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"A REVIEW ON HPLC METHOD DEVELOPMENT AND VALIDATION"

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Abstract: - High-Performance Liquid Chromatography (HPLC) is a type of column chromatography that is commonly used in biochemistry and analysis to separate, identify, and quantify active chemicals. 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 methods development and validation play important roles in new discovery, development, manufacture of pharmaceutical drugs and various other studies related to humans and animals. An analytical procedure is developed to test a defined characteristic of the drug substance or drug product against established acceptance criteria for that characteristic. This review gives information regarding various stages involved in development and validation of HPLC method. 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.

Keyword: - Chromatography, HPLC, Column Chromatography, Method Development, Method Validation.

I. INTRODUCTION

Define Chromatography: - [1] They are a define of chromatography which is a process of two or more mixture of component or chemical substances separated, identified and quantify of any components by using mobile phase or stationary phase it is also known as chromatography. The chromatography technique introduced by Mikhail tsvet in 1903.according to this technique two or more mixture of component or chemical substances are separated, identify, and quantify of any component. it is research from a analyte sample and coco3 to Administer by one glass beaker. then Administer by sample from beaker and then after added by liquid in a beaker then after some time released by three different-2 colored like yellow (Xanthophyl), orange (Carotenoid), green (Chlorophyl)

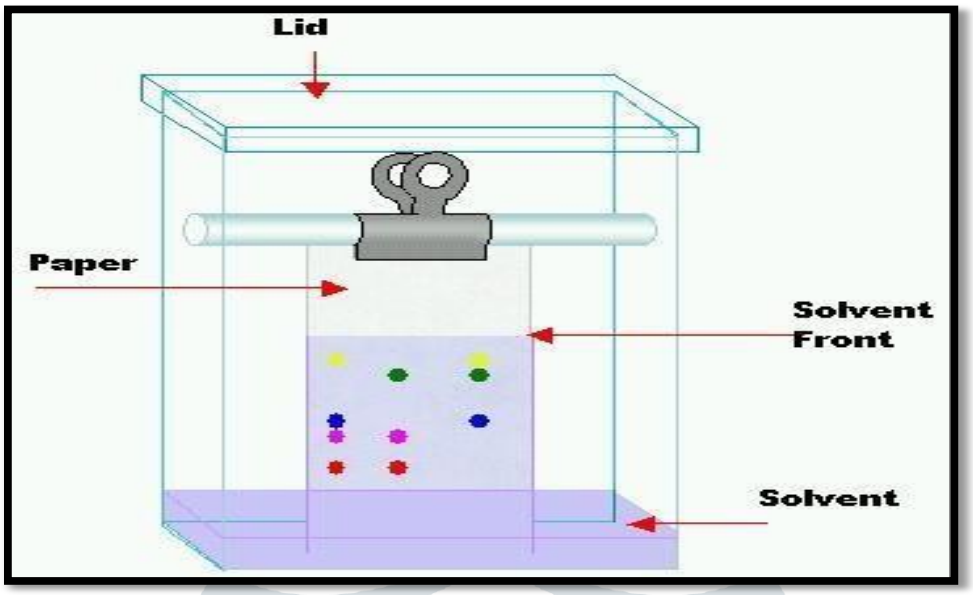


Fig.1.1 Instrumentation of chromatography

Importance of chromatography: - [2]

1) Separation of mixtures:
2) Purification
3) Identification and analysis:
4) Qualitative and quantitative analysis:
5) Environmental testing:
6) Drug development and quality control

