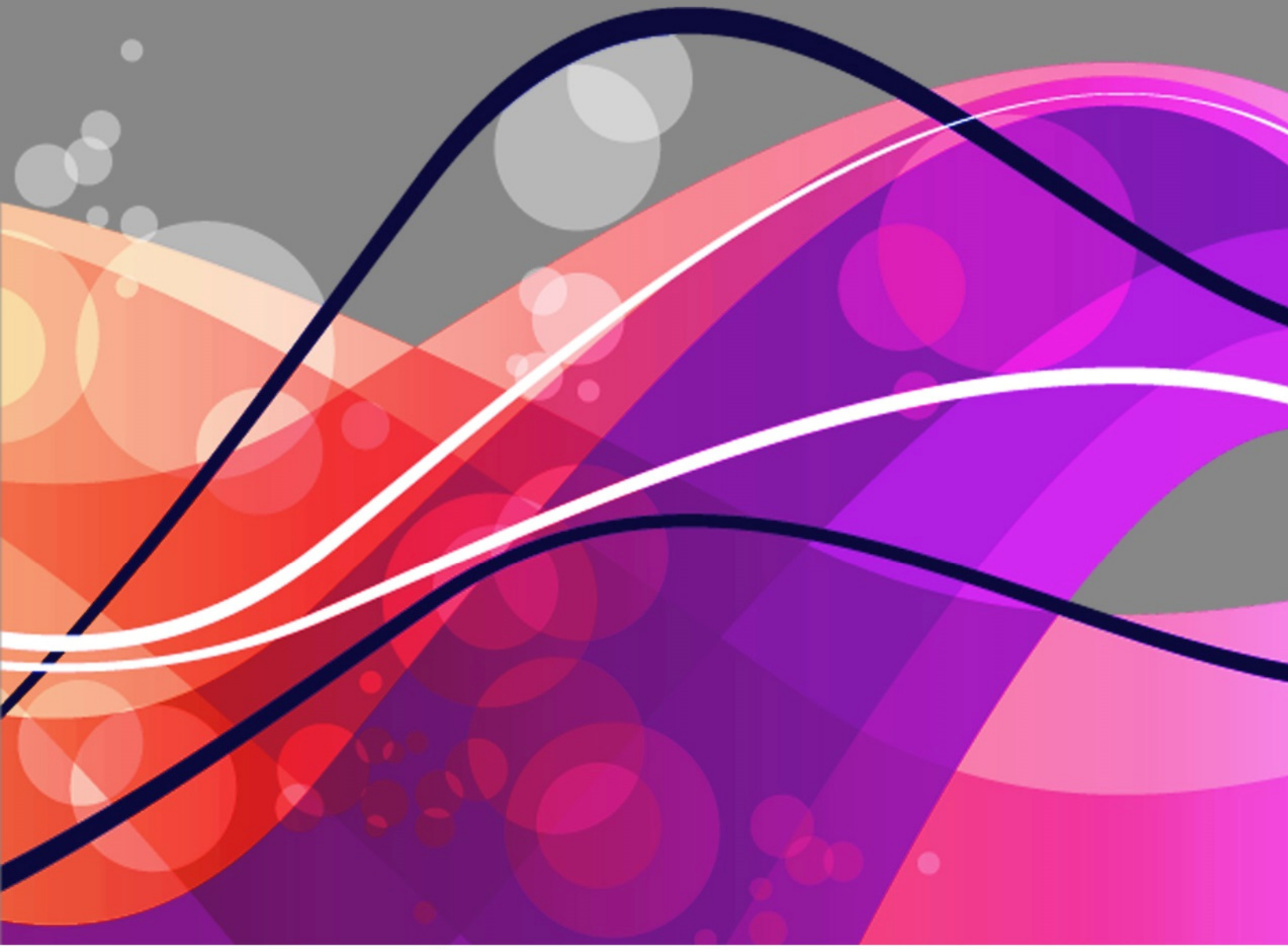
Introduction to High Performance Liquid Chromatography by Lab-Training.com



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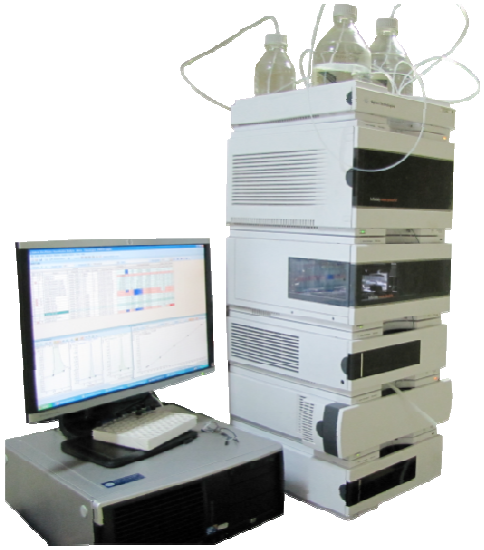

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Author's Profile

Dr Saurabh Arora is a trained pharmacist with Master's and Doctorate degrees in pharmaceutics from reputed Indian institutes NPIER and Jamia Hamdard University respectively.

Managing Director

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He has setup 2 contract laboratories and a clinical research company along with managing and growing the existing business. His organization has grown multiple folds and he has been fortunate to spearhead the growth initiatives backed by a team of over 250 employees..

Specialties: Formulation Development, Analytical Development, Chromatography, Mass spectroscopy, GMP. GLP, GCP, Laboratory designing, Residue analysis, Project management, International business and all that goes into growing and managing a business

Founder

Lab-Training.Com

Lab-Training.Com is developing and offering a series of free and paid E-Learning courses on various analytical and laboratory techniques. He is responsible for the course concepts, course content creation and review and course execution.

Founder

Food Safety Helpline

Food Safety Helpline has been established to help Food Business Operators implement the Food Safety and Standards Act

Dr Deepak Bhanot is a seasoned professional having nearly 30 years expertise beginning from sales and product support of analytical instruments. After completing his graduation and post

graduation from Delhi University and IIT Delhi he went on to Loughborough University of Technology, UK for doctorate research in analytical chemistry. His mission is to develop training programs on analytical techniques and share his experiences with broad spectrum of users ranging from professionals engaged in analytical development and research as well as young enthusiasts fresh from academics who wish to embark upon a career in analytical industry.

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- Knowledge grows when shared with others. Our belief in this has contributed immensely towards growth of our web based portal for sharing our expertise and skills.
- Knowledge does not discriminate between national boundaries color of skin, religion, caste, gender and creed.

