JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

ADVANCED TECHNIQUES OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Guided By: ParchandeK.V*

BALWANTRAO CHAVAN COLLEGE OF PHARMACY NAIGAON, DIST NANDED.

Done By: Vaishnavi Ingole.

Vaishanvi Sawle.
Komal Nagre.
Shivani Shinde.

Abstract:

High-performance liquid chromatography (HPLC) is an important analytical method commonly used to separate and quantify components of liquid samples. 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. HPLC is the most often used separation technology for detecting, Separating, and quantifying the drug. HPLC technique development and validation serve critical roles in novel drug discovery, Development, and manufacturing, as well as a variety of other human and animal investigations. This review discusses the manyProcesses involved in developing and validating an HPLC technique. The creation of an HPLC technique is influenced by the chemical Structure of the molecules, the synthetic pathway, solubility, polarity, pH and pKa values, and the activity of functional groups,

among Other factors. Accuracy, accuracy, specificity, linearity, range, limit of detection, the limit of quantification, robustness, and system Suitability testing are all included in the validation of an HPLC technique according to ICH Guidelines.

Keywords: Pressure Liquid Chromatography, Chromatography, Method validation, Method developmen

INTRODUCTION

High-Performance Liquid Chromatography, also Known as High-Pressure Liquid Chromatography, is A type of column chromatography that is Commonly used in biochemistry and analysis to separate,

Identify, and quantify active chemicals It is a popular Analytical technique for separating, identifying, and Quantifying each element of a mixture. HPLC is a Sophisticated column liquid chromatography

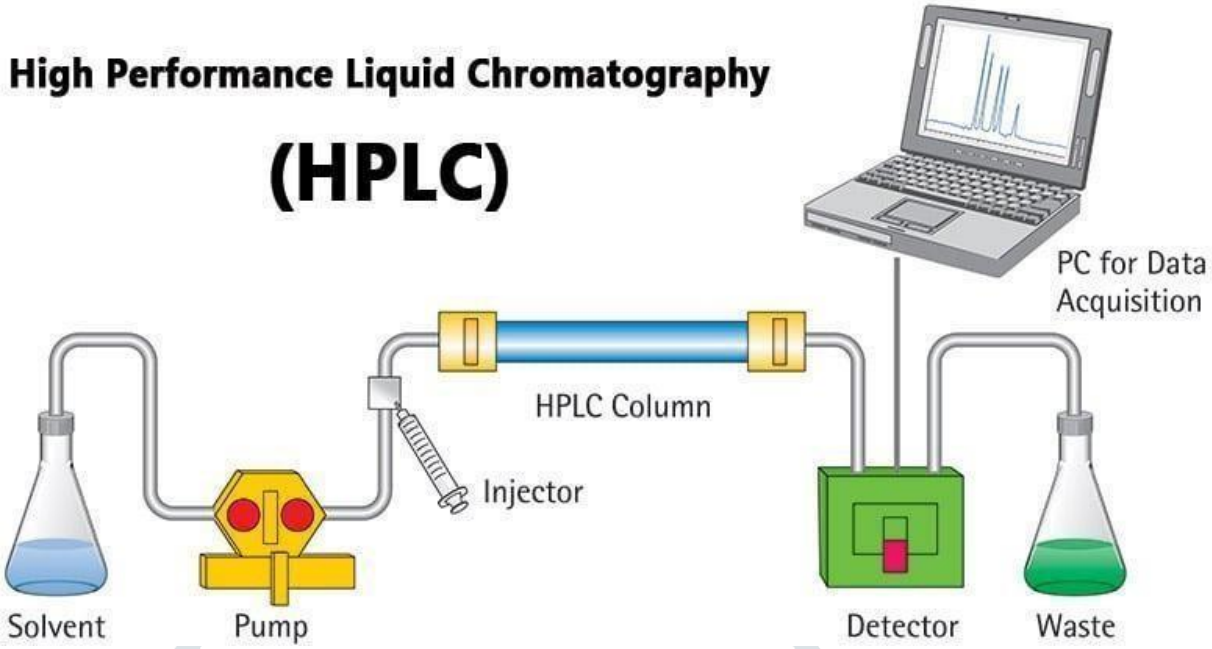
technology. The solvent normally flows through the column due to Gravity, but in the HPLC process, the solvent is pushed Under high pressures of up to 400 atmospheres so that the Sample can be separated into different constituents based On differences in relative affinities. HPLC generally Comprises a column that contains packing material (stationary phase), a pump that drives the mobile phase(s) Through the column, and a detector that detects the Molecule retention times. The retention time is affected By the interactions between the stationary phase, the Molecules being analyzed, and the solvent(s) utilized. The Samples to be analyzed are added in small quantities to the Mobile phase stream and are slowed by specific chemical Or physical interactions with the stationary phase.The Amount of retardation is determined by the nature of the Analyte as well as the composition of both the statand mobile phases. The retention time is the time it takes For a certain analyte to elute.4Any miscible combination of Water or organic liquids is a common solvent. Gradient Elution has been used to change the mobile phase Composition during the analysis. The gradient separates Analyte mixtures based on the analyte's affinity for the Current mobile phase. The nature of the stationary phase And the analyte influence the choice of solvents, additives, And gradients.Easy to fractionate the sample and purify

