Swayam Course - Analytical Techniques

Week: 5, Module 12 - High Performance Liquid Chromatography (HPLC)

Content Writer - Dr. T.Velpandian, Professor, R.P Centre, All India Institute of Medical sciences, New Delhi.

A BRIEF INTRODUCTION TO HPLC

Liquid chromatography (LC) was discovered initially as of *liquid-solid* chromatography (LSC) in late 1890s by the Russian botanist, Tswett to separate and isolate various plant pigments. The colored bands as produced on the adsorbent bed evoked the term chromatography (color writing) for this type of separation. A German organ chemist, Richard Willstätter was awarded the Nobel Prize in Chemistry in 1915, for the invention of *paper chromatography*, wherein, the separation was carried out on the basis of both partition and adsorption. In late 1930s and early 1940s, Martin and Synge introduced a form of *liquid-liquid* chromatography by supporting the stationary phase water on silica gel in the form of a packed bed and used it to separate some acetyl amino acids. They published their work in 1941 and in their paper recommended the replacement of the liquid mobile phase with a suitable gas, which would accelerate the transfer between the two phases and provide more efficient separations. Thus, the concept of *gas chromatography* was born.

Chromatography – a Separation Science

There are some basic principles which govern the separation of the analyte along with time in the given mobile phase. Among them the popular few are adsorption, partition, ion exchange, molecular sieve (gel exclusion) etc. Having hydrophilic stationary phase to separate compounds using hydrophobic mobile phase is called normal phase chromatography and having lipophilic stationary phase to separate compounds with medium polar water miscible solvents is called reverse phase. Typically, most of the drugs like compounds have log-P value less than 5, and most of them are soluble in methanol, therefore, using water soluble solvents like methanol and acetonitrile enable separation possible in the hydrophobic column. The modern day columns with chemically modified stationary phases are capable of reaching far from the primitive packed columns with absorbents like silica, chalk, charcoal, etc. The knowledge of separation is always a skilled imposition even for the expert chromatographer / scientist. Many methods need further validation before adopting it blindly for your analytical purpose.

Types of chromatography

Based on principle of separation

Adsorption chromatography

Adsorption chromatography is one of the primitive type for separation in which the stationary phase (adsorbent) is solid and the mobile phase (adsorbate) can either be liquid or gaseous in nature. Mobile phase molecules adsorb over the active site of the stationary phase and forms hydrogen bond with its hydroxyl groups. Alumina and silica are the most common adsorbents used because of their inert and porous nature which gives large surface area for adsorption.

Solute molecules are dissolved in the solution of mobile phase and travels through the length of stationary phase. Each solute molecule has differential equilibrium towards the

solution of mobile phase and stationary phase. Therefore the solute with more affinity towards mobile phase will elute first followed by the one with more affinity towards stationary phase. Most often polar compounds are separated by this technique. Thin layer chromatography, column chromatography and gas chromatography come under adsorption chromatography.

Thin Layer Chromatography

It is a type of adsorption chromatography in which stationary phase is coated on flat thin sheet or glass support and mobile phase is allowed to run anti-gravity by capillary action.

Column Chromatography

In this type of adsorption chromatography, stationary phase is packed in a column casing and the mobile phase is allowed to run through it to enable separation of compounds from complex mixtures. Flow of solvents is enabled by gravity, suction or applying pressure on mobile phase.

Gas Chromatography

In this type, carrier gas is used as a mobile phase along with varying temperature, to elute the compounds using glass capillary columns coated with stationary phase, which could be either solid or liquid.

Partition chromatography

In partition chromatography partitioning of the solute molecules between two immiscible liquids take place. The stationary phase is a non-volatile liquid which forms a thin layer over the surface of an inert matrix. The mobile phase is liquid or gaseous containing the analyte to be separated. Differential distribution of solute molecules between two immiscible liquids leads to separation. Solute molecule having high affinity towards mobile phase will be eluted first, while those with higher affinity towards stationary phase will be eluted at the end. Both non-polar and polar molecules can be separated using this technique. Paper chromatography and high performance liquid chromatography comes under partition chromatography.

Paper Chromatography

In this type of chromatography, paper is used as a stationary phase with or without special treatment and mobile phase runs by capillary action against the gravity.

Ion-exchange chromatography

In ion-exchange chromatography, retention of the analytes is based on the attraction of solute ion towards the charge sites (anionic or cationic) bound to the stationary phase. Solute ions of the opposite charge to the charged sites of the column are retained on the column and can be eluted by using the mobile phase containing salts, such as, NaCl, ammonium acetate.

Size Exclusion chromatography

In size exclusion chromatography compounds are separated on the basis of their molecular weight. It is also known as permeation or gel filtration chromatography. Hence, the affinity of solute towards the stationary phase is not the driving force towards the separation of compounds. The stationary phase is porous in nature and acts like a molecular sieve. The smaller molecules are trapped in the pores of the stationary phase, while the larger molecules simply pass through, as they are too large to enter the pores. Larger molecules therefore elute quicker than smaller molecules, i.e., the smaller the molecule, the longer the retention time. This is mainly used for the separation of proteins, carbohydrates and polymers.

Based on the column chemistry/type of mobile phase used

Affinity

In affinity chromatography the stationary phase consists of a solid material made of cellulose beads usually of different grades of agarose on which a ligand (co-enzyme or substrate) is bound covalently in such a way that it's active sites are exposed. The specific protein of interest to be purified has affinity towards the bound ligand. The buffer containing mixture of proteins is passed through the length of column and only the specific protein of interest is retained in column as it get bound to its specific ligand and all other proteins get eluted in the void volume. The ligand-protein interaction is reversible. As soon as buffer strength is changed in the mobile phase, the desired protein gets eluted. This technique is mainly used in protein purification, antigen-antibody reaction, separation of hormone receptor, enzyme purification.

Normal Phase

Normal phase chromatography is one of the readily used type of chromatography, wherein, separation of analytes is based on their affinity for the polar stationary phase such as silica. The interaction with the stationary phase happens due to hydrogen bonding or dipole-dipole type of interactions. In this type of chromatography, the mobile phase is non-polar and thus works effectively for separating analytes readily soluble in non-polar solvents. The analyte associates with and is retained by the polar stationary phase. Adsorption strength increases with increased analyte polarity. The interaction strength depends not only on the functional groups present in the analyte molecule, but also on steric factors, which allows this method to separate structural isomers.