Our world class infrastructure, manpower skills and over 25 years of experience is now accessible to web based portal as we moved on from limited classroom training provider role over the last few years.

Our e-learning courses, articles and certificate programmes have been appreciated by industries, institutions, regulatory organizations and even individuals across the globe. There are constant demands for courses and articles on techniques of analytical interest and improvement of laboratory activities. We are bound to upgrade our content keeping the needs of our clients and followers in mind. It will be our endeavor to provide leadership in this key area of development.

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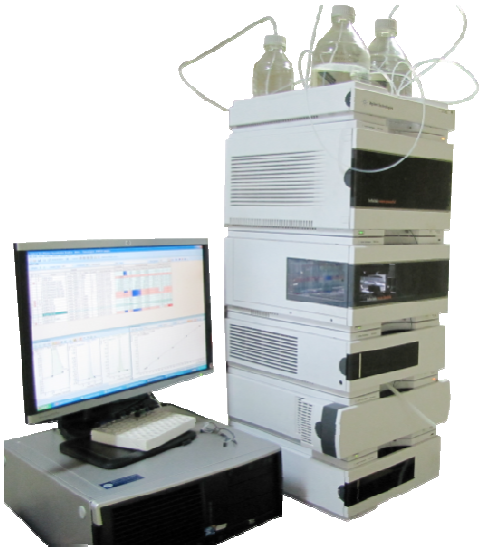
Introduction to HPLC Course and its Objectives

"It is possible to fly without motors, but not without knowledge and skill"

— Wilbur Wright



We understand that everyone has busy work schedules and today's hectic life style leaves you little or no time to refer voluminous books to learn any technique. However, for sustained growth learning has to be adopted as a lifelong habit. In an effort to make your learning task easy we embarked upon the e-Book which comprises of 10 chapters. Each chapter comprising of 200-300 words will provide functional aspects of HPLC and also present useful practical tips.

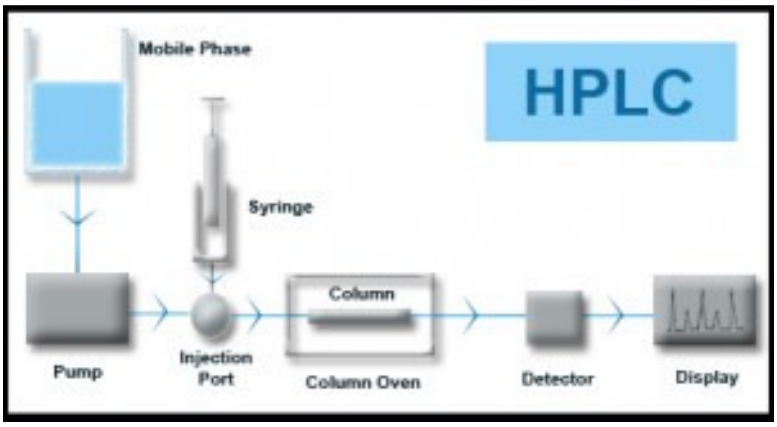
Reading a chapter and understanding it will not take more than about 10 minutes and you will get ample time to assimilate the content before you move to the next chapter. The free programme is designed to give an insight into the technique and once your interest is captivated you can opt for full time advanced online or contact programmes. Such programmes will offer additional benefits of practical exposure and interaction with our technical experts. HPLC has emerged as a major analytical technique in diverse fields such as pharmaceuticals, foods & beverages, forensics, clinical research and environmental laboratories. Growth in applications

has contributed to wide acceptance of HPLC in all areas and pharmaceuticals sector in particular which alone constitutes the largest growth segment.

General Introduction to Chromatography

"Tell me and I'll forget : show me and I may remember; involve me and I'll understand"

---- Chinese Proverb



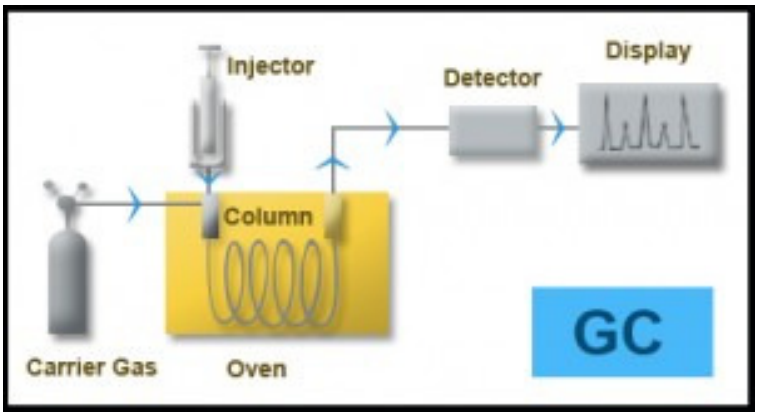
Chromatography is the most widely used laboratory technique for separation, identification and quantification of components of liquid and gaseous mixtures. Solid mixtures are also analyzed by first converting them to a liquid or gaseous state, using suitable sample preparation techniques. Differential affinity of components between the carrier and stationary phases forms the basis of separation. Components retained by stationary phase have slower migration rates than unretained or partially retained components.

You can think of the molecular mixture as a family passing by a candy store which is the stationary phase. The children will tend to get retained because of their affinity for candy, while the parents will keep on moving like un-retained molecules leading to a separation between children and parents!

Various techniques have been adapted to identify and quantify the components that migrate through the chromatographic system in a sequence depending on operating parameters. We will be discussing these detectors in-depth in chapter number 6.

In earlier days liquid mobile phase was commonly used in paper, thin layer and column chromatography. In paper and thin layer chromatography separation of components takes place

as the solvent moves along the filter paper or coated plates by capillary action. In column chromatography an empty glass tube is packed with finely powdered stationary phase and a small volume of liquid mixture is applied to top of column. Mobile phase liquid is continuously added which elutes sample components sequentially based on affinity with stationary phase. The individual components can be detected visually or with a detection system.



