving the column. The signals are converted and recorded by a data management system (computer software) and then shown in a chromatogram. After passing the detector unit, the mobile phase can be subjected to additional detector units, a fraction collection unit or the waste. In general, a HPLC system contains the following modules: a solvent reservoir, a pump, an injection valve, a column, a detector unit and a data processing unit. The solvent (eluent) is delivered by the pump at high pressure and constant speed through the system. To keep the drift and noise of the detector signal as low as possible, a constant and pulseless flow from the pump is crucial. The analyte (sample) is provided to the eluent by injection valve.

PLATE THEORY:

According to plate theory, the columns used in gas chromatography are considered to be made up of several parallel layers of discrete and continuous horizontal plates known as *theoretical plates*. During a chromatographic process, it is supposed that as the mobile phase moves over the column, the solute particles equilibrate between the mobile and the stationary phase with the solute and solvent being transferred from one plate to another in a step-wise pattern. In other words ,theoretical plate may be considered as the distance/area in the column in which equilibrium is attained by the sample in between the gaseous and liquid phase. Column efficiency can be increased by increased by increasing the number of theoretical plates. The actual number of theoretical plates,in a column can be calculated by,

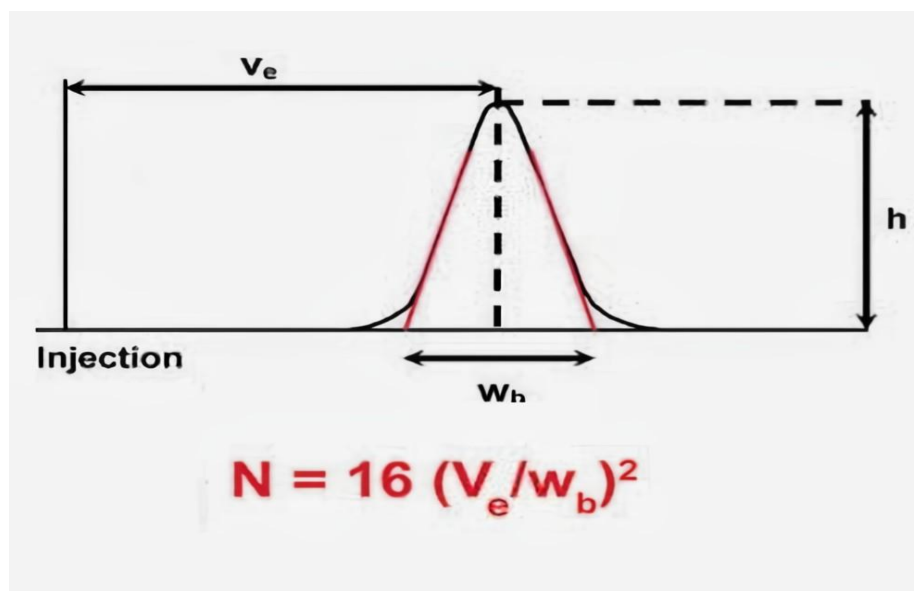
$$N = 16(t_R/W)^2$$

Where ,

N =number of theoretical plates

t_R =distance between injection port and the maximum peak height (retention time)

W =Peak width obtained upon drawing tangents from 2/3rd height of the peak up to the baseline.



Fig,no 2: Graphical Representation of Plate Theory

Plate height or Height Equivalent to Theoretical Plates (HETP) : HETP is defined as the height of the column which is required for the equilibration of the solute between the mobile and the stationary phase. N and HETP are related by the following formula.

$$HETP = \frac{L}{N}$$

where,

L =length of the column

Greater the number of theoretical plates or smaller the HETP, higher is the column efficiency.