Principle of HPLC

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) recognizes 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 to 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 (Fig. 1). 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 the injection valve.



Four Types of HPLC Columns:

1. Normal Phase Column
2. Reverse Phase Column
3. Ion-exchange Column
4. Size Exclusion Column

a. Normal Phase Column

In normal phase columns, the mobile phase should be methanol, ethanol, acetone, acetonitrile,chloroform, etc. The use of water as a mobile phase is not recommended in normal phase columns because water has a higher polarity in nature.

The normal phase column is packed with extra molar material than the mobile phase.

The eluent is dissolved in less polar material like methanol, when passes through the column and get in interacts with the high polar stationary phase, thus good separation happened.Such columns are very popular in pharmaceutical product testing.Columns containing silica are very efficient for separating non-polar and moderately polar isometric compounds.

b. Reverse Phase Column

In reverse phase columns as its name states, it is the reverse of the normal phase columns. It has a non- polar or less polar stationary phase than the more polar mobile phase. Bonded hydrocarbons like C8 and C18 and other non-polar hydrocarbons are used as stationary phase in reverse phase columns while aqueous organic solution like water-methanol or water- acetonitrile mixture is used as mobile phase.In reverse phase columns as its name states, it is the reverse of the normal phase columns Separation of sample components in reverse phase columns also occurs on the basis of the polarity of the sample components but it happens just opposite of the normal phase HPLC columns, therefore, this type of chromatography is known as

c. Ion exchange HPLC columns

Ion exchange HPLC columns have charged packing. An ion-exchange column can be either cationic or anionic. This type of HPLC column separates polar molecules based on their charge. The mobile phase is an aqueous buffer. Ion exchange HPLC columns can be used to separate many types of analytes and are commonly used for the separations of carbohydrates, amino acids, and proteins.

d. Size Exclusion Columns

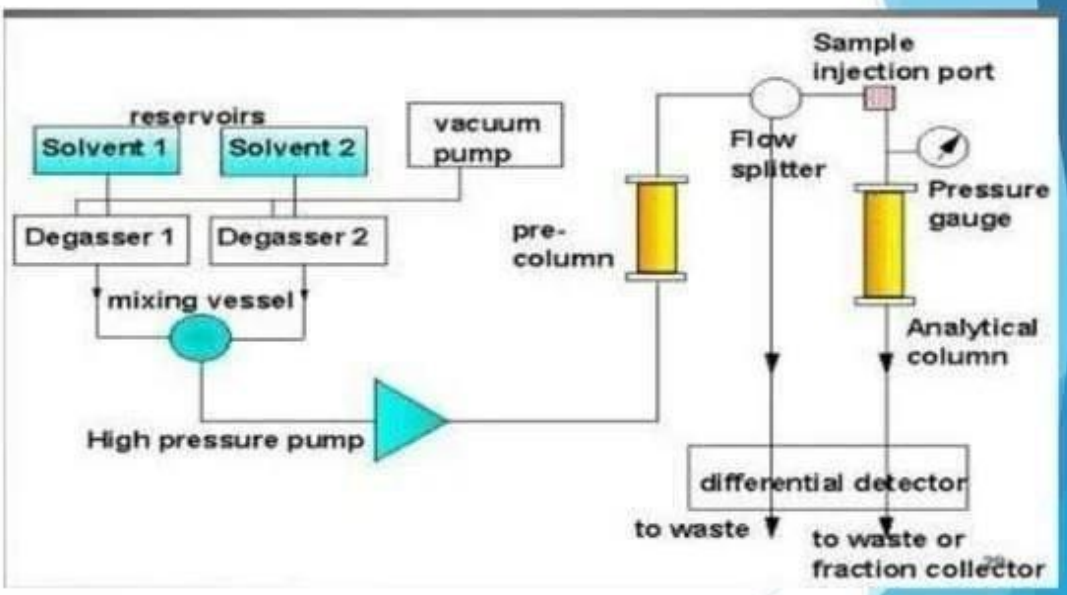
Size exclusion chromatography separates the sample using particle size. It uses a porous stationary phase that only allows small particles into the pores, leaving the larger molecules to pass through the column faster. The pore size in the stationary phase determines the retention time and elution profile of each