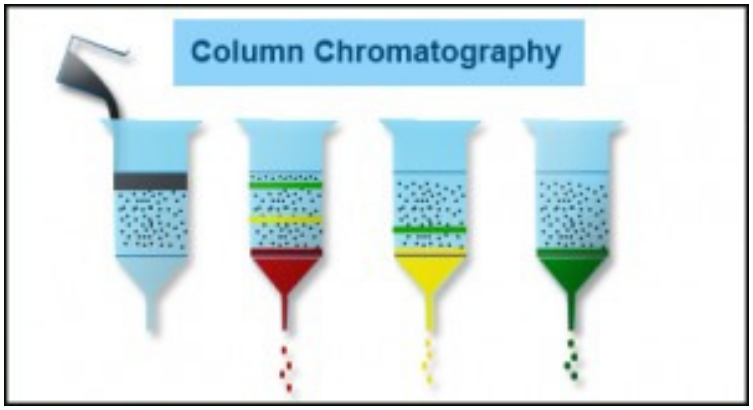
In Gas chromatography same principle is used but carrier is a gas instead of a liquid. The stationary phase is an immobilized liquid bound to an inert support or simply applied to the inner surface of a column. Gas chromatography is effectively used for analysis of gas mixtures or liquids having low boiling points. On the other hand liquid chromatography is applied for separation of thermally labile liquid mixtures or those having high boiling points.

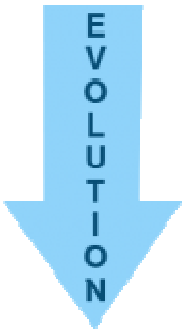
Size exclusion chromatography is based on separation of molecules on the basis of their size .Stationary phases are selected on basis of their pore size and selective retention takes place depending on the pore size of stationary phase .No chemical interaction takes place between the stationary phase and the eluting species.

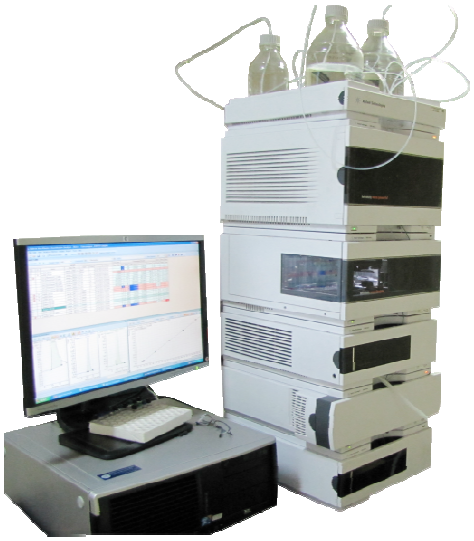
Liquid Chromatography has played a key role in development of HPLC. The next chapter takes you through the evolution of Liquid Chromatography to its present day status.

Liquid Chromatography Evolution

"Learn something new. Try something different. Convince yourself that you have no limits " *--- Brian Tracy*







Liquid Chromatography originated in early 1900 when Russian botanist Mikhail S. Tswett separated plant pigments using calcium carbonate packed glass columns. It was not until mid century that the technique was applied to develop Paper Chromatography, HPLC and GC.

HPLC was originally referred to as High Pressure Liquid Chromatography as high pressure was required to allow liquid to flow through packed columns. However, with continued advances in instrumentation and packing materials the name was changed to High Performance Liquid Chromatography leading to improvements in separation, identification, purification and quantification of complex molecules over previously known techniques.

Advances in pump technology contributed to greater control and flexibility of mobile phase composition. Isocratic operation maintains same mobile phase composition throughout the analytical run whereas gradient elution mode permits composition programming as per analysis requirements.

Column efficiency was known to increase with reduction in particle size. However, non-availability of technology for manufacture of small size particles held back progress in this direction. In 1990's particle sizes in the range of 3-5 µ m were in use. The sub – 2 µ m barrier was broken in 2003 and at Pittcon 2005, columns packed with sub - 2µ m particles were demonstrated. This development launched the era of UPLC (Ultra Pressure Liquid Chromatography) or Fast LC. It became possible to reduce column lengths and diameters to give high separation efficiencies. The advantages could be realized only after technological advancements in instrumentation which permitted operations at high pressures as well as high speed detectors and electronics for capturing fast signals and narrow peaks.

In the recent years there has been increasing interest in the synthesis and separation of Enantiomers due to their importance in biochemistry and pharmaceutical industry. Conventional chromatography could not separate Enantiomers but Chiral Chromatography offered this option for both analytical and preparative scale applications.

In the following years the growth in applications has been phenomenal in areas such as Pharmaceuticals, Life sciences, Foods, Polymers and Forensics. Pharmaceuticals industry alone accounts for 35% of all HPLC instruments and is poised to continue as the biggest end use

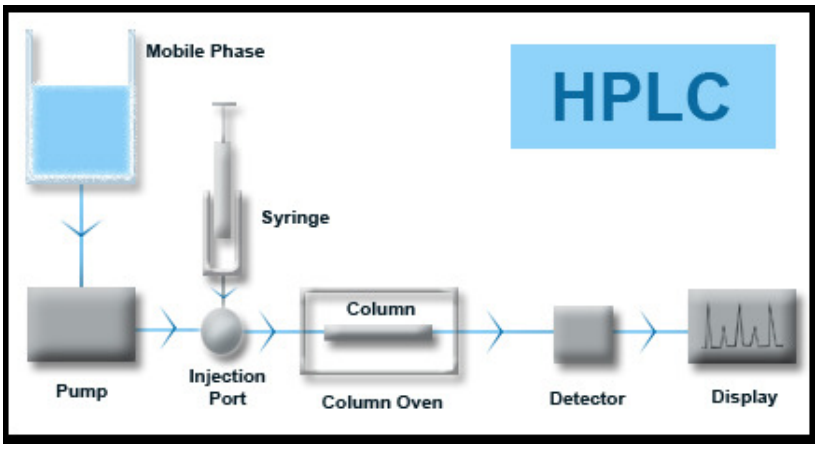
market. Major advances in LC-MS and fast LC have further expanded the scope of applications in addition to advantages of speed, sensitivity and cost saving on expensive solvents. HPLC is set to dominate the analytical instruments market in future as well. The driving force has been the large increase in number of Quality Control, Method Development and drug development laboratories. Demand has been further fuelled by emergence of CRO's and Bioequivalence studies laboratories.