Reverse Phase

Reverse phase chromatography consists of a non polar stationary phase and a moderately polar mobile phase. Stationary phase primarily consists of silica with additional straight chain alkyl groups attached to it's silanol groups to render hydrophobicity. The alkyl chain length ranges from C2 to C18 carbon and based on custom made requirements additional groups can be added to modify the column chemistry such as amino, cyano, phenyl, phenyl-hexyl, zwitterions. Non-polar compounds can be well separated by such a stationary phase as they are well retained by the column. Polar molecules are difficult to separate through such stationary phase because they are having very less affinity towards the stationary phase without special modifications.

Based on Pressure

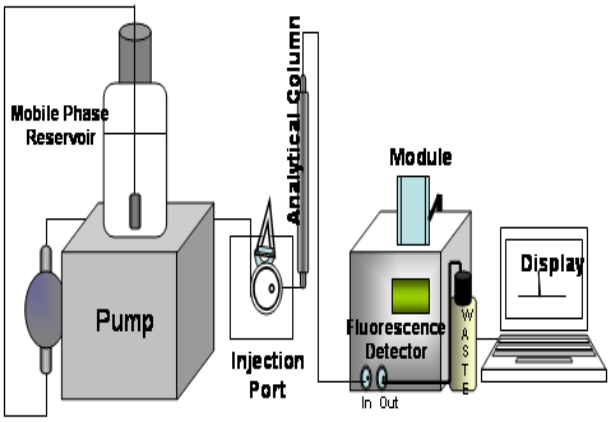
High Performance Liquid Chromatography (HPLC)

The slow speed of separation in normal gravity based liquid chromatography was converted into high performance by the use of bonded phase columns with small particle size of 2-10micron silica surface derivated with various chemistry making it polar to non-polar of various grades. The pressure required to extrude mobile phase out of these reduced particle size stationary phase was achieved by reciprocating pumps which can work at higher pressures such as 3000psi (pounds per square inch). These machines are called High Performance Liquid Chromatography (HPLC).

Ultra High Performance Liquid Chromatography (UHPLC)

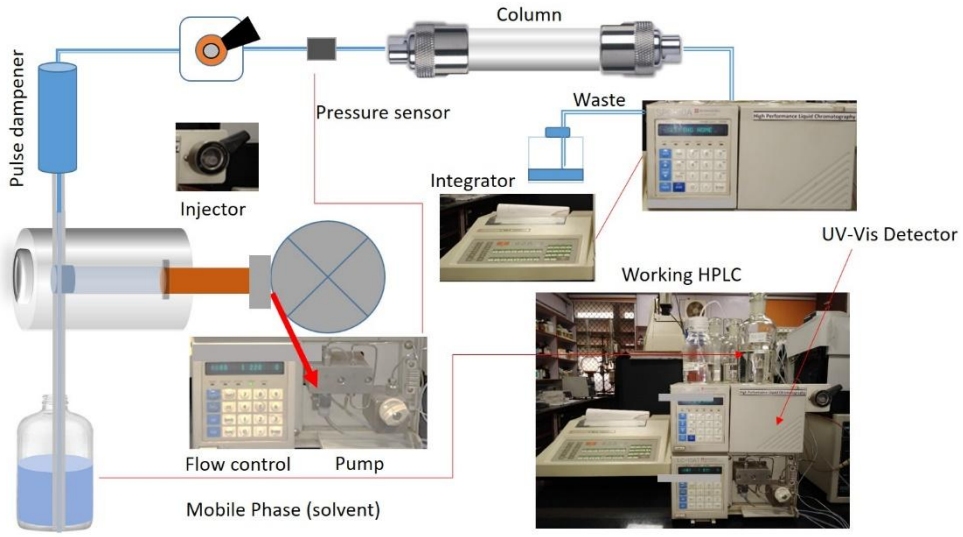
Further lowering of the particle size down to 1.5-2 micron necessitated to opt for mobile phase delivery pumps capable of delivering the required flow rate even at the pressure of 15000LBS. These machines are called ultrahigh performance liquid chromatography (UPLC or UHPLC). These UPLCs are capable of reducing the runtime with a finer separation of compounds and are preferred for connecting to mass spectrometers for precise analysis of compounds.

Basic construction of HPLC



An HPLC system consists of six basic units. The mobile phase supply system, the pump and programmer, the sample valve, the column, the detector and finally a means of presenting and processing the results.

Instrumentation



Mobile phase

It is the phase that elutes the sample from the stationary phase. Mobile phase in a HPLC system consists of a solvent system that passes through the length of HPLC system. There are two types of mobile phase ratio that can be utilized: Isocratic and Gradient. In an isocratic ratio, constant solvent system ratio is used throughout, while in gradient system, the ratio of mobile phase composition changes with respect to time. Based on the chemistry of analyte of interest, the mobile phase composition is selected. Non-polar analytes are separated by the use of organic solvents that includes methanol, acetonitrile, n-hexane, dichloromethane, tetrahydrofuran, frifluoroacetic acid etc.

Derivatizing agents are added in some of the analytes of interest for their enhanced resolution in HPLC system. The process can be pre-derivatization or post-derivatization based on the requirement. Ion-paring reagents are added in the mobile phase to enhance the analyte's affinity towards column that leads to its better resolution.

<u>Why the buffers in the mobile phase?</u>

Buffers are used to control the ionization of one or more molecules in the solution and to provide separate sharp bands. This depends on

- pH
- pKa

pKa for each ionizable function of a molecule is the pH at which equal concentrations of the ionized and unionized form exists. Organic acids have pKa around pH 4.5 and amines have pKa between pH 9.0 and 10.5. Below 2.5, organic acids exist mainly in the protonated, free acids form. Above pH of 6.5, the proton is removed and mostly the carboxylate form is present. The pH for the buffers should be 2 units above or below the pKa of the compound for buffer selection which is the key rule for separation. Some compounds have more than one ionizable functional group and show more than one pKa. Since buffers control pH best at their pKa, this case pick one close to the desired pKa.

Ionized forms are more soluble in aqueous solvents. To increase a compound's retention on a reverse-phase column, force it into its non-ionized form. Some compounds having high pKa, they are usually ionized at any pH tolerated by the silica column bed (usually pH 2-8). This

makes them very soluble and hard to resolve on a reverse-phase column. In this case, it is possible to force them into the free form by using mobile phases at pH 12.