Sample component, as each molecule diffuses into the pores to a different extent Instrumentation

INSTRUMENTATION

HPLC instrument consists of following components:

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



HPLC Pump

The solvent or MP must be through a column at high pressure up to 6000 psi. As the Particle size of SP is smaller the resistance to the flow of solvent will be high. Smaller Particle size of the SP the greater is the resistance to the flow of solvent. Hence, High Pressure is recommended.

Requirements for Pumps:

- Generation of pressure about 6000 psi.
- Pulse free output and all materials in the pump.
- It should be chemically resistant to solvent.
- Flow rate range 0.1 to 10 ml/min.
- Pump should be capable of taking the solvents from a single reservoir or more Than one reservoir containing different solvents, simultaneously.

Types of Pumps

1. Displacement Pump
2. Reciprocating Pump
3. Pneumatic Pump

Displacement pump and reciprocating pump are the constant flow rate while Pneumatic pump is constant pressure pump.

1. **Displacement Pump** –It consist of large syringe like chambers equipped with a plunger activated by a screw Driver mechanism powered by a stepping motor, so it is called as screw driven Syringe type pump. Advantage it produces a flow that tends to be independent of viscosity and back pressure. Disadvantage It has limited solvent capacity 250 ml and considerably inconvenient when solvents Must be changed

Simultaneously not working MP.

2. **Reciprocating Pump** This pump transmits alternative pressure to the solvent via a flexible diaphragm, Which in turn is hydraulically pumped by a reciprocating pump. This can be overcome By use of dual pump heads or elliptical comp to minimize such pulsation.

Advantage It has small internal volume of 35-400 ul.

Disadvantage It produces a pulsed flow which is damped because pulses appear or base line noise On the chromatograph

3. **Pneumatic Pump** It is the constant pressure pump. In this pump MP driven through the column withthe Use of pressure produced by Gas cylinder. It has limited capacity of solvent. Due to Solvent, viscosity back pressure may develop. Several injector devices are available Either for manual or automatic injection of

sample septum injector, stop flow injector. Principle HPLC principle is based on adsorption as well as partition chromatography is Depending on the nature of SP, when SP is solid; principle is based on adsorption Chromatography and if SP is liquid; principle is based on partition chromatography. It is important for determination of retention time. The time required after sample Injection maximum angle peak reaches to detector.

Mixing unite:

Most analytical scale HPLC (UHPLC) systems incorporate a solvent mixer which is designed to balance the requirements of moderate dwell volume, low noise and good mixing eefficiency

Degasser:

The degasser continuously removes dissolved gases from liquids using a special degassing membrane. It prevents The formation of gas bubbles caused by dissolved gases, which can cause the pump to

malfunction, and can cause Fluctuations in the detector baseline. The degasser also helps improve the stability and reproducibility of HPLC Analysis.

Injectors in hplc

The Injector source introduce the liquid sample into flow stream of mobile phase .typically sample volumes are 5to 20 ml .

There Are 3 Types Of Injectors 1syringe injector

In this syringe injector there is septum which is made up of elastomeric material which tolerate the pressure or withstand the pressure high as 1500psi