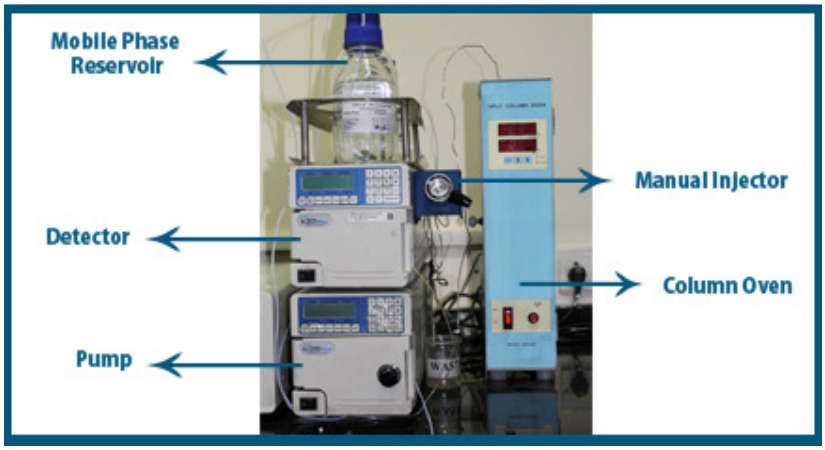
We hope that so far we have captivated your interest and now you would be looking forward to learn about the basic components of HPLC system. The next chapter will help you understand the individual parts of a HPLC system.

Introduction to High Performance Liquid Chromatography and its parts

"The only source of knowledge is experience"

--- Albert Einstein





Chromatography equipment look rather intimidating to anyone who has not handled them before, but on a closer look and as you get familiar with the equipment you realize that behind the network of wires, complex plumbing and circuitry is a simple machine with only a few major parts. Different combinations of these parts namely pumps, detectors and injectors yield an infinite number of configurations based on the application.

Just like an understanding of human anatomy makes you conscious of the vital role of each and every body organ towards your well being and vitality. Similarly you need to have a good understanding of the parts of your HPLC system to generate data of highest reliability. A conceptual understanding of the function of each component will add to your comfort level with your HPLC system. You will ensure long time usage with high reliance on output data. The present chapter is intended to serve this very purpose and in simple terms you will appreciate the role of each part and its contribution to overall system efficiency.

HPLC is a technique for separation, identification and quantification of components in a mixture. It is especially suitable for compounds which are not easily volatalised, thermally unstable and have high molecular weights.

The liquid phase is pumped at a constant rate to the column packed with the stationary phase. Before entering the column the analysis sample is injected into the carrier stream. On reaching the column the sample components are selectively retained on the basis of physico-chemical interactions between the analyte molecules and the stationary phase. The mobile phase moving at a steady rate elutes the components based on the operating conditions. Detection techniques are employed for detection and quantification of the eluted components.

We now introduce you to the significance and role of each component part of the HPLC system.

Mobile Phase

Mobile phase serves to transport the sample to the system. Essential criteria of mobile phase are inertness to the sample components. Pure solvents or buffer combinations are commonly used. The mobile phase should be free of particulate impurities and degassed before use.

Mobile Phase Reservoirs

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

Pumps

Variations in flow rates of the mobile phase effect elution time of sample components and result in errors. Pumps provide constant flow of mobile phase to the column under constant pressure.

Injectors

Injectors are used to provide constant volume injection of sample into the mobile phase stream. Inertness and reproducibility of injection are necessary to maintain high level of accuracy.

Column

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

Column Oven

Variation of temperature during the analytical run can result in changes of retention time of the separated eluting components. A column oven maintains constant column temperature using air circulation. This ensures a constant flow rate of the mobile phase through the column

Detector

A detector gives 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. Majority of the applications require UV-VIS detection though detectors based on other detection technique are also popular these days.

Data Acquisition & Control

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.

These are the main parts of a basic HPLC system more specialized equipment might also have solvent selection valves, vacuum degasser, auto samplers, column switchers, pre or post column derivatization and fraction collectors. These are all covered at length in our upcoming online certificate program on HPLC.

The first three chapters have covered general course introduction, evolution of chromatography and a brief introduction to HPLC component parts. After going through these chapters you would have felt an urge to know more about this popular technique.

You'll be pleased to know that an elaborate certificate programme is also available online. The certificate program is designed to provide you exposure to the technique through 18 sequential steps.To find more click on the link http://lab-training.com/product/join-our-certificate-course-on-hplc/

The next chapter will introduce you to the types of stationary phases used in columns for separation of compounds.

Types of Stationary Phases

"Formal education will make you a living, self education will make you a fortune "

--- Jim Rohn

In Chapter 1 an analogy was given comparing molecular mixture to a family passing by a candy store which is like the stationary phase. Children get retained because of their affinity for candy while the parents keep on moving like un- retained molecules leading to separation between them. Partitioning of sample molecules between a mobile phase and the stationary phase in the HPLC column is based on affinities which tend to hold back some molecules while allowing others to pass through freely.

The HPLC column stationary phase is where the separation occurs and is the most important part of the system. Different types of analysis are classified based on the type of stationary phase and mechanism behind the separation in the column.

The interactions are basically of three types:

- **Polar Interactions**

Differences in polarity between the sample components and the bonding entities on stationary phase result in preferential retention

- **Ionic Interactions**

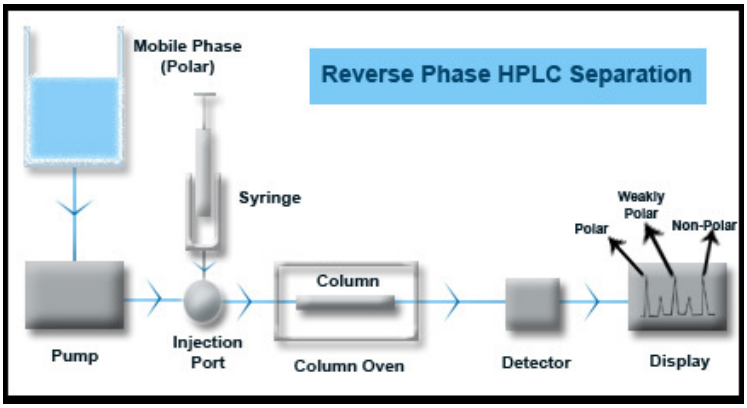
Separation based on charge properties of sample molecules. Analyte ions have affinity for oppositely charged ionic centers on the stationary phase

- **Molecular Size**

Separation takes place due to entrapment of small molecules in the stationary phase pores. Large molecules pass through first followed by elution of smaller trapped molecules.