Organic Modifiers

The ionization that causes tailing in reverse phase separations is due to ionization of the packing surface. The older the column, the more of free silanols will be present due to packing material hydrolytic degradation. These react with amines in the mobile phase through an ion-exchange interaction. This effect can be overcome by adding nonyl amine to the mobile phase during equilibration and during chromatographic runs. The amine function of this competing base or organic modifier ties up the free silanol presenting a nonpolar surface to sample amine in solution. Mobile phases used in reversed-phase chromatography are polar to ensure analyte retention. Subsequently, the mobile phase is modified by decreasing the proportion of water present which is achieved by the addition of an organic solvent modifier such as methanol or acetonitrile. This results in the retained analyte(s) to elute off the stationary phase. Changing the mobile phase composition in this way is the most effective way of achieving chromatographic resolution, with analyte elution order governed by the water solubility of the molecule and its carbon content.

Chelation

Adding the metal salt to the mobile phase can enhance the separation of compounds that serve as ligands for chelating metals.

How to remove dissolved gases from Mobile Phase?

Degassing is the process of removing dissolved gases from the mobile phase before or during use. Dissolved gas may come out of the solution in the detector cell and cause baseline spikes and noise. Dissolved air can affect electrochemical detectors by reaction or fluorescence detectors by quenching. There are different processes of the degassing.

- Heating the solvent
- Vacuum (in a vacuum flask)
- Helium sparging.
- Evacuation of a tube

Filtration: The separation of solid particles from a fluid solids suspension of which they are a part by passage of most of the fluid through a septum or membrane that retains most of the solid on or within itself. The septum is called a filter medium and the equipment assembly that holds the medium and provides space for the accumulated solids is called a filter. The fluid may be a gas or a liquid. The solid particles may be coarse or very fine and their concentration in the suspension may be extremely low (a few parts per million). Liquid filtration is used for the liquid-solids separations in the chemicals, polymer products, medicinals, beverages and foods. The simplest method of filtration is to pass a solution of a solid and fluid through a porous interface. During this process the solid gets trapped. The fluid passes through this principle relies upon the size difference between the particles making up the fluid and the particles making up the solid. For the HPLC concern 0.2 micron filter paper is used.

The vacuum filtration apparatus consists of special flasks termed as “Filter flask” that is made up of thick glass and have a side arm, which permits making a connection to a source of,

reduced pressure (a vacuum). The top opening of the suction flask accommodates a single whole rubber stopper, which in turn supports a Buchner funnel for suction filtration.

Different Grades of Water/ Quality of Water for HPLC

In chemical, biological as well as industrial laboratories, different grades of water are used.

Distilled water: Distilled water is prepared by distillation of water by boiling of the water and then condensing the steam into a clean container.

Double or triple distilled water: Multiple distilled waters are prepared by double/triple distillation of water. It is used, when single distillation does not lead to sufficiently pure water for some applications in biochemistry or trace analysis.

Deionized water: Deionized water is water that lacks ions such as cations namely sodium, calcium, iron, copper and anions such as chloride and bromide. It may contain other non-ionic types of impurities like organic compounds. The lack of ions causes the water's resistively to increase.

Ultrapure water: This is the water which is suitable for HPLC applications. Ultra pure water is prepared by subjecting distilled water (less in organic impurity with ions) or water made by reverse osmosis for the removal of organic and inorganic impurities by using ion exchange resins. This grade water can have a maximum resistance up to 18.2 MΩ·cm compared to around 15 kΩ·cm for common tap water.

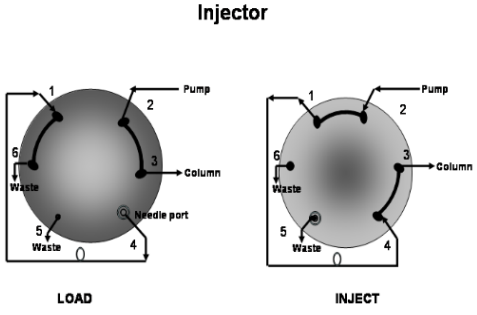
Water for Injection: Water for injection (pharmaceutical water) is a water purified by distillation or reverse osmosis. It is usually stored and distributed hot (at 80 ºC) in order to meet microbial quality requirements. It does not need to be sterile; however, the monograph specifies that WFI not contain more than 0.25 USP endotoxin units (EU) per mL. Endotoxins are a class of pyrogens that are components of the cell wall of Gram-negative bacteria (the most common type of bacteria in water).

Pump

It is made up of corrosive resistant metal fitted with a piston connected with a motor gear system to increase or decrease the flow of mobile phase.

Injector

Injector for HPLC is connected in between the pump and the analytical column. At the load mode the flow of mobile phase by-passes the injector loop and in the inject mode the loop filled with the sample containing analyte is injected in line for separation in the column. Most of the modern day HPLCs are fitted with Rheodyne™ (Cotati, California, US) injector. Samples are introduced onto column with an injection valve or loop or more frequently with a micro syringe.



Analytical column

It is the stationary phase in which the separation of analyte takes place with respect to its affinity for the mobile phase. It is the heart of HPLC system, where separations of sample components take place. Columns are subjected to natural wear mainly due

to irreversible adsorption of injected sample matrix or mechanical or chemical instabilities of stationary phase. HPLC columns are packed with very fine particles (usually a few microns in diameter), required to attain the low dispersion that give the high plate counts expected of modern HPLC. Particle sizes generally range between 3-50 microns, with 5 µm particles being the most popular for peptides. Plate counts in excess of 25,000 plates per column are possible with modern columns, however, these very high efficiencies are rarely found with real samples because of the dispersion associated with injection valves, detectors, data acquisition systems and the dispersion due to the higher molecular weight of real samples as opposed to the common test samples. Packing these small particles into the column is a difficult technical problem but even with good packing a great amount of care must be given to the column end fittings and the inlet and outlet connection to keep dispersion to a minimum. Some state of the art systems are now 'chip' based and may use no particles at all.

Silica based columns

The most common columns are packed with silica particles. For the normal phase column high grade silica is used and for the reverse phase column silanol groups of silica are blocked by the alkyl straight chains. Varying length of alkyl side chains can be added ranging from C2 to C32. Most commonly C4, C8 and C18 length carbon chains are added. End-capping is usually performed in the columns so as to give additional hydrophilicity and better separation. Custom made modifications are also made to the stationary phase by addition of phenyl, nitro, amino , cyano, phenyl–hexyl groups or even zwitterions to the silica for a wide variety of analyte separation. The beads or particles are generally characterized by particle and pore size. Larger particles will generate less system pressure and smaller particles will generate more pressure. The smaller particles generally give higher separation efficiencies. The particle pore size is measured in angstroms and generally range between 100-1000 angstroms. 300 angstroms is the most popular pore size for proteins and peptides and 100 angstroms is the most common for small molecules. Since silica dissolves at high pH, it is not recommended to use solvents that exceed pH 7. However, recently some manufactures have introduced silica-based technology that is more resistant to high pH. It is important to take note of the manufacturers' suggested use and recommendations. In addition the combination of high temperature and extremes of pH can be especially damaging to silica.