2- stop flow injector

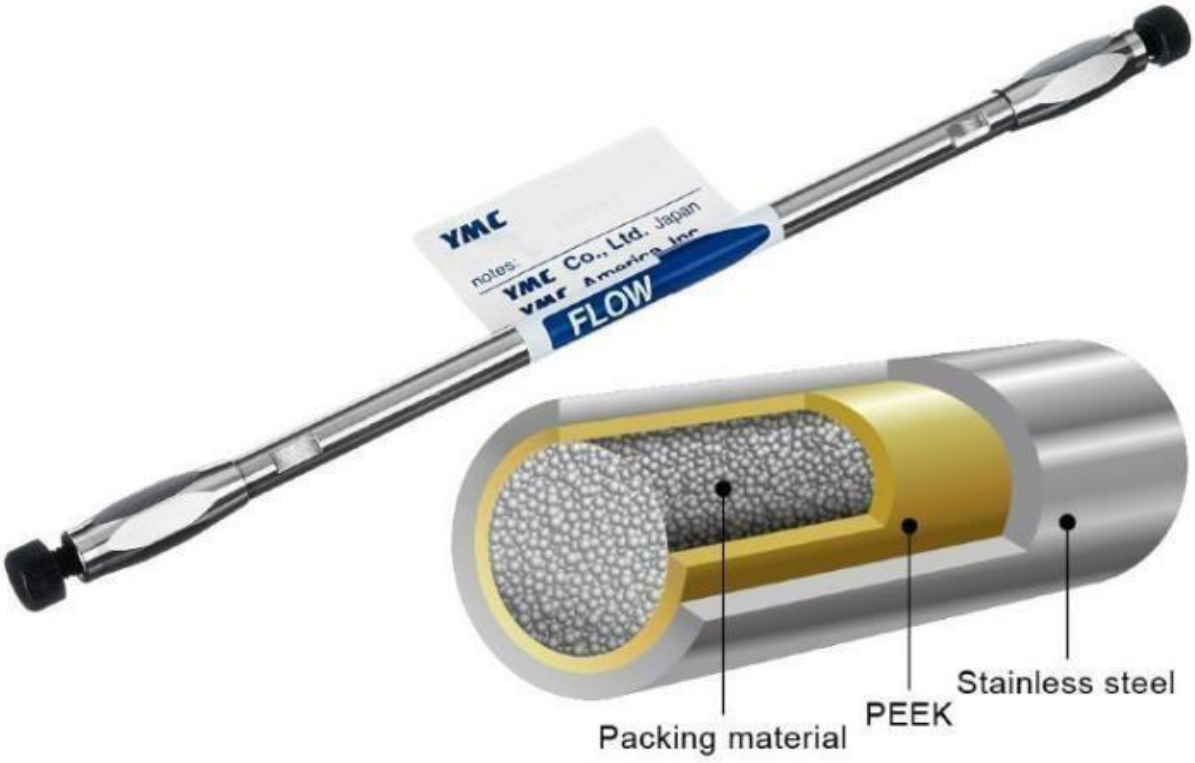
In stop flow injector the flow of mobile phase with sample is stopped for while and sample injected inside the column.

3- rheodyne or loop injector

In rheodyne Or loop injector, It is most popular automated system, for injecting, small amount of sample.This has fixed volume, loop like 20 ml or 50 ml or more

Column:

HPLC columns contain a stationary phase bonded to a support material, usually porous silica particles, to provide a large surface area. The stationary phase provides the basis for separating sample components.



In HPLC there are two types of columns main represent which are c8 and c18

C8 coloumn

C8 column: C8 column is a type of column used in reverse phase chromatography. Generally,reverse phase chromatography used a hydrophobic stationery phase.however, the solid support the stationery phase or the column containing modified silika. Here, silica is a hydrophilic stationary phase.

C8 column is a form of column present in some HPLC Apparatus. And it has octylsilane as a stationary phase and this compound in a stationary phase has 8 carbon atom in its alkayl chain. Further, it tends to retain components of the analytes less than that of the 18 column there for a compound faster in the C8 column

However it is less dence because it has the less number of carbon atom the length of the carbon chain of this compounds in the stationery phase is short moreover the non polar compound moves down the column rapidly with the C8 column. it is mainly because of the lower hydrophobicity of the C8 compound.

Column c18:

C18 column is also a form of a column that we use in HPLC Apparatus ,and it has octadecylsilane as its stationary phase . octadecylsilane (in the stationary phase) has 18 carbon atoms in its alkyl chain .further

,it tends to retain more components of the analyte when compared to C8 column. the analyte will elute slower in this column .

Main difference between column C8 and C 18

What is the Difference Between C8 and C18 Columns?

- C8 column is a form of column present in some HPLC apparatuses, and it has Octylsilane as its stationary phase. The C8 column shows a low retention time. Moreover, the analyte elutes faster in this column. It is because it has a less dense stationary phase.
- C18 column, on the other hand, is also a form of a column that we use in HPLC apparatus, but it has Octadecylsilane as its stationary phase. More importantly, this column shows a high retention time. In addition to that, the analyte elutes slowly in this column. It is because of the high dense stationary phase.
- The following infographic presents a detailed difference between C8 and C18 columns as a side-by-side comparison.
- Moreover, C18 is denser than a C8 column. And this increases the path length of the analyte through the column. Also, this allows the separation of more complex compounds. The retention time of this column is high. In addition, the hydrophobicity of the stationary phase is high. It allows the slow elution of nonpolar compounds through the column.
- Applications of this type of column are mainly in environmental science, pharmaceutical industries, chemical analysis, etc.
- C8 column is used while small RT is desired, if hydrophobicity is low, there is less retention time for non-polar analytes, therefore, the non-polar analytes or compounds separate out more quickly with the C8 column. The C8 is selected over the C18, in the reverse phase matrix where the degree of hydrophobicity is low. But, the C18 column is more accepted and broadly used because C18 silica gel interacts with a broad range of analytes, hence it is used in separation, qualitative and quantitative studies in the pharmaceutical industries, chemical analysis, and environmental science.

