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HPLC CHROMATOGRAPHY

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INTRODUCTION

Chromatography is an analytical technique for the separation of a mixture. The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase. The various constituents of the mixture travels at different speed, causing them to separate. High Performance Liquid Chromatography is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different component and leading to the separation of the component as they flow out the column.

Different wavelengths of light thereby providing a more complete profile of the plant than is typically observed with more specific types of analyses.

HPLC (High performance Liquid chromatography) is the automated, sophisticated form and improved method of TLC.

- It is a powerful analytical method equally suitable for qualitative and quantitative analytical tasks.
- HPLC is very popular for many reason such as
- Visual chromatogram,
- Multiple sample handling,
- Enables the most complicated separation,
- It is also knows as planer or Flat Bed chromatography
- HPLC is a Well Known and versatile separation method which shows a lot of advantages and options in comparison to other separation techniques.
- The method is fast and inexpensive. It does not require time consuming pre-treatments.
- HPLC is an entire concept that includes a widely standardized methodology based on scientific facts as well as the use of validated methods for qualitative and quantitative analysis.
- High performance Liquid chromatography (HPLC) is a sophisticated and automated form of TLC
- (HPLC) is a form of thin liquid chromatography (TLC) that provides superior separation power using optimized coating material, novel procedures for mobile –phase feeding layer conditional and improved application.
- The basic difference between conventional TLC and HPTLC is only in particle and pose size of the sorbents.
- The principle of separation is similar that TLC adsorption.
- It is very useful in qualitative analysis of pharmaceuticals.
- HPTLC is physical separation technique in which a sample dissolved in a liquid is injected into a column packed with small particles and it is separated into its constituent components
- HPTLC is probably the most important and widely used analytical technique for quantitative analysis of organics and biomolecules
- HPTLC is applicable to many kind of samples:
- Most useful for pharmaceuticals, biomolecules, and labile organics
- HPTLC is one type of planner chromatography and most advanced from of instrumental TLC. Now a day, HPTLC is more useful than TLC and HPLC. Because HPTLC is independent of sample application, chromatography development, etc. It is not only instrumental TLC but entire concepts that include widely standardize methodology based on validated method. It is instrument controlled by software. In this review article, we discussed about which type of instrument used in HPTLC, complete HPTLC methodology, How HPTLC better than TLC.

Planar chromatography as opposed to column chromatography (e.g. GC, HPTLC) utilizes a flat (planar) stationary phase for separation. In Thin-Layer

chromatography (TLC) this stationary phase is supported by a glass plate or a foil (plastic or aluminium). Again unlike column separations, the TLC plate constitutes an open system, which passes through the individual steps of the TLC analysis in an off-line mode.

The relative independence of sample application, chromatogram development, detection, etc. In time and location makes possible the parallel analysis of many samples on the same plate. The most advanced form of instrumental TLC is commonly called high performance thin-layer chromatography (HPTLC), but the term does not simply imply instrumental TLC on special high performance layers. HPTLC is an entire concept that includes a widely standardized methodology based on scientific facts as well as the use of validated methods for qualitative and quantitative analysis. Sophisticated instruments, controlled by an integrated software platform ensure to the highest possible degree the usefulness, reliability, and reproducibility of generated data. HPTLC is therefore the term for a method that meets all quality requirements of today analytical labs even in a fully regulated environment. Initial costs for an HPTLC system as well as maintenance, and cost per sample still remain comparatively low and all advantages derived from the planar separation principle are certainly maintained. The possibility of visual evaluation of separated samples on the plate is one the most valuable aspects of TLC. It reaches a completely new dimension in HPTLC through the use of modern techniques for generating and evaluating digital images.[1-2]

HPLC is an enhanced form of **thin-layer chromatography** (TLC). A number of enhancements can be made to the basic method of thin-layer chromatography to automate the different steps, to increase the resolution achieved and to allow more accurate quantitative measurements.

Automation is useful to overcome the uncertainty in droplet size and position when the sample is applied to the TLC plate by hand. One recent approach to automation has been the use of piezoelectric devices and inkjet printers for applying the sample.