RATE THEORY:

Sharp and symmetrical peaks of the chromatogram are indications of optimal separation in gas chromatography. This requires limited band broadening. Although N and HETP describes the column efficiency, they does not furnish any information about the effects of variables on band broadening and band separation (retention time). Van Deemeter *et.al.* proposed the rate theory to overcome this drawback of plate theory. Van Deemeter equation helps in optimizing the column efficiency and is given as,

$$HETP = A + \frac{B}{u} + Cu$$

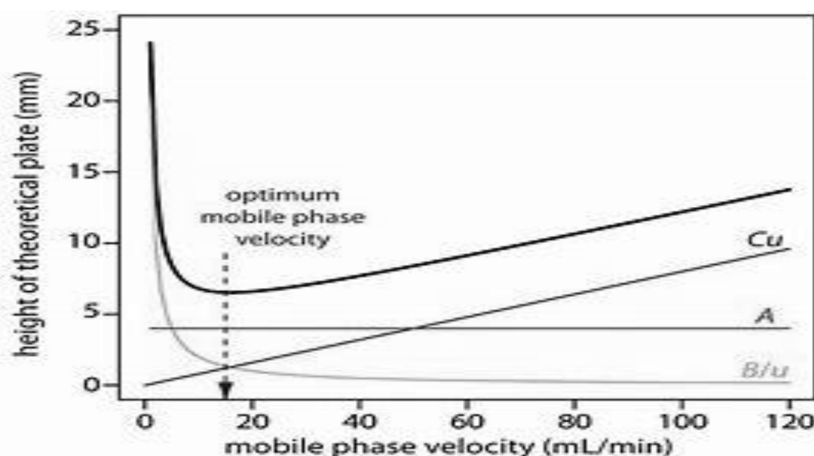
Where,

A = Coefficient of Eddy diffusion

B = Coefficient of longitudinal diffusion

C = Coefficient of mass transfer

u = Average gas velocity or flow rate (cm/sec)



Fig,no 3: Graphical Representation of Rate Theory

TYPES OF HPLC:

Types of HPLC generally depend on phase system used in the process. Following types of HPLC generally used in analysis-

❖ **Based on modes of chromatography:**

- **Normal phase chromatography**:
  Also known Normal phase HPLC (NP-HPLC), this method separates analytes based on polarity.NP-HPLC uses a polar stationary phase and a non-polar mobile phase. The polar analyte interacted with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase increases the elution time. In this technique, non -polar compounds travel and eluted first. This is because of the lower affinity between the non-polar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. Normal phase mode of separation is therefore not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute.
- **Reversed phase chromatography**:
  It is the most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode of separation, the stationary phase is non polar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. The polar compound gets eluted first in this mode and non -polar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained foe longer times and hence elute faster. The different columns used are Octa Decyl Silane (ODS)orC18, C8, C4(in order of increasing polarity of the stationary phase). An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing, and complexation) to control retention and selectivity.

❖ **Based on principle of separation:**

- **Size exclusion chromatography:**
  It is also called as gel permeation chromatography or gel filtration chromatography Mainly separates particles on the basis of size. It is also useful for determining the tertiary structure and quaternary structure of proteins and amino acids. This technique is widely used for the molecular weight determination of polysaccharides.

- **Ion exchange chromatography:**
  In ion exchange chromatography, retention is based on the attraction between solute ions and changed sites bound to the same charge are excluded. This form of chromatography is widely used in purifying water, ligand-exchange chromatography, ion exchange chromatography of proteins, high-pH anion – exchange chromatography of carbohydrates and oligosaccharides, etc…

- **Adsorption chromatography:**
  Separation of components takes place because of the difference between in affinity of compounds towards stationary phase.

❖ **Based on elution technique:**

- **Isocratic separation:**
  In this technique, the same mobile phase combination is used throughout the process of separation. The same polarity or elution strength is maintained throughout the process.
- **Gradient separation:**
  In this technique**,** a mobile phase combination of lower polarity or elution strength is used, followed by gradually increasing the polarity or elution strength.

❖ **Based on the scale of operation:**

- **Analytical HPLC:**
  Where only analysis of the samples is done. Recovery of the samples is not done.
- **Preparative HPLC:**
  Where the individual fractions of pure compound can be collected using fraction collector. The collector samples are reused.

❖ **Based on the type of analysis:**

- **Qualitative analysis:**
  Which is used to identify the compound, detect the impurities, to find the number of components, etc.
- **Quantitative analysis:**

Which is done to determine the quantity of the individual or several components in a mixture. This can be done by comparing peak area of the standard and sample.