	C8 Column	C18 Column
Definition	C8 column is a form of column present in some HPLC apparatuses, and it has Octylsilane as its stationary phase.	C18 column is a form of column, we use in HPLC apparatus that has Octadecylsilane as its stationary phase
Stationary Phase	The stationary phase of C8 column contains Octylsilane	The stationary phase of C18 column contains Octadecylsilane.
Retention Time	C8 column shows a low retention time.	C18 column shows a high retention time
Carbon Atom	The number of carbon atoms in C8 column is 8.	The number of carbons atoms in C18 column is 18.
Elution	Analyte elutes faster in C8 column	Analyte elutes slower in C18 column.
Density	C8 column has a less dense stationary phase.	C18 column has a high dense stationary phase.
Hydrophobicity	C8 stationary phase has a low hydrophobicity.	C18 stationary phase has high hydrophobicity.
Movement of Nonpolar Compounds	Nonpolar compounds move down the column rapidly.	Nonpolar compounds move down the column slowly.

Considering the above-stated differences, it is easy to identify some of the major ones.

1. C18 has 18 carbon atoms while C8 has only 8 carbon atoms.
2. C18 has a longer carbon chain, but C8 has a shorter one.
3. C18 has higher retention while C8 has shorter retention.
4. C18 has higher hydrophobicity, but C8 has lower hydrophobicity.

With the above-listed HPLC column comparison, it is easier to know the appropriate column that one should use when performing different types of HPLC.

Detectors

A detector in HPLC is placed at the end of the system. Its work is to analyse the solution which is eluting from the Column. The concentration of individual component of the Analyte is proportional to the

electronic signal coming out of theComponent of the mixture
Features of Detectors Used In HPLC

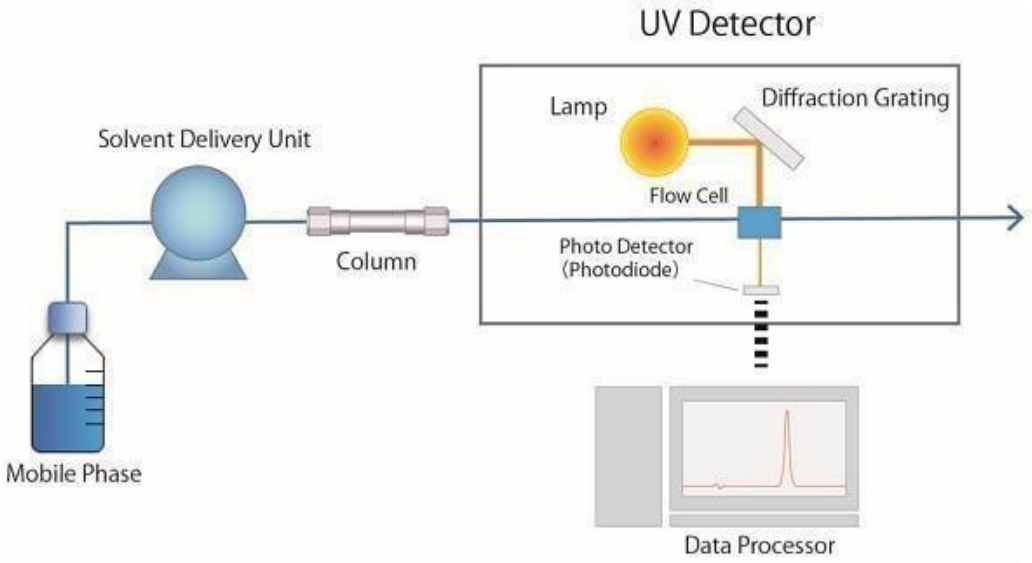
- It must show response for all the components in the Mixture.
- It must show a linear response to the concentration of The analyte.
- Temperature variation must not affect the response.