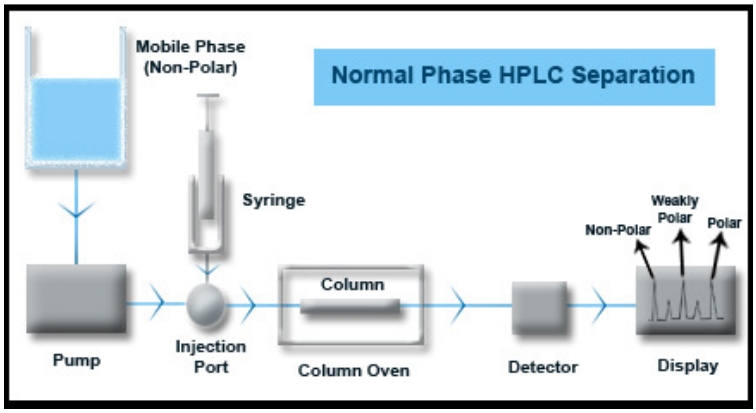
Now we shall briefly discuss the types of HPLC separations based on such interactions.

Reverse Phase HPLC Separations



Majority of HPLC applications are covered under reversed phase chromatography. Stationary phases mostly comprise of non polar alkyl hydrocarbons such as C-8 or C-18 chains bound to Silica or other inert supports. **C18 columns actually can handle more than 60% of the applications in most HPLC labs.** Mobile phase is polar and the elusion order is polar followed by less polar and weakly polar or non-polar compounds in the end.

Normal Phase HPLC Separations



Normal Phase separations are the opposite of reverse phase separations. The stationary phases are polar having either plain silica or organic compounds such as amino, cyano, etc., groups bound to silica based supports. Mobile phases are non-polar such as hexane, heptane, etc. with

small quantity of polar modifiers such as methanol, ethanol, isopropanol, etc. The elution order is non-polar molecules followed by weakly polar and polar molecules in the end.

Ion Exchange Chromatography

Synthetic organic resins are normally employed for separation or water soluble ionizable compounds. Anion exchangers have positive centres on surface and are used to separate compounds having sulfonate, phosphate or carboxylate groups. Cation exchangers have negative centers on the surface and are used to separate basic substances such as amines. Cross-linked styrene divinylbenzene is typical base material with charged groups linked to phenyl rings. Charges on packing material attract oppositely charged molecules from mobile phase and release them in inverse order of the attraction forces. Separation of components can be controlled by control of pH of mobile phase, temperature, ionic composition and addition of modifiers.

Size Exclusion Chromatography

Separation takes place on basis of molecular size of molecules. Small molecules get trapped in the stationary phase pores and exit after the large molecules. There are no chemical or ionic forces involved in the separation process. Such phases are available with silica or zirconium backbones with heavily cross-linked polymers and are used for separation of large molecules such as polysaccharides, peptides, proteins and polymers.

Column length with the same stationary phase has significant effect on separation .Long and wide columns can take higher sample loads and provide higher resolution. On the other hand shorter columns reduce analysis time resulting in lower mobile phase consumption.

After an understanding of stationary phase it is necessary to understand mobile phases which primarily serve to carry the sample through the HPLC system.

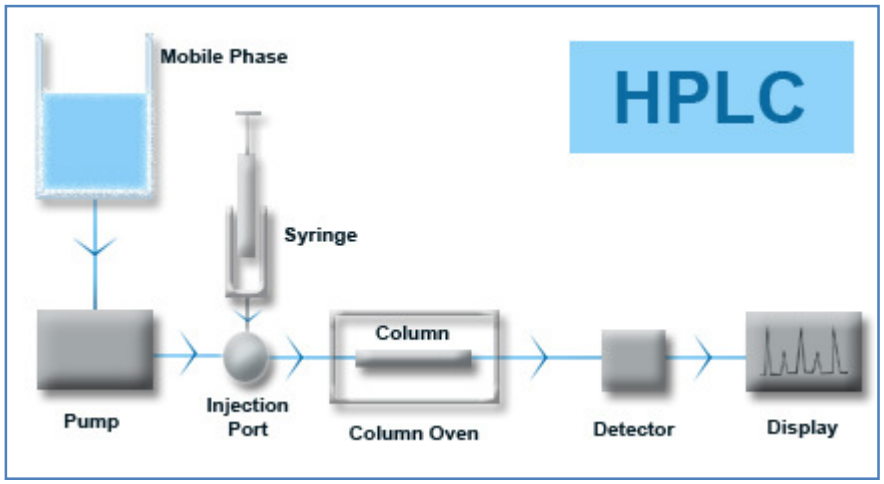
Useful Tip

Never use a column as a stirrer and take care not to subject to mechanical shock as the packing can be irreversibly disturbed.

Types of Mobile Phases

"A man is but the product of his thought, what he thinks, he becomes"

---- M.K.Gandhi



In the previous chapter you were introduced to the stationary phases which serve to retain selectively the sample components. After separation these components need to be transported to the detector for detection and quantification. Mobile phase is the life line of HPLC system as it transports the sample from the injector to the detector and its characteristics such as composition, pH etc, have a profound effect on separation of sample components. The mobile phase should have the following desirable characteristics to carry out this important function.

- Affordable cost
- Non hazardous
- Inertness towards sample constituents and stationary phase
- Sample components should be miscible fully with the mobile phase
- Detector should not respond to mobile phase or to changes in the mobile phase composition as in gradient elution

Mobile phases generally consist of water – organic solvent, aqueous buffer or mixtures of organic solvents with or without modifiers. In isocratic mode the composition of the mobile

phase remains unchanged throughout the analytical run whereas in the gradient elution mode mobile phase composition changes through programming of the pump.

Choice of mobile phase depends on the mode of HPLC operation.

Mobile Phases in Reverse Phase chromatography

In reverse phase applications water is usually the base solvent. Other polar solvents such as Methanol, Acetonitrile or Tetrahydrofuran are added in fixed or varying proportions. pH is adjusted by buffers to modify separations of ionizable solutes. Ion-pairing reagents also enhance separation selectivity of charged analytes by increasing retention on hydrophobic bonding phases.

Mobile Phases in Normal Phase Chromatography

Typically non polar solvents such as hexane, heptane, iso-octane are used in combination with slightly more polar solvents such as isopropanol, ethyl-acetate or chloroform. Retention increases as the amount of non polar solvent increases in the mobile phase.

Mobile Phases in Ion Exchange Chromatography

Aqueous salt solutions are generally used as mobile phases. Moderate amounts of water miscible polar organic solvents such as methanol can be added to buffered mobile phases. Solvent strength and selectivity can be adjusted by control of pH, buffer and salt concentrations.