The spot capacity (analogous to peak capacity in **HPLC**) can be increased by developing the plate with two different solvents, using **two-dimensional chromatography**. The procedure begins with development of sample loaded plate with first solvent. After removing it, the plate is rotated 90° and developed with a second solvent.

HPTLC (high-performance thin layer chromatography) is a sophisticated form of TLC, which provides superior separation efficiency. The HPTLC concept includes validated methods for qualitative and quantitative analysis, and fulfils all quality requirements for use in fully regulated environments.[3-4]

The process steps of HPTLC are identical to classical TLC. The main difference between them is in the characteristics of the separation plate. HPTLC plates are based on optimized silica gel 60 with a significantly smaller particle size than used for classical TLC. This allows a higher packing density and a smoother surface. Hence, sample diffusion is reduced, resulting in compact bands or spots. Furthermore, the smaller particle size and thinner layer significantly increase detection sensitivity and analysis speed.

Analysis of pharmaceutical compounds and newer drugs is commonly used in all the stages of drug discovery and development process. These analytical techniques provide more accurate and précised data, not only supporting drug discovery and development but also post market surveillance. Pharmaceutical analysts work regularly to improve the reliability of existing techniques to cope up the demands for better chemical measurements. Modern pharmaceutical analysis is mainly dominated by costlier instrumental analysis. Hence, many analysts' focus is on developing newer applications, discoveries, and new methods of analysis to increase the specificity and sensitivity of a method.

Analytical methods used in drug analysis are diversified and are still being improved to find better solutions to satisfy manufacturers and institutions that test drug quality. Official documents dealing with the problem of QC of pharmaceutical products recommend diversified analytical techniques, with chromatographic methods playing a significant role in pharmaceutical analysis.

Thin layer chromatography studies are among the key identity tests in most pharmacopoeia monographs. Pharmacopoeia standards are typically used by industry as a basis for meeting QC requirements and current good manufacturing practices (CGMPs). An extension of TLC is high-performance thin layer chromatography (HPTLC) is robust, simplest, rapid, and efficient tool in quantitative analysis of compounds. HPTLC is an analytical technique based on TLC, but with enhancements intended to increase the resolution of the compounds to be separated and to allow quantitative analysis of the compounds. Some of the enhancements such as the use of higher quality TLC plates with finer particle sizes in the stationary phase which allow better resolution. The separation can be further improved by repeated development of the plate, using a multiple development device. As a consequence, HPTLC offers better resolution and lower Limit of Detection (LODs).[5,6]

Visual detection is suitable for qualitative analysis, but a more specific detection method is needed for quantitative analysis and for obtaining structural information on separated compounds. UV, diode-array and fluorescence spectroscopy, mass spectrometry (MS), Fourier-transform infrared (FTIR), and Raman spectroscopy have all been applied for the *in situ* detection of analyte zones

on a TLC plate. Van Barked and co-workers have recently described couplings of TLC to atmospheric pressure chemical ionization and electrospray ionization. In both couplings, a special surface sampling probe is used for extracting the analyte on-line from the TLC plate to MS analysis.

The usage of HPTLC is well appreciated and accepted all over the world. Many methods are being established to standardize the assay methods. HPTLC remains one step ahead when compared with other tools of chromatography.[7]

One of the available chromatographic techniques is HPTLC, which is used for the identification of constituents, identification and determination of impurities, and quantitative determination of active substances. The use of modern apparatus such as video scanners, densitometers, and new chromatographic chambers, and more effective elution techniques, high-resolution sorbents with selected particle size or chemically modified surface, the possibility of combining with other instrumental methods, and development of computer programs for method optimization all make HPTLC an important alternative method to HPLC or gas chromatography. Specifically, HPTLC is one of the ideal TLC techniques for the analytical purposes because of its increased accuracy, reproducibility, and ability to document the results, compared with standard TLC. Because of this, HPTLC technologies are also the most appropriate TLC technique for conformity with GMPs. Today the comprehensive use of TLC in pharmaceutical analysis is demonstrated by the great number of articles published in this field.[13]

HPTLC remains one of the most flexible, reliable, and cost-efficient separation technique ideally suited for the analysis of botanicals and herbal drugs. Used with standardized procedures, it guarantees reproducible results, a vital element in the routine identification of complex fingerprints of plant extracts and pharmaceutical products. It has established itself as the method of choice for handling complex analytical tasks involving herbal drugs and botanicals. The unique combination of state-of-art instrumentation, standardized procedures, and solid theoretical foundations enables it to deliver reliable, CGMP-compliant results time after time.