- It must be independent of eluent composition (gradient).
- It must be capable of tracing even lower concentrations.
- The peaks must not be widened.
- It must produce stable and reproducible signal.
- It should be non destructive

Type of detector :

1. UV-Visible detector

UV-Vis detector in HPLC diagram It is a non-destructive type of detector. That means it does not destroy the sample which is being detected. It is useful for aromatic and unsaturated components. Preparative HPLC is possible by using a UV detector.



Principle: When the solution containing the sample component dissolved in the mobile phase is placed under UV radiation, if the sample component contains conjugation, it will absorb the UV radiation and the transmitted radiation will be detected. If the solution contains the component, it will absorb the UV radiation and if it has only the mobile phase, it will not absorb any radiation.

Working:

A low-pressure mercury lamp is used as a UV source.The solution comes from the column into the sample cell and the reference compound contains the solution containing the desired compound.

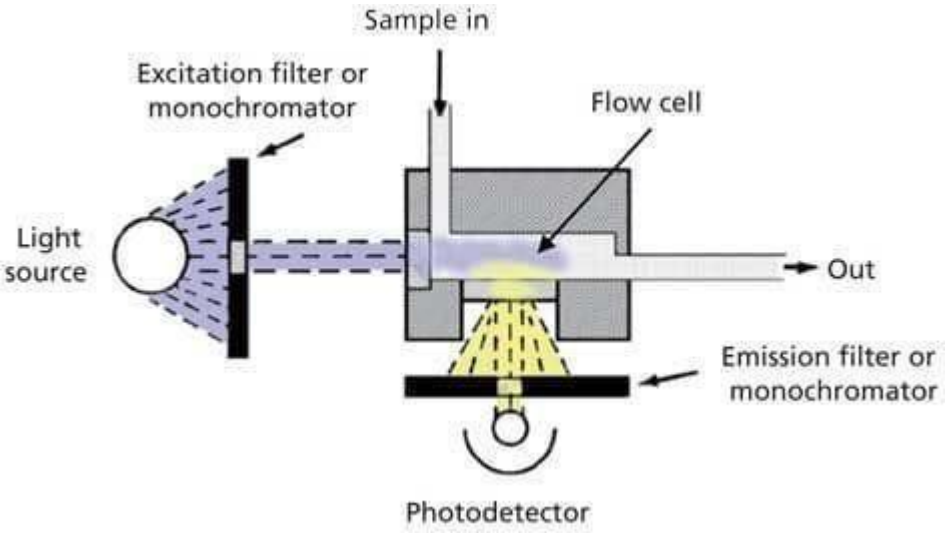
The UV radiation passes through the cells and the transmitted radiation is measured.

There are two ways of measuring the transmitted radiation: fixed wavelength detector and variable wavelength detector.

Fixed wavelength detector uses a monochromator connected to a photodiode detector which measures the absorption at one wavelength.

A variable wavelength detector uses filters that measure the absorbance at a wide range of wavelengths.

2. Fluorescence detector



Principle: The fluorescence detector in HPLC works on the principle of photodensitometry where the compound is detected by derivatizing it with a fluorescence dye that has the same absorption

wavelength as that of the compound of interest.

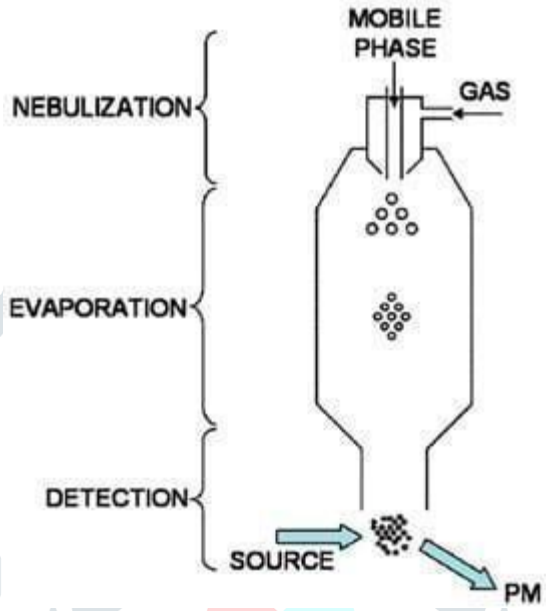
Working:

For compounds that do not show fluorescence, we use a fluorescent dye. It can absorb UV radiation and show fluorescence.

When the solution from the column contains the analyte, it will absorb the UV radiation and get excited. And while going back to the ground state, it can show the fluorescence which Is then detected by the photocell.