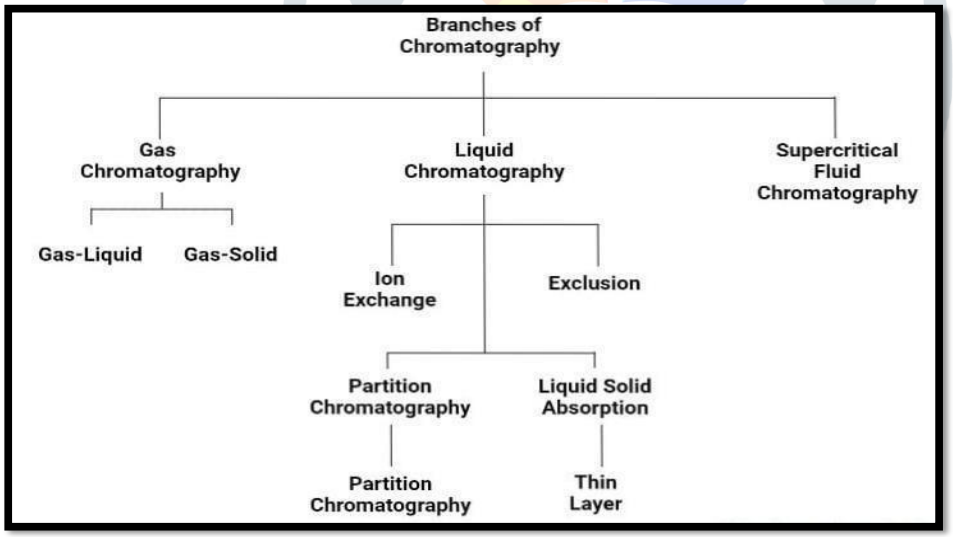


Fig. 1.2. Branches of chromatograph

Define HPLC: - [3, 4]

High-performance liquid chromatography, also known as high-pressure liquid chromatography, is a type of column chromatography that is commonly used in biochemistryand analysis to separate, identify, and quantify active chemicals. it is a popular analytical technique for separating, identifying, and quantifying each element of a mixture.

the modern pharmaceutical industry, high performance liquid chromatography (HPLC) is themajor and integral analytical tool applied in all stages of drug discovery, development, and portability.

The goal of HPLC method is to try & separate, quantify the main drug, any reaction impurities,all available synthetic intermediates and any degradants.

High performance liquid chromatography is now one of the most powerful tools in analyticalchemistry. it has the ability to separate, identify, and quantify the compounds that are presentin any sample that can be dissolved in a liquid. HPLC is the most accurate analytical methodswidely used for the quantitative as well as qualitative analysis of drug product and used for determining drug product stability.

The analytical instrumentation is widely used in biochemistry lab, and research and development, pharmaceutical industry and institutes. the HPLC are a powerful analytical technique two or more mixture of component or chemical substances are separated, identify,and quantify of any component by using mobile phase or stationary phase.

Importance of HPLC: - [5]

The high-performance liquid chromatography (HPLC) is a critical analytical technique in chemistry, biochemistry, pharmacology, and other fields. it is widely used to separate, identify, and quantify components in a mixture. here's why HPLC is important:

1. It is a high precision and accuracy

2. It is a high sensitivity

3. It is a wide range of applications

4. It is a fast analysis time

5. It is a quantitative analysis

6. It is a purity assessment

7. It is a chemical stability

8. It is a component separation

Advantages of HPLC: -

1) Simple, rapid, reproducible

2) It is High performance

3) It is high sensitivity

4) It is rapid process and time saving

5) It is high separation capacity

6) Its stationary phase and mobile phase are chemically inert

7) This is wide varieties of stationary phase The important for qualitative and quantitativeanalysis.

Application of HPLC: -

1) Checking the purity of compound.
2) Isolation and purification of biologically active natural products.
3) Used for separation of antibiotic from broth mixture.
4) Bio monitoring of pollutants.
5) Pharmaceutical quality control.
6) Identification of steroids in blood, urine etc.
7) Identification of steroids in blood, urine etc.

The Phases of HPLC: -[6]

1. **Mobile Phase: -**
2. This is the phase that moves through the system. it can be a liquid (as in liquid chromatography)or a gas (as in gas chromatography). the mobile phase carries the sample mixture through the stationary phase, enabling different components to be separated based on their affinity with the stationary phase.