INSTRUMENTATION:

HPLC instrument consists of following components:

- Pump
- Mixing unit
- Solvent degassing
- Injector
- Column
- Detectors
- Application

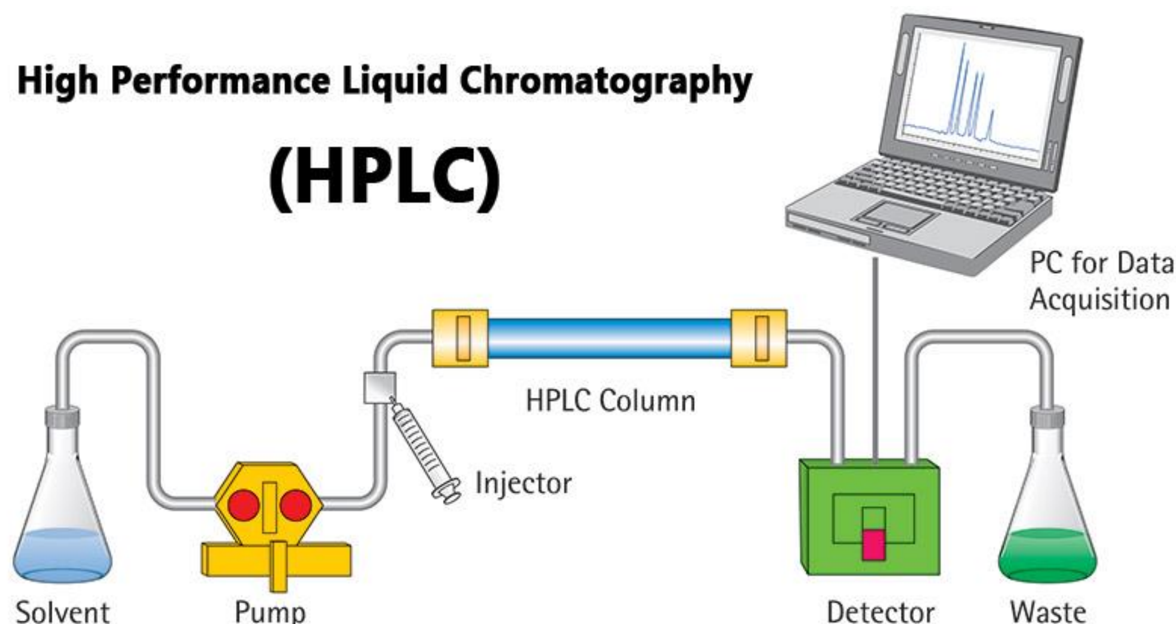


Fig no 4: Diagrammatic Representation of Instrumentation of HPLC

PUMP

The role of the pump is to force a liquid (called mobile phase) through the liquid chromatography at a specific flow rate, expressed in millilitres per min (ml/min). Normal flow rates in HPLC are in the 1-2ml/min range. During the chromatographic experiment, a pump can deliver a constant mobile phase composition (isocratic) or an increasing mobile phase composition (gradient). Best for the analysis of complex samples.

TYPES OF PUMPS:

Mainly three types:

- Constant flow reciprocating pump
- Syringe type pump
- Pneumatic pump

1.CONSTANT FLOW RECIPROCATING PUMP

The term reciprocating describes any continually repeated backwards and forwards motion widely used type of pump.

2.SYRINGE OR DISPLACEMENT TYPE PUMP

Consists of large syringe like chamber. Suitable for small bore column.

3.PNEUMATIC PUMP

Gas is used to pressurize the mobile phase present in a collapsible solvent container.

MIXING UNIT

It is used to mix solvents in different proportions and pass through the column. Mixing of solvent is done either with a **static mixer** which is packed with beads or a **dynamic mixer** which uses magnetic stirrer and operates under high pressure.

SOLVENT DEGASSING

Several gases are soluble in organic solvents. When solvents are pumped under high pressure, gas bubbles are formed which will interfere with the separation process, steady baseline and the shape of the peak. This can be done by following techniques:

- Vacuum filtration
- Helium purging
- Ultra-sonification

INJECTOR

The injector serves to introduce the liquid sample into the flow stear the mobile phase. Typical sample volumes are 5-20 microlitres. The injector must also be able to withstand the high pressure of the liquid system. Types of injectors:

- Septum injectors
- Stop flow
- Rheodyne injector

COLUMN

It is the heart of the chromatograph. The most common material used for column packing is **silica gel**. Changing a column will have the greatest effect on the

resolution of analytes during method development. Generally, modern reverse phase HPLC columns are made by packing the column housing with spherical silica gel beads which are coated with the hydrophobic stationary phase. The stationary phase is introduced to the matrix by reacted a chlorosilane with the hydroxyl groups present on the silica gel surface. In general, the nature of stationary phase has the greatest effect on capacity factor, selectivity, efficiency and elution. There are several types of matrices for support of the stationary phase, including silica, polymers, and alumina. Silica is the most common matrix of HPLC columns. Silica matrices are robust , easily derivatised, manufactured to consistent sphere size, and does not tend to compress under pressure. Silica is chemically stable to most organic solvents and to low pH systems.