High-throughput analysis using HPLTC is being aimed at the rapid analysis of large numbers of compounds. This field has been expedited by the requirement to provide analytical support for multiple drug targets emerging from the field of molecular biology, human genetics, and functional genomics. Further, drivers for development have been in the support for the analysis of large compound libraries arising from parallel and combinatorial chemistry, and economic pressure to reduce time-to-market for new drug candidates.

PRINCIPLE OF HPLC

The principle of separation in normal phase mode and reverse phase mode is adsorption. When a mixture of component is introduced into a HPLC column, they travel according to their relative affinities towards the stationary phase. The component which has more affinity towards the adsorbent travels slower and the component which has less affinity towards the stationary phase travel faster. Since no two components have the same affinity towards the stationary phase, the components are separated

- Stationary phase have small particulate size and high surface areas.
- Columns 20 cm or less
- Mobile phase pumped at high pressures of 200Bar, 3000 psi
- Flow rates 1-3 cm3 per min

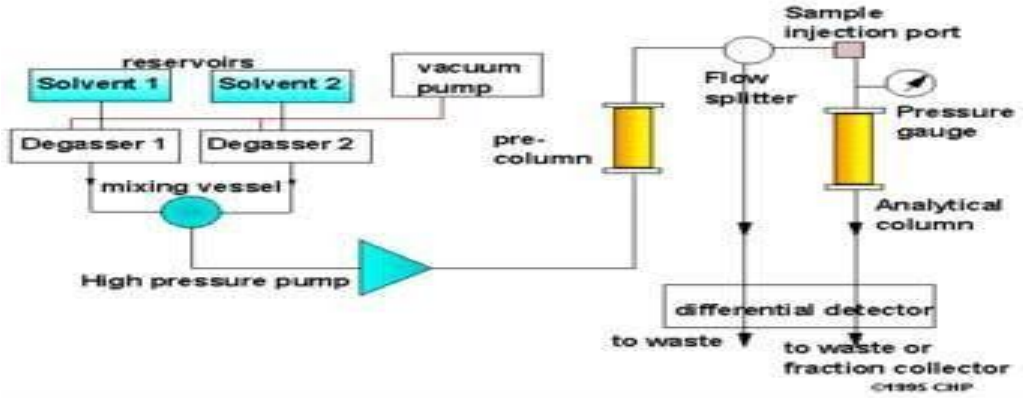
The process involves the interaction of the compound in the analyte (which travels along with a mobile phase) across an immobile surface (stationary phase).

The compounds bind at specific regions of stationary phase based on certain physical and chemical properties. These bound molecules are then eluted with a suitable buffer and same collected with time. These are.

- polarity
- Charge
- Molecular weight
- Present of functional group

1. And component move according to their affinities towards the adsorbent.
2. The component with higher affinity toward adsorbent travels slowly.
3. And the component with lesser affinity towards the stationary phase travels faster.
4. Thus the components are separated on a chromatographic plate according to their
5. Same theoretical principle of TLC (Adsorption chromatography) i.e. the principle of separation is adsorption.
6. Mobile phase flow by capillary action effect.
7. And component move according to their affinities towards the adsorbent.
8. The component with higher affinity toward adsorbent travels slowly.
9. And the component with lesser affinity towards the stationary phase travels faster.
10. Thus the components are separated on a chromatographic plate according to their affinity and separation also based on their solubility in mobile phase.
11. The mobile phase solvent flows through because of capillary action. The components move according their affinities toward the adsorbent. The component with more affinity toward the stationary phase travels slower. The component with lesser affinity towards the stationary phase travels faster. Thus the components are separated on a chromatographic plate.