Mobile Phases for Size Exclusion Chromatography

The mobile phase composition is not varied as the detector is sensitive to such changes.

Choice of mobile phase is dependent on its ability to dissolve sample and maintain consistent viscosity at operating temperature. High polarity solvents such as acetone, alcohols, DMSO and water are not used with polystyrene packings. Ionic strength is maintained by addition of salts.

Precautions in usage of Mobile Phases

Observation of following precautions will save your valuable time and enhance laboratory throughput

- Use HPLC grade water and solvents
- Degas solvents to prevent bubble formation
- Use same method of mixing solvents every time as mixing is not always additives
- Mobile phase changeover should be gradual and wash with solvent of intermediate polarity
- After use of buffer solutions always wash with water to prevent damage to pump parts by crystalline deposits on drying of buffers
- Do not store solvents in plastic container due to possible leaching of plasticizers
- Glass containers are not suitable for aqueous mobile phases with pH > 8.0 as metal ions will leach from glass. Stainless steel containers are suitable in such cases
- Solvent reservoir should have small opening and should be covered
- Oxidizable solvents such as chloroform or THF should be protected with the nitrogen gas cover
- Use solvent inlet filters in reservoirs. Periodically clean solvent lines by back flushing or sonication

The next chapter will introduce you to the commonly used detectors in HPLC

Useful Tip

It is good practice to filter solvents before use as even HPLC grade solvents may contain suspended impurities.

Types of Detectors

"A day spent without learning something is a day wasted"

--- Anonymous

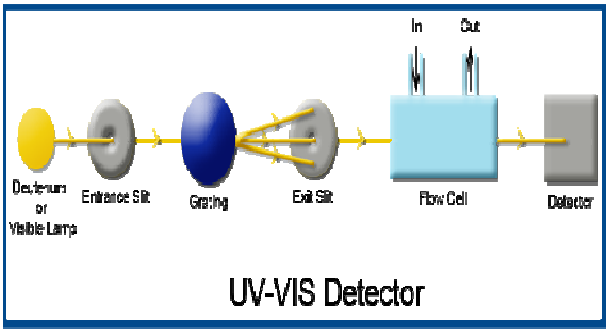
A detector can be compared to a gate watchman who verifies the visitors before permitting them entry inside a building. The chromatographic detector is capable of establishing both the identity and concentration of eluting components in the mobile phase stream. A broad range of detectors is available to meet different sample requirements. Specific detectors respond to a particular compound only and the response is independent of mobile phase composition. On the other hand the response of bulk property detectors is dependent on collective changes in composition of sample and mobile phase.

The desirable features of a detector are:

- Sensitivity towards solute over mobile phase
- Low cell volumes to minimize memory effects
- Low detector noise
- Low detection limits
- Large linear dynamic range

Specific Detectors

UV-VIS Detector



UV-VIS Detector is the most commonly used detector. Its response is specific to a particular compound or class of compounds depending on the presence of light absorbing functional groups of eluting molecules. Some compounds which do not have such light absorbing groups can give suitable response after post column derivatization to introduce light absorbing entities.

Photo Diode Array Detector

Incorporation of large number of diodes which serve as detector elements makes possible simultaneous monitoring of more than one absorbing component at different wavelengths. This provides benefit of time saving and cost reduction on expensive solvents.

Fluorescence Detector

Fluorescence detection offers greater sensitivity than a UV-VIS detector. However, the number of naturally fluorescent compounds is smaller in comparison to light absorbing compounds. This limitation is overcome by post column derivatization.

Mass Spectroscopic Detector

Mass spectroscopy offers very high sensitivity and selectivity. Detection is based on fragmentation of molecules by electric fields and separation on basis of mass to charge ratios of fragmented molecules. LC –MS technique has opened up new application areas due to advantages of resolution and sensitivity.

Bulk Property Detectors

Refractive Index Detector

The response is dependent on changes in refractive index of eluting compounds in the mobile phase. The mobile phase itself should have refractive index different from the sample. Gradient programming is not possible due to resulting changes in refractive index of mobile phase. The detector is less sensitive than UV-VIS detector. Temperature control is necessary as it has high temperature sensitivity. Typical applications are in Size Exclusion Chromatography.

Electrochemical Detector

Based on electrochemical oxidation or reduction of sample on electrode surface. It is, however, sensitive to changes in composition or flow rate of mobile phase.

Light Scattering Detectors

Light scattering detectors are useful for detection of high molecular weight molecules. After removal of mobile phase by passing through a heated zone the solute molecules are detected by light scattering depending on molecular sizes.

Useful Tip

Never exceed pressure abruptly or exceed prescribed pressure limits as it can damage detector cell walls.

The last three chapters covered the stationary phase – solute interactions. mobile phase requirements, and detectors commonly used in HPLC. You will find an elaborate treatment on these and other related topics in the certificate programme.

Please revisit the link http://lab-training.com/product/join-our-certificate-course-on-hplc/. You're sure to get answers to most of your questions without going through voluminous texts and references.

Types of Pumps

"Optimism is the faith that leads to achievement, nothing can be done without hope and confidence"

---- Helen Keller

In the last chapter you appreciated the crucial role played by a detector in the HPLC system. In this chapter you will be introduced to the pump which provides continuous and consistent flow of mobile phase through the HPLC system. A pump can be compared to the human heart which continuously pumps blood throughout the body but though the human heart can withstand changes in blood pressure within specified limit due to stress and strain the HPLC pump is required to deliver flow of mobile phase at constant pressure and flow rate. Changes in both these parameters can lead to errors in the results. In simple language the HPLC pump has to have ruggedness and at the same time should be able to provide reproducible flow characteristics run after run. The operational pressure limits have a vast range depending upon analysis requirements. In normal analytical operation the pressure can vary between 2000 – 5000 psi but in applications covered under UHPLC mode operating pressure can be as high as 15000 – 18000 psi.

An ideal pump should have the following desirable characteristics :

- Solvent compatibility and resistance to corrosion
- Constant flow delivery independent of back pressure
- Convenience of replacement of worn out parts

- Low dead volume for minimum problems on solvent changeover

Three commonly used pump types are Syringe type pumps, Constant pressure pumps and Reciprocating piston pumps.

Constant pressure pumps provide consistent continuous flow rate through the column with the use of pressure from a gas cylinder. Valving arrangement allows rapid refill of solvent chamber. A low pressure gas source is needed to generate high liquid pressures.