3. **Stationary phase:**
This Phase does not move; it stays fixed in place within the chromatography system. the stationary phase can be a solid or a liquid supported on a solid. different components of the sample mixture interact with the stationary phase to varying extents, causing them to travel atdifferent rates and leading to separation.

4. **Column chromatography: -[7]**
The column chromatography is one of the most useful methods for the separation and purification of both solids and liquids. This is a solid liquid technique in which the stationaryphase is a solid & mobile phase is a liquid.

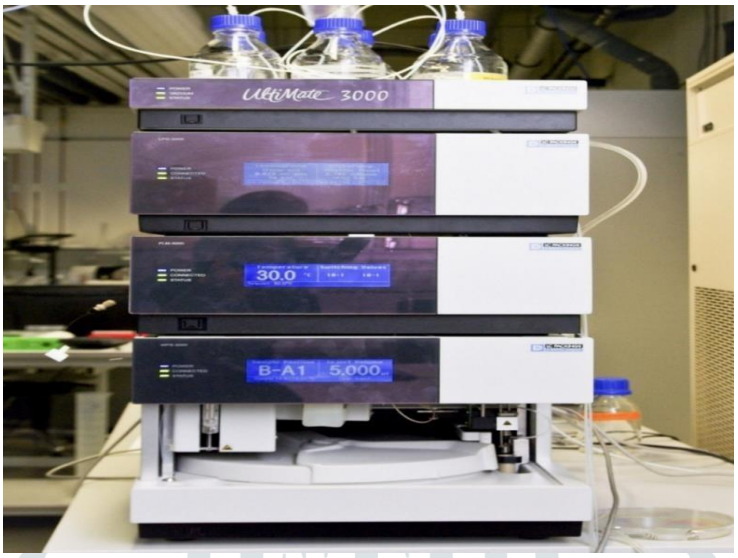


Fig. 1.3 Analytical instrumentation of HPLC

1. Parts of instrumentation: -[9]

1) Solvent reservoir:

These are inert containers for mobile phase storage and transport. generally, transparent glass bottles are used to facilitate visual inspection of mobile phase level inside the container. stainless steel particulate filters are provided inside for the removal of particulate impurities in the mobile phase if any.

2) Pump:

The variations in flow rates of the mobile phase affect the elution time of sample components and result in errors. pumps provide a constant flow of the mobile phase to the column under constant pressure.

3) Sample injector:

It is injectors are used to provide constant volume injection of the sample into the mobile phase stream. inertness and reproducibility of injection are necessary to maintain a high level of accuracy.

4) Column:

The column is a stainless-steel tube packed with a stationary phase. it is a vital component and should be maintained properly as per supplier instructions for getting reproducibility and separation efficiency run after run.

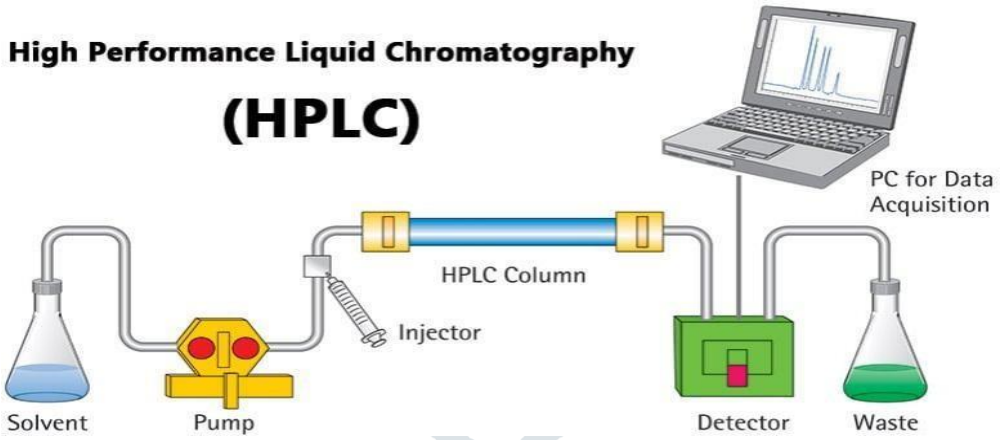
5) Detector:

The detector gives a specific response for the components separated by the column and also provides the required sensitivity. it has to be independent of any changes in mobile phase composition. the majority of the applications require uv-vis detection, though detectors based on other detection techniques are also popular these days.