INTRUMENTATION OF HPLC



- The main parts of HPLC comprises of the following;
- Pump
- Injector
- Column
- Detector
- Data system

PUMP

The main function of HPLC pump is to force a liquid (which is also known as mobile phase) via a liquid chromatograph at a specific flow rate, expressed in milliliters per min (mL/min). The solvent is passed through the column using high pressure.

INJECTION

The injector introduces the liquid sample into the flow stream of the mobile phase. It is mainly used to place a sample into the solvent stream flowing through the column.

Column;

Column is considered to be the most important part of the chromatograph which separates the sample components using various physical and chemical parameters. It comprises of a metal housing in a tube shape which is packed with tiny beads that have an affinity for the chemicals that are being analyzed. The chemical interacts with the beads as soon as they flow through the column in a solvent. The column also has a filter which lets the solvent flow through but keeps the beads from leaving the column.

DETECTOR;

This is used for detecting individual molecules that comes out from the column. There are different varieties of detector that are used for deciding when the chemical should exit from the column.

Some of the detectors used in HPLC includes: Ultraviolet and visible detectors.

- Fluorescence detectors
- Electrochemical detectors Photodiode array detectors Refractive index detectors

DATA SYMTEM

This is a computer that helps control all the modules of the HPLC instrument, takes the signal from the detector and uses it to determine the time of elution (retention time) of the sample components (qualitative analysis) and the amount of sample (quantitative analysis.

TYPES OF HPLC

Type of HPLC generally depend on phase system used in the process.[3,4] Following types of HPLC generally used in analysis.

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.

Reversed phase chromatography: Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. RPC operates on the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar analyte, and the non-polar stationary phase. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand in the aqueous eluent.

Size exclusion chromatography: Size exclusion chromatography (SEC), 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 charged sites bound to the stationary phase. Ions of 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

Bio-affinity chromatography: Separation based on specific reversible interaction of proteins with ligands. Ligands are covalently attached to solid support on a bio-affinity matrix, retains proteins with interaction to the column-bound ligands.

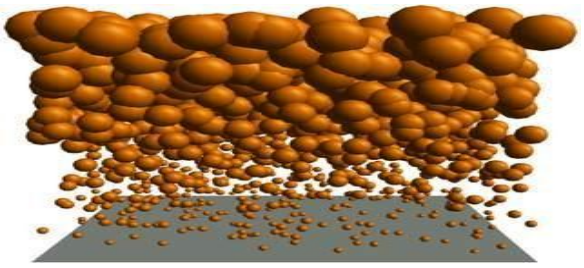
Proteins bound to a bioaffinity column can be eluted in two ways: • Biospecific elution: inclusion of free ligand in elution buffer which competes with column bound ligand. • Aspecific elution: change in pH, salt, etc. which weakens interaction protein with column-bound substrate. Because of specificity of the interaction, bioaffinity chromatography can result in very high purification in a single step (101000-fold).

PARAMETERS

For the accurate analysis of a compound, there are some parameters which are used as a standard for a particular compound. If there is a change occurs in the parameters the result may be affected greatly. The most commonly used parameters are internal diameter, particle size, pore size, pump pressure. For different compounds the parameters can be changed according to their nature and chemical properties.

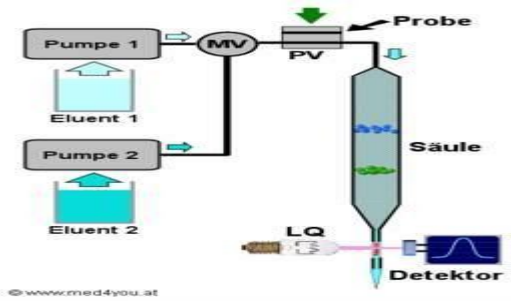
Internal diameter: The internal diameter (ID) of an HPLC column is a critical aspect that determines quantity of analyte that can be loaded onto the column and also influences sensitivity. Larger columns are usually seen in industrial applications such as the purification of a drug product for later use. Low ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity.

Particle size: Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). Smaller particles generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter square.