Types: Lichrosorb®: Totally porous, irregularly formed silica gel

Lichroshere®: Totally porous, spherical, particle size 5µm and 10µm suitable for acidic, neutral or weakly basic compounds used frequently in analytical fields.

Supersphere®: Totally porous, spherical, 4µm high performance carrier.

Polymer based columns

Polymer based column have higher pH stability compared to silica based column, but their mechanical stability is compromised, thus, leading to lesser column age. The pressure and solvent compatibility may vary with the type of polymer used. General rules followed for silica based column care are not followed for polymer based column. Manufacturer instructions need to be followed for such columns.

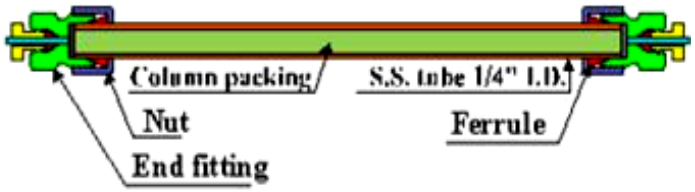
Chiral columns

Chiral separation is based on retention of one enantiomer over another in the sample mixture and cause enantio-selective separation. Chiral columns are used for separation of proteins, polysaccharides, antibiotics, chiral synthetic polymers, dextrin etc

Carbohydrate Column

These are used for analysis of carbohydrates especially dietary products. It is based on ligand-exchange chromatography on polymeric phases based on sulphated polystyrene divinyl benzene, loaded with Ca2+, Na+ or Pb2+. Furanoses and pyranoses are bound to the metal cations via co-ordination complexes. The affinity for carbohydrates increases in the order of Na+<Ca2+<Pb2+. Separation is carried out at 80-90º C, using water as solvent.

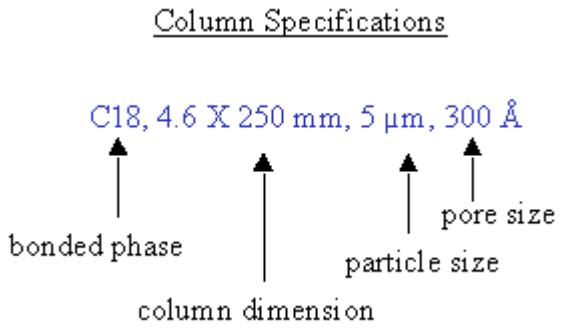
HPLC Column Components and Specifications



a. Column dimension (size)
b. Particle size and pore size
c. Stationary phase

a. Since columns are tubular, column dimensions usually take the following format, internal diameter X length (4.6mm X 250mm).

b. The stationary phase is generally made up of hydrophobic alkyl chains ($-CH_2-CH_2-CH_2-CH_3$) that interact with the analyte. There are three common chain lengths, C4, C8, and C18. C4 is generally used for proteins and C18 is generally used to capture peptides or small molecules.



For therapeutic drug monitoring most of the drugs are analyzed by using reverse phase C-18 column. Basically, C-18 column is packed with silicone coated with eighteen carbon chain to

make the bed more hydrophobic. Columns meant for quantitation are made up of stainless steel often connected with a guard column.

Criteria for selection of column and mobile phase for HPLC analysis

Type of Compounds	Mode	Stationary Phase	Mobile Phase
Neutral, Weak acids, Weak bases, Moderately hydrophobic	Reverse Phase	C18, C8, C4, cyano, amino, Phenyl, phenyl hexyl	Water/ organic along with pH modifiers
Ionics, Bases, Acids	Ion pair	C18, C8	Water/organic along with ion pair reagents
Highly hydrophillic compounds	HILIC	ZIC –HILIC chemistry	Organic phase with water
Highly hydrophobic compounds	Normal phase	Silica, Amino, Cyano, Diol	Organic solvents
Ionics inorganic ions	ion exchange	Anion or cation exchange resins	Aqueous / buffer counter ion
Proteins and high molecular weight compounds	Size exclusion	Silica gel polystyrene	Gel filtration- water based Gel permeation- organic

Detector

The success of the high-pressure technique depends on the use of highly sensitive detectors because of small sample sizes. This requires the use of small-volume detectors, typically 10-100µl. The most widely used detectors are the refractive index monitor and the ultraviolet absorption photometer. The former detector monitors are refractive indices of the column effluent and pure solvent. Although RI detectors respond to most substances, they are not highly sensitive. Ultraviolet (UV) detectors can be 1000 times more sensitive than RI detectors. These UV detectors can be of continuously variable or fixed wavelength. Radioactivity detectors are extremely useful for β- emitting labeled compounds. Fluorescence properties of the compounds are also utilized for its successful detection. Electrochemical detectors are helpful in the estimation of neurotransmitters at the level of picograms.

Integrators

These are various types of integrators available for interpretation of the signal from the detector to quantify the response. Initially, calculations were made from the tracings of the chart recorders but it has been replaced with modern data integrators and computer aided software.

Types of detectors and their principles

Refractive Index Detector

It is a universal detector having low sensitivity for the analyte. It detects based on the refractive index of the analyte as compared to the mobile phase. Conventionally, it is used for the analysis of carbohydrates.

Ultraviolet – Visible Detector

It is a sensitive detector most used for the drug analysis. The ultraviolet absorption of an analyte is a parameter used for its detection after separating it from other matrix interferences. UV absorption depends upon the basic structure of an analyte. The sensitivity of the instrument for

a particular compound is highly depend upon its molar extinction coefficient. At many occasions, simple derivatization techniques are employed to make the analyte to absorb more in a particular ultra-violet wavelength.

Photodiode array detector

It is a hybrid detector which is capable of monitoring all the visible to ultraviolet wavelengths of the incident beam continuously. The detector is having array of diodes instead of photomultiplier tube or photodiode, which is capable of monitoring from 200nm -800nm. The bundle of data generated is transformed into three dimensional plot having time in 'x' axis, absorbance in 'y' axis and wavelength in 'z' axis. This detector is slightly less sensitive to single wave length UV or tunable UV-visible detector, however, capable of matching the peak spectrum with the standard to confirm the peak purity.

Fluorescent Detector

This detector works based on the fluorescent property (inherent or induced in the analyte by derivatization). Typically, this is more sensitive as compared to UV detector however, the major limitation is that all compounds do not possess fluorescence. It is often powered with xenon light source which is capable of emitting light both in ultraviolet as well as in visible range.