6) Data acquisition & control:

The modern HPLC systems are computer-based and software controls operational parameters such as mobile phase composition, temperature, flow rate, injection volume and sequence, and also acquisition and treatment of output.

Fig. 1.5 Components of an HPLC instrumentation



Method development of HPLC:-[13]

An Analytical procedure is developed to test a defined characteristic of the substance against acceptance criteria for that the characteristics. In the development of a new analytical procedure, the choice of analytical instrumentation and methodology should be based on the and scope of the analytical method. Analytical procedures developments are primarily intended purpose based on a combination of mechanistic understanding of the basic methodology and prior experiences.

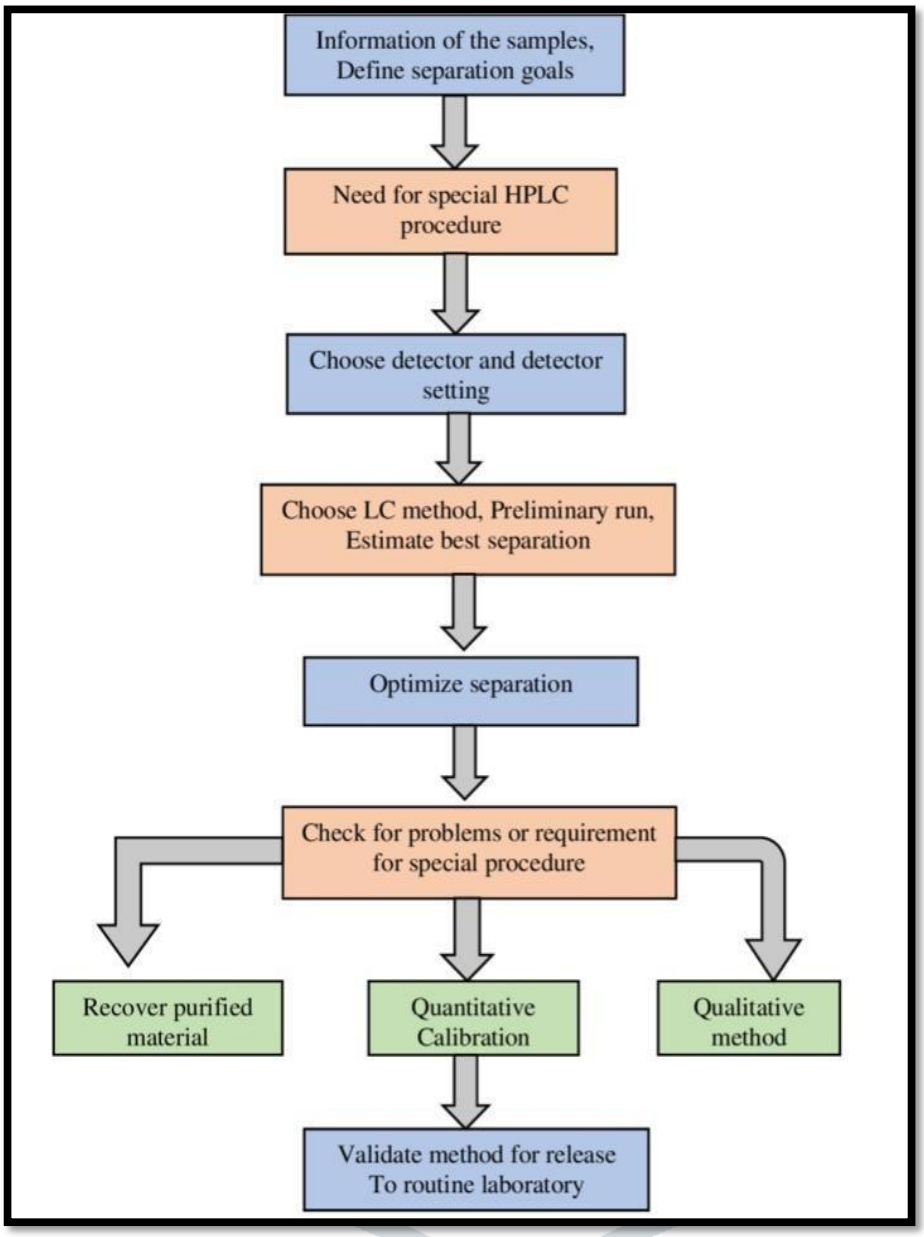


Fig. 1.6 Steps involved in HPLC method development

Methods development involves the following steps:

1) Understanding the Physicochemical properties of the drug molecule.2)Selection of chromatographic conditions.

3)Developing the approach of analysis.

4)Sample preparations

3) Method optimization

4) Method validation

1) Understanding the physicochemical properties of the drug molecule:[14]

The physicochemical properties of a drug molecule play an important role in method development. for method development one has to study the physical properties like solubility,polarity, pka and PH of the drug molecule. polarity is a physical property of a compound. it helps an analyst, to decide the solvent and composition of the mobile phase. in a nonpolar covalent bond, the electrons are shared equally between two atoms. a polar covalent bond isone 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 i.e.,materials with similar polarity are soluble in each other. selection of diluents is based on thesolubility of analyte. The analyte must be soluble in the diluents and must not react with any ofthe diluent components. pH and pka plays an important role in HPLC method development. Theph value is defined as the negative of the logarithm to base 10 of the concentration of the hydrogen ion

PH = - log10[h3o+].