Pore size: Many stationary phases are porous to provide greater surface area. Small pores provide greater surface area while larger pore size has better kinetics especially for larger analytes. Pore size defines an ability of the analyte molecules to penetrate inside the particle and interact with its inner surface. This is especially important because the ratio of the outer particle surface to its inner one is about 1:1000. The surface molecular interaction mainly occurs on the inner particle surface.

Pump pressure: Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible flow rate. Modern HPLC systems have been improved to work at much higher pressures, and therefore be able to use much smaller particle sizes in the columns (< micrometres).



APPLICATION OF HPLC

The information that can be obtained using HPLC includes identification, quantification, and resolution of a compound. Preparative HPLC refers to the process of isolation and purification of compounds. This differs

from analytical HPLC, where the focus is to obtain information about the sample compound.

Chemical Separations It is based on the fact that certain compounds have different migration rates given a particular column and mobile phase, the extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase.

Purification: Purification is defined as the process of separating or extracting the target compound from a mixture of compounds or contaminants. Each compound showed a characteristic peak under certain chromatographic conditions. The migration of the compounds and contaminants through the column need to differ enough so that the pure desired compound can be collected or extracted without incurring any other undesired compound.

Identification Generally assay of compounds are carried using HPLC. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels which the assay will be performed.

Other Application

1-	**PHARMACEUTICAL/ BIOCHEMICAL**
•	Pharmaceutical quality control
•	Shelf-life determinations of pharmaceutical products
•	Identification of counterfeit drug products
•	Complex molecules separation
2-	CLINICAL
•	Analysis of antibiotics and blood substances
•	Detection of endogenous neuropeptides in brain extracellular fluids
3-	FOOD AND FLAVOR
•	Sugar analysis in fruit juices
•	Ensuring soft drink consistency and quality.

Advantages and Disadvantages of HPLC

High-performance liquid chromatography is one of the most popular and widely accepted separation techniques of chromatography, in which the mobile phase containing a sample of complex mixture pumped with a certain pressure through the column, which already filled with the adsorbent material, analytes interact with the adsorbent according to their properties and detector detects the retention time, area, and height of the peak. Compared to other technologies, HPLC has both advantages and disadvantages, let's check it.

The advantage of HPLC

HPLC offers a rapid, automated and highly precise method to recognize certain chemical components in a sample.

High-performance liquid chromatography offers a fast and precise quantitative analysis.

A gradient solvent system can be applied in certain methods. It is highly reproducible.

HPLC can be upgraded to mass spectroscopy (MS).

The HPLC is very rapid, efficient, and delivers high resolution as compared to other chromatographic techniques, such as TLC, column chromatography, and paper chromatography.

Manages all areas of analysis to increase productivity
The disadvantage of HPLC:

HPLC can be an expensive method, it required a large number of expensive organics, needs a power supply, and regular maintenance is required.

It can be complicated to troubleshoot problems or develop new methods.

The lack of a universal detector for HPLC, however, the UV-Vis detector only detects chromophoric compounds.

The separation in High-performance liquid chromatography has less efficiency than GC.
It is more difficult for the beginner.

HPLC pump process reliability relies on of cleanliness of the sample, mobile phase, and proper operation of the system.

A capsule is a form of solid dose in which the one or more medicine and excipients is enclosed in a hard or soft soluble shell, generally as gelatin.

The Capsule is a common form of dose for oral administration, let's check some Advantages and Disadvantages of Capsules.

Advantages of Capsules

- Capsules can mask the odor and taste of unpleasant medicines and can be simply administered.
- Easy to swallow with water.
- Fewer excipients are required than tablets.
- Easily and rapidly digested.
- It's easy to handle and take.

- The capsule is economical than other dosage forms.
- Appearances of capsules are attractive.
- The gelatin shell can give protection of the drug from light.
- The shells of capsules are physiologically inert.
- The gastrointestinal tract and digested easily and quickly.

Disadvantages of Capsules

- Medicines that absorb water from the hygroscopic capsule shell, make it brittle and therefore are not appropriate for the capsules filling.
- Concentrated solutions requiring previous dilutions are inappropriate for the capsule since irritation of the stomach.
- Not appropriate for highly efflorescent.
- For storage purpose, it required Special conditions.

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