Column length: varies from 5-30cm

Column diameter: ranges from 2-50mm

Particle size: from 1-20µ

Particle nature: spherical, uniform sized, porous materials are used.

DETECTORS

HPLC detectors monitor the elute as it leaves the column. Produce an electronic signal proportional to the concentration of each separated component. It is very crucial in trace analysis and highly sensitive. It will give fast responses. Detector is a very important part of HPLC. Selection of detector depends on the chemical nature of analytes, potential interference, limit if detection required, availability and /or cost of detector. UV-Visible detector is versatile, dual-wavelength absorbance detector for HPLC. This detector offers the high sensitivity required for routine UV- based applications to low level impurity identification and quantitative analysis. Photodiode array (PDA) Detector offers 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 component with limited or no UV absorption. Multi-wavelength fluorescence detector offers high sensitivity and selectivity fluorescence detection for quantitating low concentrations of target compound. The most widely used detection methods are:

- Spectrophotometers
- Fluorometers
- Electro-chemical detectors
- Mass spectrophotometer
- Refractive index detector

ADVANTAGES:

- Separation of volatile and non-volatile components.
- It is simple, rapid, reproducible
- Quick analysis
- High resolution
- High sensitivity
- Rapid process and hence time saving
- High performance
- Having high resolution and separation capacity
- Stationary phase was chemically inert
- Wide varieties of stationary phase
- Mobile phase was chemically inert
- Less requirement of mobile phase in developing chamber.
- Early recovery of separated components
- Early visualization of separated component
- Having good reproducibility and repeatability
- It is an analytical technique important for validation of product, quality control studies of product.
- Used both for analytical and preparative purpose.
- Accuracy and precision
- Important for qualitative and quantitative analysis

DISADVANTAGES:

- High cost
- Complex to operate
- Tedious to detect co-elution

APPLICATIONS:

- Environmental factors:
  - Detection of chemicals compounds/contaminant in water and air quality
  - Chemical exposure in the workspace/environment
  - Pesticides, herbicides, phenols, polychlorinated biphenyls (PCBs)
- Forensics chemistry
  - Identification & determination of abuse drugs in blood, urine etc.eg: cocaine, steroid, ketamine etc.
  - Quantification of drugs, poisons, blood alcohol, narcotics
  - Forensic analysis like textile dyes, chemicals, etc.
- Industrial application:
  - Identification & determination of cosmetics-active ingredient content, purity, impurities and stability study.

- Analysis of preservative, surfactants, and stability study.
- Organic chemicals like polymers
- Artificial sweeteners, antioxidants, additives.
- Thermally unstable compounds such as trinitrotoluene, enzymes

- Drug discovering
- Clinical analysis
- Cosmetic analysis
- Structural determination
- Biochemical genetics
- Therapeutic drug monitoring
- Diagnostic studies
- Food analysis
- Modern applications are mainly in pharmaceutical field
- Most widely used in agrichemicals i.e., analysis of pesticides in cleaning water.
- Chromatography separation of anions can be carried out by using ion exchange ion pair chromatography and ion exclusion chromatography.
- RP-HPLC has biggest impact on the separation of oligo peptides and proteins.
- The various applicability, speed, sensitivity of HPLC is the most popular chromatography technique used for purification and all types of biological molecules.