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) Selection of chromatographic conditions:[15]

The

during the early stages of method development, a set of beginning conditions (detector,column, and mobile phase) is chosen to generate the sample's first "scouting" chromatograms. these are typically based on reversed phase separations on a c18 column with UV detection. atthis point, a choice should be taken between establishing an isocratic or a gradient method.

. Selection of column:[16]

The first and most significant stage in method development is the selection of the stationaryphase or column. it is impossible to develop a robust and reproducible procedure without theavailability of a stable, high-performance column. columns must be stable and reproducible to avoid difficulties caused by irreproducible sample retention during technique development. ac8 or c18 column made of particularly purified, less acidic silica and specifically intended forthe separation of basic chemicals is generally suitable for all samples and is strongly recommended. the key ones include column diameters, silica substrate qualities, and bondedstationary phase characteristics. due to a variety of physical properties, silica-based packing is preferred in the majority of today's HPLC columns.
The hardware, matrix, and stationary phase are the three primary components of an HPLC column silica, polymers, alumina, and zirconium are some of the matrices used to sustain thestationary phase. the most common matrix for HPLC columns is silica. silica matrices are strong, easily derivatized, produced in constant sphere sizes, and do not compress under pressure. most organic solvents and low PH solutions are chemically stable to silica. one disadvantage of a silica solid support is that it dissolves above pH 7 .

Selection of chromatographic mode:[17]

The chromatographic modes are determined by the molecular weight and polarity of the analyte. all case studies will concentrate on reversed-phase chromatography (rpc), which is the most prevalent technique for tiny organic compounds. rpc is frequently used to separate ionizable substances (acids and bases) using buffered mobile phases (to keep the analytes from becoming ionized) or ion-pairing reagents.