Evaporation Light Scattering Detector

Since 1980 the detection of lipids have been revolutionized by the introduction of ELSD. The detector works by measuring the light scattered from the solid solute particles remaining after nebulization and evaporation of the mobile phase. For native lipids (not derivatized), the light-scattering detector (ELSD) is far more useful for on-line lipid quantification than the commonly used UV detector.

This type of detector can be used for all solutes having a lower volatility than the mobile phase. In the ELSD, the mobile phase enters the detector, is evaporated in a heated device and the remaining solute is finally detected by the way it scatters light. The intensity of the light scattered from solid suspended particles depends on their particle size. Therefore, the response is dependent on the solute particle size produced. This, in turn, depends on the size of droplets generated by the nebulizer and the concentration of solute in the droplets. The droplet size produced in the instrument nebulizer depends on the physical properties of the liquid and the relative velocity and flow-rates of the gas and liquid stream.

Three important steps can be defined during the working of the instrument, steps which are located in three different parts of the detector: the nebulizer which transforms the whole liquid phase flowing from the HPLC column into fine droplets. The larger the droplet size, the higher the temperature needed to evaporate the liquid phase. The bigger the residual solute particles the more intense the scattered light.

Then evaporation of the effluent where the droplets are carried by the gas flow into the heated area located before the detection chamber. Practically, a temperature in the range 40-60°C is sufficient to evaporate solvents used in HPLC of lipids where high percentages of water or polar solvents are frequently used. Lastly Detection where sample particles pass through a flow cell where they are hit with an incident light beam, the amount of light scattered being measured using a photomultiplier and an electronic device. In some instrument a secondary gas inlet is used to concentrate the particles in the center of the detection chamber.

Applications of ELSD includes its utility in lipid analysis to separate the molecular species of lipids especially triacylglycerol and also it is easy to make rapid adjustments during method development since change in solvent has virtually no effect on the base line.

Electrochemical Detector

The electrochemical detector responds to substances that are either oxidizable or reducible and the electrical output results from an electron flow caused by the chemical reaction that takes place at the surface of the electrodes. The detector normally has three electrodes, the working electrode (where the oxidation or reduction takes place), the auxiliary electrode and the reference electrode (which compensates for any change in the electrical conductivity of the mobile phase) There are two modes of operation coulometric detection and ampiometric detection. If the reaction at the electrode surface exhausts all the reactant and the current becomes zero, the total charge that passes will be proportional to the mass of solute detected. For obvious reasons this process is called coulometric detection. If the mobile phase is flowing past the electrodes, the solute will be continuously replaced as the peak passes through the detector. While there is solute present between the electrodes, a current will be maintained (albeit varying in magnitude). The process is called ampiometric detection. There are a number of electrode configurations, each having claims to special attributes; the electrochemical detector has been reported to be very flow sensitive for compounds like catecholamines etc.

Mass detector

Liquid chromatography coupled with mass spectrometry (LC/MS) is one of the most powerful analytical tools used in pharmacokinetic studies of pharmaceuticals and is thus the most frequently used technique in the field of bioanalysis. An LC/MS detector consists of three major components: an ion source that generates ions at atmospheric pressure, a mass analyser which filters ions, and a detector that detects ions. As chromatographic peaks elute from the LC column and transfer to the ion source, two main processes occur. First, the ion source produces charged molecules or ions, and second the mobile phase is removed. Once the ions are created, they are extracted from the ion source and transferred to the mass analyser. Sample ions are then filtered by the quadrupole mass analyser according to their mass-to-charge ratio (m/z) prior to detection.

Working principle of HPLC

Mobile phase varies according to the column chemistry and type of analyte undergoing separation using that column. As adsorption and partition principles are employed in the separation in bonded phase silica columns, appropriate combination of solvents must be selected based on their miscibility index and viscosity. If it is aqueous based separations, additional factors such as ion paring reagents and buffers for maintaing the pH can be added to enable the fine separation of analyte of interest from the complex matrices like blood, urine, plant, tissue extracts and environmental samples.

Prepared mix of mobile phase is allowed to come back to room temperature as mixing Acetonitrile and water would be an endothermic event and mixing methanol with water would be an exothermic event which is expected to alter the final volume of the resultant mixture. These mobile phases are usually sonicated or vacuum filtered to reduce dissolved oxygen as its low solubility is expected to produce microbubbles during high pressure generated against the resistance of the column resulting in increased baseline noise.

The efficiency of the column for the analytical separation is expressed by the factor called Height Equivalent to Theoretical Plates (HETP). Plate counts more than 25,000 plates per column has been achieved in modern columns, however, these very high efficiencies

are rarely found with real samples because of the dispersion or distortion due to instrument design. Maintaining the flow rates of 0.5-1ml in 2µm columns for very high HETP is complicated with the generation of high pressure at room temperature. Therefore, column oven heating systems are used by some instrument manufacturers to reach such limits. However, the most successful model is increasing the capability of the pump to reach pressures up to 15000 psi while maintaining the flow rate of 0.5-1ml per minute in ultrahigh performance liquid chromatography systems.

van Deemter equation (1956) explains the plate number as a function of column property and the experimental conditions employed-

HETP = A+B/v + Cv

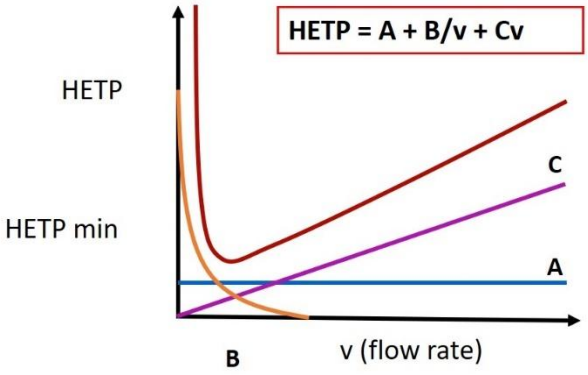
HETP- Height Equivalent Theoretical Plate

A- Eddy's diffusion or multipath effect of the solvent inside the column

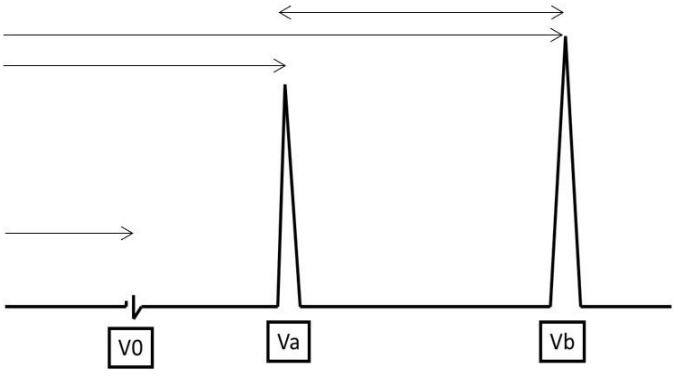
B- Random molecular/longitudinal diffusion