Syringe Type Pumps are suitable for small bore columns. Constant flow rate is delivered to column by a motorized screw arrangement. Solvent delivery rate is set by changing voltage on the motor. These pumps deliver pulseless flow independent of column backpressure and changes in viscosity but major disadvantages are limited solvent capacity and limitation on gradient operation

Reciprocating Piston pumps deliver solvent(s) through reciprocating motion of a piston in a hydraulic chamber. On the back stroke the solvent is sucked in and gets delivered to the column in the forward stroke. Flow rates can be set by adjusting piston displacement in each stroke. Dual and triple head pistons consist of identical piston chamber units which operate at 180^0 or 120^0 phase difference. The solvent delivery of reciprocating pump systems is smooth because while one pump is in filling cycle the other is in the delivery cycle. High pressure output is possible at constant flow rate and gradient operation is possible. However, pulse dampening is required for further elimination of pressure pulses.

We now have a fair understanding of different pumps and their operation principles and our next post will deal with injectors for injection of samples into the flowing mobile phase stream.

Useful Tip

After using buffers do not allow salts to dry as these can damage pump components. Always prime with water before shutting the system.

Types of High Performance Liquid Chromatography Injectors

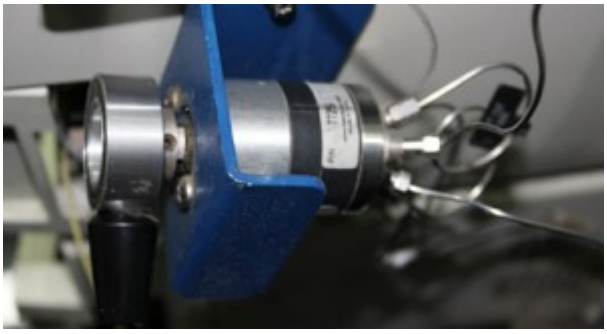
"If you want to increase your success rate, double your failure rate"

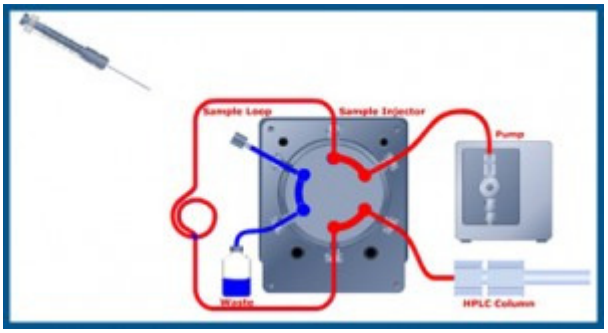
--- Thomas Watson, Sr. founder of IBM

Understanding of injectors is as important as that of pumps in the last session. Injectors serve to introduce required sample volume accurately into the HPLC system.

Sample injection into the moving mobile phase stream in HPLC is quite different from injection into a gas stream in Gas Chromatography as precise injection is required against high back pressure. In such a situation it is not possible to simply inject using a syringe alone.

Manual injection(Rheodyne/Valco injectors)





Injection is done through specially designed 6-port rotary injection valve. The sample is introduced at atmospheric pressure by a syringe into a constant volume loop. In the LOAD position the loop is not in the path of the mobile phase. By rotating to the INJECT position the

sample in the loop is moved by the mobile phase stream into the column. It is important to allow some sample to flow into waste from loop so as to ensure there are no air bubbles in the loop and previously used sample is completely washed out to prevent memory effects.

Automatic Injection

Automatic injection improves laboratory productivity and also eliminates personal errors. Present day advanced HPLC systems are equipped with an auto injector along with an auto sampler. The software programmes filling of the loop and delivery of the sample to the column. The computer also controls the sequence of samples for injection from vials kept in numbered positions of the auto sampler. It is important to adopt precautions to ensure consistency of results and also prolong the service life of the automated system.

- Prime injector with solvents to be used but it should be ensured that solvent is compatible with solvent used earlier.
- Needle wash between samples will prevent carry over between injections.
- Before start and at end of analysis ensure tubing is completely washed of buffers or previously used solvents.
- Do not forget to feed the vial number correctly on auto sampler rack and list out the sequence correctly in the computer.

We have now gained an understanding of HPLC and its systems. The next session will discuss some application areas particularly in analysis of Pharmaceuticals and Foods.

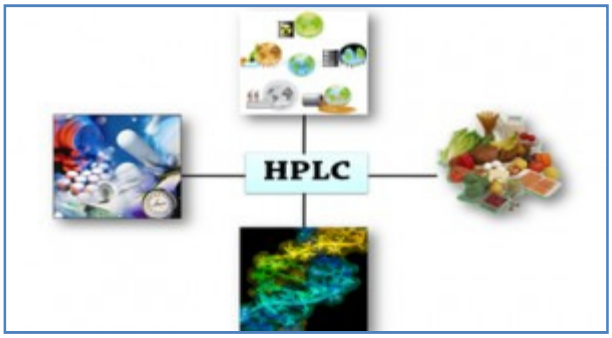
Useful Tip

Injector tubes and loops should be rinsed sufficiently before subsequent analysis to prevent cross contamination.

Applications of High Performance Liquid Chromatography

"Success doesn't come to you, you go to it"

--- Marva Collins



After having gained exposure to High Performance Liquid Chromatography systems and their components we now introduce to typical applications.

HPLC has contributed to analytical solutions in diverse fields such as pharmaceuticals, foods, life sciences, environment, forensics, etc. In the present chapter we shall discuss some application areas in pharmaceuticals and foods.

Pharmaceuticals

High Performance Liquid Chromatography provides reliable quantitative precision and accuracy along with a high linear dynamic range to allow determination of API and related substances in a single run. A convenient method for sample preparation for solid dosage forms is dispersion in water or aqueous media modified with acetonitrile or methanol .HPLC offers several possibilities for separation of chiral molecules into their respective enantiomers.These include precolumn derivatization to form diastereomers. Alternately, specialty columns prepared with cyclodextrins or special chiral moieties as stationary phases maybe used .In short HPLC, particularly reverse phase HPLC is the most popular choice for quantitative analysis in the pharmaceutical industry.