When a compound is not naturally fluorescent, the fluorescent dye is used to detect the emission. Examples of fluorescent dyes: Dansyl chloride for amino acids and phenols for protein hydrolysates.

3. vaporative light scattering detector



Principle: When the solution from the column enters the detector, the solvent evaporates and leaves the solute as a solid particulate matter from which a light beam is passed. If the solution contains the

analyte, the light will scatter, and if not then the light will not scatter.

Working:

The solution of the mobile phase containing the analyte passes from the column and it reaches the detector.

It passes through a spray system which atomizes the column eluent into small droplets and the solvent is allowed to evaporate.

The solute remains In the form of particulate matter and it gets suspended in the atomizing gas.

These suspended solute particles pass through a light beam and the light scattered from the particles is viewed at a 45° angle using a pair of optical fibers.

The scattered light then falls on the photomultiplier tube detector and the output is electronically processed.

4. Bulk property HPLC detectors

The bulk property HPLC detectors are the detectors that detect the presence of solute in the solution by relying on the property of the bulk solution and not the solute alone. There are two detectors in this

category: Refractive index detector and electrochemical detector.

5. Refractive index detector

Principle: Refractive index detector which is also known as a differential refractometer works on the principle of the difference in the refractive index of the pure mobile phase and the mobile phase containing solute.

Working:

This detector is a universal detector because it can detect any solute present in the mobile phase and it is not specific for a certain solute.

It contains two cells: one containing a pure mobile phase and another containing the eluent from the column.

If the eluent contains any solute dissolved in it, the refractive index of the overall solution differs from that of the pure mobile phase.

When such a difference is noted, it is represented as a peak in the HPLC chromatogram.

However, this detector is not suitable for gradient elution where the composition of the mobile phase keeps on changing throughout the analysis.

6. Electrochemical detector

Principle: The electrochemical detector detects the presence of electrochemically active groups in a compound by the generation of electric current.

Working:

Two electrodes are placed and the potential difference is applied across the electrodes. When the solution containing an electrochemically active substance enters the detector, it reaches the surface of electrodes via diffusion.

Due to the potential difference applied, an oxidation reaction of the electrochemically active substance takes place which releases electrons and protons.

These electrons and protons are responsible for the generation of electric current across the electrodes and thus the presence of the analyte in the solution is confirmed.

But for this detector to work, the analyte component must contain electrochemically active groups. Examples of such groups are hydroxyl, amine, methoxy, phenol, thiol, carbohydrates,

Detector Used In Infrared (IR) Spectroscopy – Principle, Working with Diagrams December 26, 2022

In “Intrumental Methods of Analysis” Post navigation

Previous PostNext Post

VALIDATION PARAMETERS

As per ICH guidelines Q2 (R1)

1) Accuracy
2) Specificity
3) Linearity
4) Precision
5) Limit of detection (LOD)
6) Limit of quantification (LOQ)
7) Range
8) Robustness

Accuracy: Accuracy is expressed as the nearness of agreement between the values Found and values that are already available. It can also be defined as the closeness Valuevalu Between the true value and the observed value. It is sometimes as trueness, and it Could be determined by using at least 9 determinations over a maximum of 3 Concentrations over the specified range.

Specificity: For every stage of development, the analytical technique should Demonstrate specificity. The technique should have the power to unequivocally assess The analyte of interest whereas within the

presence of all expected parts, which can Encompass degrades, excipients/ sample matrix and blank peaks.

Linearity: Linearity may be characterized as the capacity of an analytical techniqueTo produce outcomes which are directly related to the concentration of an analyte in The sample solution.

Limit of Detection: Lowest quantity of an analyte which may be detected by the Chromatographical separation. However, it is not necessary that this quantity will Quantify as a precise value. A blank resolution is injected and peak to peak Quantitative noise relation we have to calculate from blank chromatograms. Then, Calculate the concentration at the signal to quantitative noise relation is

concerning3.1

Limit of Quantification: It is characterized by the least quantity of an analyte that Can be quantified ith exactness and precision.

Precision: The exactness of an analytical procedure expresses the nearness of Agreement between a groups of measurement obtained from different sampling of a Uniform sample under prescribed conditions. Precision may be taken into Consideration at 3 levels.

Range: It can be characterized as the interval amongst upper and lower quantities of Analyte in the sample. Minimum of the specified range to be 80 % to 120 % of the Sample for the assay test.

Robustness: It is characterized by the level of ability of an analytical technique, to Stay similar by minute purposely change in the technique parameter. The different Technique parameters which can be

modified in HPLC are pH, drift rate, temperature of column and mobile phase composition.