STANDARD OPERATING PROCEDURE OF HPLC:

Procedure:

1. Before switching on the instrument do ensure the valves are in closed position.
2. Switch ON the PC and switch ON the instrument in the order of detector, pump 1, pump 2.
3. Auto-calibration will start and end when λ reaches 254nm and a lamp will glow at the top right corner of detector.
4. Pumps will show READY when calibration is completed.
5. Double click HPLC shortcut key and ensure that serial computer port is connected or not.
6. Ensure that suction heads are dipped in containers with mobile phase.
7. Purging is done to remove air bubbles from suction tubes and inlet tubes. Plunger is turned 45° to anti-clockwise direction.
8. After purging is done close the inlet tube opening.
9. Repeat the same for next pump.
10. Set initial flow rate as 1ml/min and maximum as 4ml/min.
11. Washing of the column should be done for 15 minutes with water or methanol before sample injection. After washing the flow rate can be adjusted for both solvents or for one solvent.
12. Press pump run, continue for 15 minutes and then click pump STOP.

Instructions:

- All dilutions should be done in HPLC grade apparatus and with HPLC grade solvents.
- The solvents should be suitably degassed and filtered using membrane filters.
- After each sample injection a wash period of minimum15 minutes should be done with mobile phase.
- Always check the pressure of pumps and flow rate.

METHOD VALIDATION FOR HPLC ANALYSIS

Validation of an analytical method is the process by which it is established by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application.

Validation is required for any new or amended method to ensure that it is capable of giving reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories. The type of validation program required depends entirely on the particular method and its proposed applications.

Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice. Use of equipment that is within specification, working correctly and adequately calibrated is fundamental to the method validation process. Analytical methods need to be validated or revalidated

- Before their introductions into routine use.
- Whenever the conditions change for which, the method has been validated.
- Whenever the method ids changed. Typical parameters recommended by FDA, USP, & ICH

Steps in the method validation process:

- Method validation and further method development
- Final method development and trail method validation
- Formal method validation and report generation

- Formal data review and report issuance

Validation is complete when we;

1. Demonstrate that you have met all the acceptance criteria.
2. Clearly document the results in a cGMP compliant fashion
3. Show how the acceptance criteria in a final methods validation report, including references to raw data, all of which have been reviewed and approved by the appropriate personnel including peers, management and QA.

Method validation is required for:

- For assuring the quality of the product.
- For achieving the acceptance of the products by the international guidelines.
- It is mandatory requirement for accreditation as per ISO 17025 guidelines
- A mandatory requirement for registration of any pharmaceutical product or pesticide formulation.

DESCRIPTION OF METHOD VALIDATION:

As per USP and BP, it consists of;

- Precision
- Accuracy
- Limit of detection
- Limit of quantitation
- Specificity
- Linearity
- Range
- Robustness
- System suitability
- Retention factor

As per ICH, it contains;

- Precision
- Accuracy
- Limit of detection
- Limit of quantitation
- Specificity
- Linearity and range
- Ruggedness
- Robustness

ACCURACY:

- The accuracy is the closeness of the test results obtained by the method of the true value.
- Accuracy should be established across its range.
- It is assessed using a minimum of 9 determinations over a minimum of 3 concentration levels.
- The acceptance criterion for accuracy is the Relative Standard Deviation (RSD) for all the recovery values should not be than 2%.

How to determine?

Accuracy is measured by spiking the sample matrix of interest with a known concentration of analyte standard and analysing the sample using the "method being validated". The procedure and calculation for accuracy will be varied from matrix to matrix and it will be given in respective study plan or amendment to the study plan.

PRECISION:

The precision of an analytical method is the degree of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample.

The RSD for all the assays of sample preparations should not be more than 2%.

Precision: - Repeatability
Intermediate precision
Reproducibility

- **Repeatability: -** Expresses the precision under the same operating conditions over a short interval of time. It is also termed *intra-assay precision.*
- **Intermediate precision: -** it expresses within -laboratories variations: different days, different analysts, different equipment etc.
- **Reproducibility:** - It expresses the precision between the laboratories.

How to determine?

It is measured by injecting a series of standards or analysing series of samples from multiple samplings from a homogenous lot. From the measured standard deviation (SD) and mean values, precision as relative standard deviation(%rsd) is calculated.

$$\%rsd = \frac{SD}{mean} \times 100$$

SPECIFICITY:

The ability to assess unequivocally the sample in the presence of components that may be expected to be present-impurities-degrading agents-excipients, Specificity must be demonstrated for: -Identification-impurities test-assay test. The RSD for all assays of sample preparations should not be more than 2%. For the chromatographic methods developing a separation involves demonstrating specificity, which is the ability of the method to accurately measure the analyte response in the presence of all potential sample components.