Common application areas in pharmaceutical analysis are :

- Assay
- Related Substances
- Analytical Method Validation
- Stability Studies
- Compound Identification
- Working Standards

Foods

High Performance Liquid Chromatography has brought desirable advantages in the field of food analysis. Food matrices are generally complex and extraction of analytes is not an easy task. To further complicate matters both desirable and undesirable components are often found in trace levels and classical extraction and analysis does not provide the required levels of accuracy and precision. HPLC offers viable solutions due to vast choice of stationary phases and mobile phase options. Common applications in foods are :

- Fat soluble vitamins (A,D,E and K)
- Water soluble vitamins (B-complex vitamins such as B1, B2, B3, B6, Folic acid, Pantothenic acid, B12, VitaminC)
- Residual pesticides such as 2, 4-D and Monochrotophos.
- Antioxidants such as TBHQ, BHA and BHT.
- Sugars: Glucose, Fructose, Maltose and other saccharides
- Cholesterol and sterols
- Dyes and synthetic colours.
- Mycotoxins such as Aflatoxins B1,B2,G1,G2,M1,M2and ochratoxin
- Amino acids
- Residual antibiotics
- Steroids and flavanoids
- Aspartame and other artificial sweeteners.
- Active ingredients of farm produce such as allin in garlic and catachin in tea extracts.

Top 10 Interview questions on High Performance Liquid Chromatography

"Education costs money, but then so does ignorance"

--- Claus Moser

Your understanding and pickup of a topic is gauged by your ability to answer questions related to basics and practical aspects. We now provide you an opportunity to familiarize yourself with some typical questions that you may face in job interviews involving extensive use of High Performance Liquid Chromatography systems

Q1. What are the main differences between High Performance Liquid Chromatography and Gas Chromatography?

A.

- In HPLC the mobile phase is a liquid whereas in GC the mobile phase or carrier is a gas.
- HPLC is useful for analysis of samples which are liable to decompose at higher temperatures. GC involves high temperatures so compounds are stable at such temperatures.
- GC is applied for analysis of volatile compounds whereas non volatile compounds can be easily analyzed on HPLC
- GC cannot be used for analysis of high molecular weight molecules whereas HPLC has applications for separation and identification of very high molecular weight compounds
- HPLC requires higher operating pressures than GC because liquids require higher pressures than gases for transport through the system
- HPLC columns are short and wide in comparison to GC columns

Q 2. Which type of High Performance Liquid Chromatography technique is most widely used?

A. Reverse phase Chromatography has the widest range of applications. The stationary phase comprises non polar organic chains bound to inert silica surface and mobile phase comprises of aqueous or aqueous-organic mixtures comprising of polar solvents of varying degrees of polarity. The elution sequence is polar followed by less polar and least polar or non polar compounds eluting last through the column.

Q3. What is the separation principle in Size Exclusion Chromatography?

A. In size exclusion chromatography the separation does not involve chemical interactions between eluting molecules and stationary phase. The separation takes place on the basis of molecular size with larger molecules eluting first and small molecules in the end. Small molecules are retained longer in the pores of the stationary phase therefore they get eluted last.

Q4. Why is it necessary to degass the mobile phase?

A. Mobile phases entrap air from the atmosphere and this trapped air gets released as small bubbles under high pressures encountered during the HPLC analysis. Such bubbles can lead to noise in detector response or hinder flow of mobile phase through columns. In order to overcome such problems degassing of mobile phase becomes essential

Q5. Which is the most commonly used detector in High Performance Liquid Chromatography and why?

A. The most commonly used detector in HPLC is the UV-VIS detector. The reason for its predominant use is that it gives specific response to a particular compound or class of compounds. Most of the organic compounds absorb at specific wavelengths covered in the available wavelength range of the detector.

Q6. What do you understand by a bulk property detector and a specific property detector?

A. A bulk property detector responds to some property of mobile phase and sample combination passing through it at any point of time such a Refractive index or Electrochemical detector whereas a specific property detector is responsive only to the characteristic property of the eluting molecule and is independent of changes in mobile phase composition such as UV-Vis and Fluorescence detectors.

Q7. What do you understand by Isocratic and Gradient elution?

A. When the composition of the mobile phase is not changed through the chromatographic run the operation is termed as isocratic. It can involve a single solvent or a mixture of two or more solvents mixed in a fixed proportion. In gradient operation the composition at start of run is programmed to change at a predetermined rate and the composition at the end of run is different from the composition at the start.

Q8. What are the desirable features of a High Performance Liquid Chromatography detector?

A. The desirable features of a detector are

- Sensitivity towards solute over mobile phase.
- Low dead volume to eliminate memory effects
- Low noise
- Low detection limits
- Large dynamic linear range

Q9. What do you understand by theoretical plate concept and how HETP affects the separation of HPLC column?

A. Plate theory concept was introduced to explain efficiency of columns. The concept assumes that a state of instantaneous equilibrium exists between the concentration of solute in stationary phase and the mobile phase and further the column is imagined to be divided into a number of theoretical plates. Any analyte spends a finite time in each plate and this is the equilibrium time.

Smaller the plate height the greater is the number of plates in a given length (HETP) and better is the column resolution.

Q10. What are the benefits of Fast LC or UHPLC?

A. Fast or UHPLC technique makes use of small particles below 2 µ size Use of such particle sizes result in high resolution and as small columns can be used it results in completion of analysis in much less time thereby reducing consumption of expensive solvents.

We hope you had a great learning experience through the introductory free e-learning HPLC course. I shall remain in contact with you for our offerings on advance versions of the HPLC e-learning courses and subsequent introduction covering other analytical techniques.

Conclusion

We believe that you enjoyed the free e- course on HPLC. The course provided you an insight into the components of HPLC system and their individual contribution towards the overall accuracy and precision of your results. Apart from a general introduction the course was designed keeping in mind the requirements of the HPLC user. Without going into mathematical treatment of the subject an attempt has been made to convey the basics concepts and offer practical tips on effective utilization of the HPLC system.

The last chapter of the course provides answers to 10 common questions that you may be faced with as you move up your career ladder. However, learning is a lifelong process and there will be several unanswered questions and queries which will be coming up in your mind from time to time. Our suggestion to you would be to post such queries or comments on the site and we shall try to offer clarifications to the best of our ability based on our expertise and experience.

In case the e- course has awakened your desire to go deeper into the subject you are welcome to join the Certificate Course on HPLC which is available round the year. For more details on this advanced treatment of the subject go to the http://lab-training.com/product/join-our-certificate-course-on-hplc/

Once again we take the opportunity to thank you for your interest. Please feel free to participate actively by contributing articles in areas off your interest and offer your valuable comments and suggestions.