STEPS INVOLVED IN METHOD DEVELOPMENT

1. Physicochemical properties of the active compound 2. Column selection
3. Shape and particle size effect
4. Common stationary phases
5. Mobile phase solvent type
6. Buffer selection
7. Selection of detectors
8. Instrumental set up
9. Preparation of sample solution for method development
10. Optimization of method

ADVANTAGES OF HPLC CHROMATOGRAPHY:-

HPLC has high resolution and speed of analysis.

- High surface area.
- It has high pressure gradient.
- It has wide range of stationary phases.
- Precise flow rate control.
- Senstive detection methods.
- Low sample method requirement.
- Accurate peak identification using HPLC.

DISADVANTAGE OF HPLC CHROMATOGRAPHY:-

- HPLC has high cost.
- High quality components are needed.
- The solvents and columns used in HPLC are expensive.
- Regular maintenance and calibration is needed which add extra cost.
- Sophisticated software is required for data analysis.
- Research and development cost

Limitations of HPLC

Cost: Despite its advantages, HPLC can be costly, requiring large quantities of expensive organics. Complexity HPLC does have low sensitivity for certain compounds, and some cannot be detected as they are irreversibly adsorbed. Volatile substances are better separated by gas chromatograp

Applications of HPLC

The HPLC has developed into a universally applicable method so that it finds its use in almost all areas of chemistry, biochemistry, and pharmacy.

Analysis of synthetic polymers

- Analysis of pollutants in environmental analytics
- Determination of drugs in biological matrices
- Isolation of valuable products
- Product purity and quality control of industrial products and fine chemicals
- Separation and purification of biopolymers such as enzymes or nucleic acids
- Water purification
- Pre-concentration of trace components
- Ligand-exchange chromatography
- Ion-exchange chromatography of proteins
- High-pH anion-exchange chromatography of carbohydrates and oligosaccharides

Conclusion:

High Performance Liquid Chromatography is one of the most widely used analytical techniques. Using this technique, it is possible to produce very pure compounds. HPLC is useful both at the laboratory as well as clinical

level and provides accurate, precise results with increased specificity. In this paper, the authors have tried to conclude that HPLC is a reproducible and versatile chromatographic method for analyzing drug products, having a wide range of applications in both qualitative and quantitative estimation of various biological and drug molecules. Along with this, a number of patents and research have also proved the applicability of the HPLC technique in the healthcare sector in various areas, which paves the path for many successful prospects of this analysis technique.

REFERENCES

1. Rao BV, Sowjanyal GN, Ajitha A, Uma V, Rao M. A review on Stability indicating HPLC method development. World Journal of Pharmacy and Pharmaceutical Sciences.

2. Rasaiah JC. Molecular Theory of Solutions By Arieh Ben-Naim (The Hebrew University, J3erusalem, Israel). Oxford University Press: Oxford, New York. 2006.

3. Kumar Bhardwaj S. A Review: HPLC Method Development and Validation. International Journal of Analytical and Bioanalytical Chemistry 2015;

4..Y.chemguide . High performance Liquid Chromatography (HPLC)

5. Yadav MK, Jaiswal Y, Srivastava S, Yadav S, Yadav P. A Short review On: High performance liquid chromatography. International Journal of Creative Research Thought 2021

6. Gokul M, Shalini S, Satishkumar D,. HPLC Method Development and Validation, International Journal of Advance Research, Ideas and Innovations in Technology.2021

7. Sharma S, Goyal S, Chauhan K,. A Review on Analytical Method Development And Validation, International Journal of Applied Pharmaceutics. 2018

8. Sanap G, Zarekar N, Pawar S,. Review on Method Development and Validation, International Journal of Pharmaceutics and Drug Analysis. 2017

9. Patil P, HPLC Method Development – A Review, Journal of Pharmaceutical Research and Education, 2017

10. Chikanbanjar N, Semwal N, Jyakhwa U, A Review Article on Analytical Method Validation, Journal of Pharma Innovation. 2020

11. Ramole R, Baile M, Jain A, A Review: Analytical Method Development and Validation, A Multifaceted Review Journal in The Field of Pharmacy. 2022

12. Chilukurni S, Sahoo N, .A Review on Stability Indicating Method Development And Validation Using HPLC, American Journal of Phytomedicines and Clinical Therapeutics. 2021

13. Shinde M, Kumar S, Mallik A, Jyothi N, A Review on HPLC Method Development and Validation, EPRA International Journal of Research and Development. 2021