How to determine?
It is checked by examining chromatographic blanks (from a sample that is known to contain no analyte) in the expected time window of the analyte peak. And the raw data for selectivity will be recorded in the raw data in approved formats.

LINEARITY:
The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to the concentration of the analyte in the sample. Linearity is usually demonstrated by the analysis of various concentrations of the analyte across the indented range and represented graphically. The relationship between the concentration (in %) of drug in sample area should be linear in the specified range and the correlation should not be less than 0.9.

How to determine?
It is determined by injecting a series of standards of stock solution/diluted stock solution using the solvent/mobile phase, at a minimum of five different concentrations in the range of 50-150% of the expected working range. The linearity graph will be plotted manually/using Microsoft excel or software of the computer (concentration vs. Peak area response) and which will be attached to respective study files.

ROBUSTNESS:
The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during use. The RSD for the samples should not be more than 2%. It also provides some indication of the reliability of an analytical method during normal usage. Parameters that should be investigated are percent organic content in the mobile phase or gradient ramp; pH of the mobile phase; buffer concentration; temperature; and injection volume. These parameters may be evaluated one factor at a time or simultaneously as part of a factorial experiment. The chromatography obtained for a sample containing representative impurities when using modified parameters should be compared with the chromatography obtained using the target parameters.

SYSTEM SUITABILITY:
The simplest form of an HPLC system suitability test involves a comparison of chromatogram trace with a standard trace. This allows a comparison of the peak shape, peak width, baseline resolution. System suitability testing is an integral part of many analytical procedures the tests are based on the concepts that the equipment, electronics, analytical operation and samples to be analysed constitute an integral system that can be evaluated as such. Typically, the process involves making five injections of a standard solution and evaluating several chromatographic parameters such as resolution, area % reproducibility, number of theoretical plates and tailing factor.

RUGGEDNESS:
The ruggedness of an analytical method is the degree of reproducibility of the test results obtained by the analysis of the same samples under a variety of conditions, such as; day to day variations, laboratory to laboratory, reagent kit to kit, instability of analytical reagents. Different source of reagent, elapsed assay, time, assay temperature conditions. Ruggedness is the measure of reproducibility of test result under the variation in conditions normally expected from analyst to analyst. The criteria of the ruggedness is the RSD should be not more than 2%.

RANGE:
Range of the analytical procedure is the interval between the upper and the lower concentration of the analyte for which it has been demonstrated that the analytical procedure has a suitable precision, accuracy, and linearity. For assay the range is usually not less than 80 to 120% of the test concentration. The range is determined using data from the linearity and accuracy studies. Range criteria for an assay method is the at the acceptable range will be defined as the concentration interval over which linearity and accuracy are obtained per previously discussed criteria and that yields a precision of 3% RSD.

LIMIT OF DETECTION:
It is the smallest quantity of an analyte that can be detected, but not necessarily quantified. It is the lowest amount of analyte in a sample which can be detected, but not necessarily quantitated as an exact value. Main approaches for the calculation are visual evaluation, signal to noise ratio, standard deviation of the response and the slope of the calibration curve.

How to determine?
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: -
  a) May be used for non-instrumental methods but may also be used with instrumental methods.
  b) 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: -
  a) This approach can only be applied to analytical procedures which exhibit baseline noise.
  b) Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte.
  c) A signal-to-noise between 3or 2:1 is generally considered acceptable for estimating the detection limit.

LIMIT OF QUANTITATION:

The quantitation limit is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. Used particularly for the determination of impurities and/or degradation products. Various approaches are based on visual evaluation, signal to noise, Standard Deviation of Response and Slope.

How to determine?

Several approaches for determining the quantitation 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: -
  c) May be used for non-instrumental methods but may also be used with instrumental methods.
  d) 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: -
  d) This approach can only be applied to analytical procedures which exhibit baseline noise.
  e) Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte.
  f) A signal-to-noise between 10:1 is generally considered acceptable for estimating the detection limit.

RETENTION FACTOR OR CAPACITY FACTOR:

The capacity factor is a measure of how long each component is retained on the column.

RELATIVE RETENTION/SEPARATION:

It depends on the peak separation depends on the component's interaction with the stationary phase.

VALIDATION OF HPLC

The goal of equipment validation is to produce constant result with minimal variation without compromising the product and performance of equipment.