Buffer selection:[18]

It is a different buffer, such as potassium phosphate, sodium phosphate, and acetate, were tested for system compatibility factors and overall chromatographic performance

General consideration for buffer selection:[19]

Phosphate dissolves more easily in methanol/ water than in acetonitrile/water or thf/water. some salt buffers are hygroscopic, which can cause chromatographic alterations such as enhanced tailing of basic chemicals and possibly selectivity discrepancies. in general,ammonium salts are more soluble in organic/water mobile phases. trifluoroacetic acid degrades

with time. it is a volatile substance that absorbs at low Uv wavelengths. microbial growth canoccur quickly in buffered mobile phases containing little or no organic modifier. the growthbuilds on the inlets of the columns and can impair chromatographic performance. at PH morethan 7, phosphate buffer increases silica dissolution and significantly reduces the lifetime ofsilica-based HPLC columns. organic buffers should be utilized, if possible, at Ph levels higherthan 7. ammonium bicarbonate buffers are typically prone to PH shifts and are only stable for24 - 48 hours. because Of carbon dioxide emission, the PH of this mobile phase tends to become more basic. after preparing the buffers, they should be filtered using a 0.2-m filter degassing of mobile phases is required.

Buffer concentration:[20]

A Buffer concentration of 10-50 mm is usually sufficient for small molecules. in general, a buffer should not contain more than 50% organic material. this will be determined by the typeof buffer as well as its concentration. the most frequent buffer systems for reversed-phase HPLC are phosphoric acid and its sodium or potassium salts. when testing organophosphatechemicals, sulfonate buffers can be used instead of phosphonate buffers.

Selection of mobile phase:[21]

The mobile phase influences resolution, selectivity, and efficiency. the composition of the mobile phase (or the strength of the solvent) is critical in RP-HPLC separation. acetonitrile (acn), methanol (me oh), and tetrahydrofuran (thf) are regularly used solvents in RPHPLC, withUV cut-offs of 190, 205, and 212nm, respectively. these solvents are miscible with water. during technique development, an acetonitrile-water mixture is the ideal initial choice for the mobilephase.

Selection of detectors:[22]

The detector is a critical component of HPLC. the detector to be used is determined by the chemical composition of the studies, potential interference, the detection limit required, detector availability, and/or detector cost. UV detectors, fluorescence detectors, electrochemical detectors, refractive index (ri) detectors, and mass spectrometry (MS) detectors are examples of commercial detectors used in lc. the detector used is determined by the sample and the goal ofthe analysis.

3. Developing the approach of analysis.[23]

The initial stage in developing an analytical method for Rp-HPLC is to select various chromatographic parameters such as mobile phase, column, mobile phase flow rate, and mobile.

phase ph. all of these characteristics are chosen based on trials, and they are then compared tothe system suitability parameters. typical system suitability parameters include, for example, a retention time of more than 5 minutes, a theoretical plate count of more than 2000, a tailingfactor of less than 2, a resolution of more than 5, and a percent r.s.d. of the area of analyte peaksin standard chromatograms of no more than 2.0 %. in the case of simultaneous estimation oftwo components, the detection wavelength is usually an isosbestic point. the laboratory combination is also analyzed to determine the practicability of the suggested method for simultaneous estimation. following that, the marketed formulation is analyzed by diluting it up to the concentration range of linearity. with time. it is a volatile substance that absorbs at low uv wavelengths. microbial growth canoccur quickly in buffered mobile phases containing little or no organic modifier. the growth builds on the inlets of the columns and can impair chromatographic performance. at PH morethan 7, phosphate buffer increases silica dissolution and significantly reduces the lifetime ofsilica-based HPLC columns. organic buffers should be utilized, if possible, at PH levels higherthan 7. ammonium bicarbonate buffers are typically prone to PH shifts and are only stable for24 -48 hours. because Of carbon dioxide emission, the PH of this mobile phase tends to become more basic. after preparing the buffers, they should be filtered using a 0.2-m filter degassing of mobile phases is required.

4. Sample preparation.[24]

sample preparation is an essential part of HPLC analysis, intended to provide a reproducibleand homogenous solution that is suitable for injection onto the column. the aim of sample preparation is 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 without affecting sample retention or resolution. samplepreparation begins at the point of collection, extends to sample injection onto the HPLC column

5. Method optimization.[25]

This is identifying the "weaknesses" of the method and optimize the method through experimental design. understand the method performance with different conditions, different instrument setups and different samples.