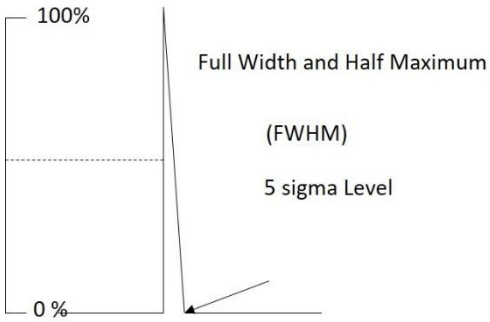
C- Mass transfer within particle caused by mobile phase

v- Flow rate of the mobile phase



Peak parameters/System Suitability Tests





Capacity Factor (K'): It is a measure of the relative retention of each peak on the column.

K' = (Va- Vo)/Vo

For a better separation capacity factor must be >1.

Separation Factor (α): It represents relative retention time between separated peaks.

α = (Va-Vo) / (Vb-Vo)

For a better separation separation factor must be >1 (1.1-2)

Efficiency Factor (N): It represents the degree of sharpness of the separated peak on the column.

$N = 5.54 (Va/w_{0.5h})^2$ Where width of a peak w is calculated at FWHM.

Factor N for analytical separation must be 100-10000 (more gives more efficiency)

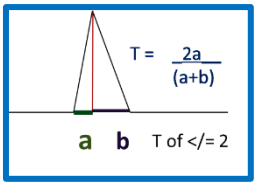
Resolution (R) (Snyder and Kirkland equation): It gives combined measure of separation including efficiency and selectivity.

$$R = 2\frac{t_2 - t_1}{w_2 + w_1}$$

Or

$$R = \frac{1}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\sqrt{N}\right)\left(\frac{K'}{1+K'}\right)$$

Tailing Factor (T):



Quantitative analysis

Quantification of compounds are performed on the basis of either considering peak height or peak area. In a well resolved chromatographic peak the peak area/height increases with respect to the increase in concentration and is then analyzed. There are two methods for sample analysis that includes external calibration and internal calibration.

External standards

With external standards multiple concentrations of the standards are injected, areas are measured and a calibration curve is plotted. Unknowns are then run, and areas are calculated and compared to the calibration curves to determine the amount of each compound present.

Internal standards (IS)

With external standards, known amounts of an internal standard are added to each known concentration of standard compounds and areas or peak heights response factors relative to those of the internal standards are calculated. When unknowns are run the same amount of internal standard is added to the unknown sample, and relative areas or heights are calculated based on the response factors to the internal standard from the calibration curves. Internal standards are usually used to correct for variation in injection size due to different operators and injection techniques. Internal standard can be used to correct extraction variation.

Calibration

A calibration curve is produced by analyzing different concentrations of the pure drug with constant amount of IS and from the chromatogram. Ratio (R) for each concentration of the analyte is calculated:

$$R = \frac{\text{Area of the drug}}{\text{Area of the IS}}$$

This ratio is plotted against the concentration of the pure drug (R/Concentration of the drug). The slope of this plot is the response factor.

Calculation for External Calibration Curve

$$\text{Single point calculation} = \frac{\text{Unknown area}}{\text{Known area}} \times \text{Known Concentration}$$

or plot

Area of known concentration in Y axis and concentration in X axis

Plot $Y = mx + c$ or $Y = mx$

Also correlation coefficient should be recorded to know about the linearity of the method.

HPLC system- General Maintenance

Pump

- A pump delivers flow between 10 µL/min and 10 mL/min which requires considerable pressure. Incase it does not delivers change in pressure, allow quick assessment of blocked frits, or columns or change in retention times.
- Pump seals wear more quickly than other pump parts hence change in every three to six months is recommended. It may result from sloughing seals or contaminated material. Buffer crystals built up from evaporated mobile phase also will accelerate wear.
- Pump seal life can be extended by filtering the mobile phase solvents to remove the particles responsible for accelerated seal wear.
- Efficiency of check valve deteriorates in case of contamination with mobile phase, pump seal, wear material, and build-up of salts in the valve.
- Ensure use of 0.22µm or finer filter on the solvent intake line. Care should be taken to prevent contamination with dust particles as even a few hours of exposure to air can detoriate the system affecting the check valve.
- The outlet check valve should be flushed with 50 ml of clean HPLC grade IPA or water. Also in severe cases with 20 % nitric acid and sonicate for a maximum of 20 minutes. Follow this by flushing the valve with 50 ml of HPLC grade water.
- The sapphire, ruby or ceramic ball and seat in check valves may sometimes tend to bond to each other when allowed to dry out, this is common with use of acetonitrile.

Software

- Always open the HPLC system first and then its software to initialize
- Check for the proper communication between the software and the HPLC system
- Always use original software for data acquisition and should not be copied to other systems
- Do not fiddle with the software unnecessary it might remove any major options
- Always refer to the manual or the vendor before using any new options
- In case of changing the auto-sampler plate type corresponding change in the software should be made otherwise it might damage the system

Hamilton Syringe

- Hamilton syringe is available in various different volumes as 10µl, 25µl, 100µl, 1000µl, 5000µl etc. proper care ensure the long term use of these syringes.
- These syringes have accuracy to within ±1% of nominal volume with a precision of 1% at 80% of the total volume.
- The syringe should be rinsed thoroughly after use with deionized water, acetone, or another solvent compatible with the sample. Allow the syringe to air dry. Avoid prolonged immersion of the syringe while cleaning.
- Remove the syringe piston and wash it with mild detergent water. Flush the syringe barrel with mild detergent water incase it needs a thorough cleaning. If it is too dirty, use 3M nitric acid to clean it. Nitric acid wash must be reserved only for Hamilton rescue and not for normal use.
- After washing with acid, it should be rinsed thoroughly with distilled water till reaches neutral pH and rinse with methanol/ acetone before storing it.
- Incase the needle gets clogged do not attempt to clean it by forcing liquid or compressed air through the syringe. Excessive pressure will split the glass barrel.
- Pump the plunger with the syringe needle immersed in the fluid to be transferred. This will expel any trapped air in the needle and syringe.
- Incase the plunger is removed from the syringe barrel by chance, wipe it carefully with a lint-free tissue. Reinsert the plunger into the barrel and pump deionized water or acetone through the needle and syringe.
- In case the adhesives used to cement needles and other terminals are clogged which may result in frozen plungers and plugged needles. Cleaning should be ensured by non-alkaline, non-phosphate and non-detergent based solvents.