QUALIFICATION:

Qualification is a subset of the validation process that verifies module and system performance prior to the instrument being placed on line.

- *DESIGN QUALIFICATION (DQ):*
  For setting the functional and performance specifications and can be very simple for similar equipment e.g. Just another HPLC system.
- *INSTALLATION QUALIFICATION(IQ):*
  For performing and documenting the installation in the selected user environment and provides safety, service requirements.
- *OPERATIONAL QUALIFICATION(OQ):*
  For testing the equipment in the selected user environment to ensure that it meets our defined functional and performance specifications.
- *PERFORMANCE QUALIFICATION(PQ):*
  For testing, that the system consistently performs as intended for the selected application. Periodic calibration /maintenance. Must be signed by the user. May use method system suitability checks as part of PQ.

PARAMETERS INVOLVED IN VALIDATION:

1. Mean (X_i)
2. Standard deviation (SD)
3. Relative standard deviation (RSD)
4. Correlation co-efficient (R)
5. Linear regression.

MEAN:

The average result is calculated by summing the individual results and dividing the sum by the number (n) of individual values.

$$X_i = x_1 + x_2 + x_3 \ldots\ldots /n$$

Where,

$x_1, x_2, x_3 \ldots$= values of individual results

N = Number of individual results

STANDARD DEVIATION:

Its is the root mean square deviation of values from their average.

$$SD = \{\sum(X - Xi)/n - 1\}^{1/2}$$

Where,

$\sum$ =sum of observations

Xi = mean

X = individual observation value

(X-Xi) = deviation of a value from mean

n = number of observations

RELATIVE STANDARD DEVIATION:

Relative standard deviation (RSD) is a defined as the standard deviation expressed as the percentage of mean

RSD =(SD/Xi)×100

Where,

SD =standard deviation

Xi = mean

CORRELATION COEFFICIENT:

- The correlation co-efficient (R) is used to estimate the relationship of two random variables.
- It provides a measure of the strength and direction of the correlation varying from +1 to -1
- Positive values indicate the two variables are positively correlated, meaning the two variables vary in the same direction.
- Negative values indicate that the two variables are negatively correlated, meaning the two variables vary in the contrary direction.
- Values close to +1 or -1 reveal the two variables are highly related.

LINEAR REGRESSION:

A regression is a statistical analysis assessing the association between any two variables. It is used to find the relationship between two variables.

y = a +bx

where, a =intercept

b =slope

CONCLUSION:

In recent years development of the analytical methods for identification, purity evaluation and quantification of drugs has received a great deal of attention in the field of pharmaceutical analysis. This review describes HPLC method development and validation in general way. A general and very simple approach for the HPLC method development for the separation of compounds was discussed. Knowledge of the physicochemical properties of the primary compound is of utmost importance prior to the any HPLC method development. The selection of buffer and mobile phase composition (organic and pH) plays a dramatic role on the separation selectivity. Final optimization can be performed by changing the gradient slope, temperature and flow rate as well as the type and concentrations of mobile phase modifiers. Optimized method is validated with various parameters (e.g., specificity, precision, accuracy, detection limit, linearity, etc.) per ICH guidelines.

The growing pharmaceutical industry demands various analytical methods for various pharmaceutical products. To ensure quality of the product, it is necessary that the analytical method used for assuring quality should give accurate and predictable results. For this the method need to be validated. The HPLC methods are the preferred methods of analysis due to their responsiveness. It can be concluded from the entire review that HPLC is a versatile, reproducible chromatographic technique for the estimation of drug products. It has wide applications in different fields in term of quantitative and qualitative estimation of active molecules. The method development and validation of HPLC was found to be accurate, precise, and reliable. The method was proposed for the quality control studies of various pharmaceutical dosage forms and to find out the efficacy or therapeutic activity. It could be effectively separated the drugs and further studies should be preferred to evaluate the stability of pharmaceutical formulation. The advantages of HPLC were high selectivity, sensitivity, economic, less time consuming and low limit of detection. Whenever the method is changed and the change are outside the scope of the original method. If no previous methods exist for the analyte in the literature, work from analogy to investigate compounds that are similar in structure and properties. HPLC method should be developed within the GMP and GLP environments using the protocols set out in ICH guidelines.

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