Methods validation: -[26]

The Need to validate an analytical method is encountered by analysis in the pharmaceutical industry on an almost daily basis, because adequately validated methods are a necessity for approvable regulatory filings.

The objective of validation of analytical procedures is to demonstrate that it is suitable for itsintended purpose. the discussion of the validation of analytical procedures is directed to thefour most common types.

- Identification tests.
- Quantitative tests for impurities content.
- Limit tests for the control of impurities.
- Quantitative tests of the active moiety in samples of drug substance or drug product or otherselected components in the drug product.

Methods need to be validation and revalidation.

- Before their introduction into routine use.
- Whenever the condition change for which the method has been validated e.g. instrument withdifferent characteristics.
- Whenever the method is changed and the changes are outside the original scope of the method.

Validation parameters specificity:[27]

Specificity is the ability of the method to measure the analyte in the presence of other relevantcomponents those are expected to be present in a sample. analytical techniques that can measure the analyte response in the presence of all potential sample components should be used for specificity validation.
It is not always possible to demonstrate that a single analytical procedure is specific for a particular analyte. in this case a combination of two or more analytical procedures is recommended to achieve the necessary level of discrimination. a frequently used technique in pharmaceutical laboratories is high performance liquid chromatography (HPLC) and to someextent gas chromatography (gc). in practice, a test mixture is prepared that contains the analyte and all potential sample components.
The result is compared with the response of the analyte. in pharmaceutical test mixtures, components can come from synthesis intermediates, excipients and degradation products. generation of degradation products can be accelerated by putting the sample under stress conditions, such as elevated temperature, humidity or light. specificity in liquid chromatography is obtained by choosing optimal columns and setting chromatographic conditions, such as mobile phase composition, column temperature and detector wavelength. besides chromatographic separation, the sample preparation step can also be optimized for best selectivity.

It is a difficult task in chromatography to ascertain whether the peaks within a sample chromatogram are pure or consist of more than one compound. the analyst should know howmany compounds are in the sample which is not always possible. therefore, the target compound peak should be evaluated for purity.

Accuracy and recovery:[28]

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value andthe value found. thus, accuracy is a measure of the exactness of the analytical method.

Precision:[39]

The precision of an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogeneous sample under the prescribed.

Repeatability:[30]

Repeatability expresses the precision under the same operating conditions over a small interval of time. repeatability is also termed intra-assay precision.

Intermediate precision:[31]

The intermediate precision expresses within-laboratories variation: different days, different equipment, etc. intermediate precision is determined by comparing the results of a method run within a single laboratory over a number of days. a method's intermediate precision may reflect discrepancies in results obtained from

- It is different operators
- It is inconsistent working practice
- It is different instruments
- It is standards and reagents from different suppliers
- The columns from different batches
- They are a combination

The objective of intermediate precision validation is to verify that in the same laboratory themethod will provide the same results once the development phase is over.

Reproducibility: -[32]

Reproducibility expresses the precision between laboratories (collaborative studies usually applied to standardization of methodology). The objective of reproducibility is to verify that the method will provide the same results in different laboratories. The reproducibility of an analytical method is determined by analyzing aliquots from homogeneous lots in different laboratories with different analysts. In addition, typical variations of operational and environmental conditions that may differ from, but are still within, the specified parameters of the method are used. Validation of reproducibility is important if the method is to be used indifferent laboratories. Factors that can influence reproducibility include differences in roomtemperature and humidity, or equipment with different characteristics such as delay volume of an HPLC system, columns from different suppliers or different

batches and operators with different experience and thoroughness.

CONCLUSION: -

The development of analytical methods for drug identification, purity evaluation, andquantification has received a lot of attention in the field of pharmaceutical analysis in recentyears. This review provides a general overview of HPLC method development and validation. A general and very simple approach to developing HPLC methods for compound separationwas discussed. Knowledge of the physiochemical 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 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.

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