General HPLC Column Care

The correct use of an HPLC column is extremely important for the life time of a column and therefore for the benefit of your HPLC analysis. The following pages will give you some guidelines for the use, cleaning and storage of HPLC columns. These guidelines depend on the one hand on the nature of the chromatographic support (silica, polymers or others) and on the other hand on the surface chemistry of the corresponding stationary phase. Silica based columns

General guidelines

Silica is the ideal support for HPLC columns. It offers a large mechanical stability, excellent physicochemical surface properties, a wide range of bonding chemistries and is compatible with a broad range of organic solvents. However, the following points are extremely important to know when working with silica based HPLC columns.

• pH stability

In general, HPLC columns are stable within a pH range of 2 to 8. If you are measuring a pH value, the measurement must be done in the aqueous media before mixing the eluent with organic solvents. Modern HPLC columns can be used outside that pH range. The new bonding chemistries allow use down to pH 1 for some stationary phases. However, please check vendor's product information before using silica based column outside the pH range of 2 to 8. However, best lifetimes are obtained between pH 2.0 and pH 6.8.
Stationary phases based on ultra pure silica gel can also be used at higher pH ranges, up to pH 11, depending on the chemical nature of the modifier used in the mobile phase. Large bases (like Pyrolidine) are not able to attack the surface of the silica and therefore can be used at higher pH values.

• Mechanical stability

Stationary phases based on silica are mechanically very stable. The packed columns show no pressure limit and can be used at more than 40 MPa (6000 psi) without any problem. However, please avoid pressure shocks on the column. Pressure shocks lead to channeling in the column, which results in peak splitting in the corresponding chromatogram.

• Mobile phases (Eluents)

Silica based stationary phases are compatible with all organic solvents in the above mentioned pH range. Please use the highest quality solvents available (HPLC grade). Also, please filter all prepared buffer through a 0.4 µm filter before using them in your HPLC system. Always keep in mind; your column is the best filter!

To avoid irreversible adsorption at the column head, you should always use a pre-column. The use of a pre-column increases the life time of a column dramatically. In addition, a pre-column can filter solid parts stemming from pump seals or injection rotors. An alternative to a pre-column is an in-line filter. These filters are attached directly to the column. These filters get rid of solid parts in the eluent but will not avoid irreversible adsorption of organic impurities.

Proper storage of HPLC columns

- Columns should never be stored in the buffer solution
- Columns should be flushed with pure water to prevent microbial growth
- Then flush about 20 column volumes of any aprotic solvent (acetonitrile / methanol)
- Finally flush the column with 20 column volumes of 50% acetonitrile – water or methanol – water

Equilibration time

The equilibration time of a column depends on the column dimensions. In general, a column is equilibrated after flushing with 20 column volumes.

Regeneration of a column

Irreversible adsorption of impurities stemming from the matrix on the column head can cause changes in selectivity or peak splitting. Often those “dirty columns” can be regenerated by applying the following protocols (Flow rate: 0.2-0.5 ml/min).

Regeneration of RP packings

RP- packings are C18, C8, C4, C1, C30, CN or Phenyl stationary phases.

- Firstly, flush the column with 20 column volumes Water → then with flush 20 column volumes Acetonitrile → then flush 5 column volumes Isopropanol→ followed by flushing 20 column volumes Heptane→ then flush column with 5 column volumes Isopropanol→ then flush the column with 20 column volumes Acetonitrile

Regeneration of NP (Normal Phase) packings

NP-packings are Silica, Diol, Nitro and Amino stationary phases.

- Firstly, flush the column with 20 column volumes Heptane → then 5 column volumes Isopropanol → followed by 20 column volumes Acetonitrile → then flush 20 column volumes Water → then 20 column volumes Acetonitrile → flush 5 column volumes Isopropanol → lastly flush 20 column volumes Heptane

Regeneration of Ion Exchange Packings

Ion exchange packings are Anion or Cation exchangers (WCX, SCX, WAX or SAX)

- Initially flush the column with 20 column volumes of the same eluent, but double the buffer concentration → follow the regeneration protocol for RP packings (see above) → flush with 20 column volumes of Water → finally equilibrate the column now to the original conditions.

Column Maintenance

General

- Ensure that the plunger seal is washed frequently
- Always increase pressures/ flow rates slowly in stepwise manner
- After removing the column its ends should be capped properly or covered with parafilm® and then stored
- Column should be held carefully and should not slip or fall from hand
- After finishing the run, column should be given proper washing before storing

Column washing

- After the use of matrix (Plasma/blood) column should be washed properly for the next usage.
- Flush 10 column volumes of ultrapure (Milli-Q) water at the flow rate of 1ml /min if buffer is one of the components of the mobile phase before shifting to non-aqueous solvents.
- Wash the column with pure HPLC grade methanol (100%) in 1 ml/min flow rate for 5 hrs
- Switch over to 50% acetonitrile-water or methanol-water for 30 minutes
- Bring the temperature of the column oven to standard temperature if column washing under increased temperature

- 1% DMSO washing can be done to remove the protein load in the column for 1 hr

Column Fixing

- Select the column as dedicate for the particular estimation
- De-screw the end-fittings
- Properly secure the column in the direction as mentioned
- Avoid over-tightening of the ferrule/PEEK
- Properly cover the column end-fittings with ferrule or with parafilm
- Always store the column in 50% acetonitrile or methanol
- Avoid storing the column in buffer as it is detrimental to the column
- Use only the recommended buffers and temperature as per the manufacturers instructions
- Avoid the usage of drastic chemicals/solvents (eg. More acidic or basic buffers, chloroform, ethyl acetate, corrosive solvents) which could hydrolyze the packing materials of the column
- Flush always 0.2µ filtered solvents
- Read necessary information's about the column before use
- Make the entry in the log book before and after use

Column Performance Check

- Stabilize the column in 50% acetonitrile-water or methanol water at the flow rate of 1ml/min for 20 minutes
- Open the general method
- Inject 20µl standard ace-benz or ace-benz-toluene mixture (10µl acetone + 10µl Benzene + 5µl toluene → 10 ml pure methanol)
- Analyze the peak at 254nm
- Compare it with the earlier documented performance
- Document in the ABC check folder as well as in the HPLC log book
- Use only one direction as per the manufactures instruction

HPLC Method development

Standard Operating Procedure for Starting a Method

Step1: Standard Preparation: Appropriate standard is chosen either on the basis of structure similarity, similar elution pattern (RT, λmax) or literature reports. The standard is dissolved in appropriate solvent (usually methanol, acetonitrile, water in case of reverse phase chromatography) to obtain known concentration of the drug (eg: 1mg/ml). The standard is diluted with the vehicle to 1µg/ml, which is the starting concentration, for plotting the standard curve. After carefully preparing the standard, it is stored under specified conditions of light and temperature.

Step2: Sample Preparation: Prepare the sample using standard protocol of de-proteination, concentration, weighing and re-constitution in appropriate solvent. The sample is neatly labeled and prepared in triplicate for injection.

Step3: Mobile Phase Preparation: Appropriate solvents (HPLC grade) for mobile phase are measured, mixed and de-gassed. The solvent delivery tubing are inserted into the bottle(s)

holding the mobile phase and then sealed, to avoid any vaporization of solvents. The bottle is neatly labeled for content, date of preparation, volume etc.

Step4: Starting the System: Attaching the appropriate column to the system is the most crucial and tedious step. Care has to be taken that the column is attached in the right direction (along with flow direction), frit ends are tightly screwed to avoid any possibility of leakage, and adjusted into the column oven, if the method demands.
Following this, the system is switched on in the order of-gradient controller, pump, detector and computer, printer. The column oven is not switched on till the mobile phase is flowing at full speed. Appropriate system windows are opened to start the software and allow the system to connect to all hardware. Simultaneously, the solvent line is cleared of any residue salts, air bubbles by drawing up to 5ml of the mobile phase.
Precaution has to be taken here, that the system has been appropriately stored (preferably in methanol:water -50:50) and is in a compatible condition with the mobile phase that is currently being run.
The pump is stepped up in graded manner so that the mobile phase runs at the flow rate of 1ml/min, or as the method conditions specify. The system is allowed to equilibrate for at least 20 min with simultaneous monitoring.
After ensuring column performance, the system is now ready for operation. Following standard protocol the standard curve is plotted by injecting at least five concentrations in triplicate. The sample is injected, starting with lowest concentration. The data is analyzed using the external calibration method.

Column Performance Check

- Stabilize the column in 50% acetonitrile-water or methanol water at the flow rate of 1ml/min for 20 minutes
- Open the general method
- Inject 20µl standard ace-benz or ace-benz-toluene mixture (10µl acetone + 10µl Benzene + 5µl toluene → 10 ml pure methanol)
- Analyze the peak at 254nm
- Compare it with the earlier documented performance

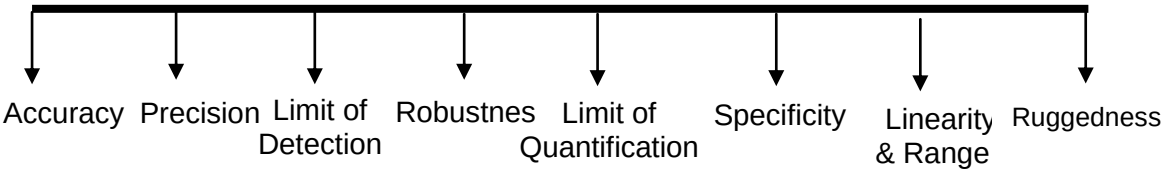
Use only one direction as per the manufactures instruction

HPLC Method Validation

Method validation is performed to ensure that any analytical methodology is accurate, specific, reproducible and rugged over the specified range. Regulated laboratories need to perform method validation in order to be in compliance with FDA regulations. According to 1987 guideline (Guideline for Submitting Samples and Analytical Data for Methods Validation), the FDA designated the specifications in the current edition of the United States Pharmacopoeia (USP) as those legally recognized when determining compliance with the Federal Food, Drug, and Cosmetic Act.

The USP Eight Steps of Method Validation are often referred as "analytical performance parameters" as per ICH guideline on the "Validation of Analytical Methods: Definitions and Terminology is available. The difference in USP and ICH terminology is for the most part, one of semantics, however, with one notable exception. ICH treats system suitability as a part of method validation, whereas the USP treats it as separate. The following are the eight parameters for any method validation

Method Validation



Steps for Method Validation

1. Accuracy

Accuracy is the measure of exactness of an analytical method, or the closeness of agreement between the value which is accepted either as a conventional, true value or an accepted reference value and the value found. It is measured as the percent of analyte recovered by assay, by spiking samples in a blind study. For the assay of the drug substance, accuracy measurements are obtained by comparison of the results with the analysis of a standard reference material, or by comparison to a second, well-characterized method.

Concentration Added (μg/ml)	Concentration Obtained (μg/ml)	CV (%)	RE (%)
Intra-day			
1.0	1.05	7.6	105.1
3.0	3.11	4.0	103.8
25.0	26.55	5.0	106.2
40.0	43.68	0.6	109.2
Inter-day			
1.0	1.06	4.8	106.4
3.0	3.05	3.2	101.7
25.0	25.55	3.3	102.2
40.0	41.44	5.0	103.6

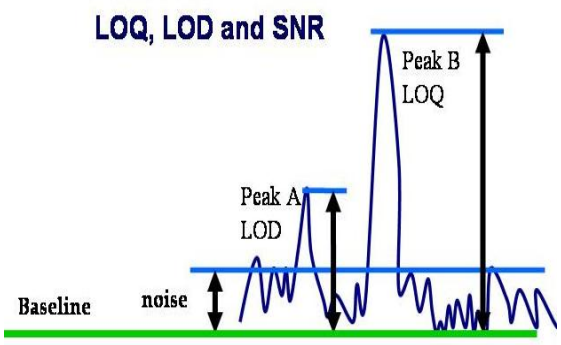
As per ICH guideline the data should be collected from a minimum of nine determinations over a minimum of three concentration levels covering the specified range (for example, three concentrations, three replicates each).

2. Precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation for a statistically significant number of samples. As per ICH guidelines precision should be performed at three different levels: repeatability, intermediate precision, and reproducibility.

3. Limit of Detection

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value. It is expressed as a concentration at a specified signal-to-noise ratio, usually two- or three-to-one. The ICH has recognized the signal-to-noise ratio convention, but also lists two other options to determine LOD: visual non-instrumental methods and a means of calculating the LOD.



LOD can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula: LOD = 3.3(SD/S). The standard deviation of the response can be determined based on the standard

deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y-intercepts of regression lines. The method used to determine LOD should be documented and supported, and an appropriate number of samples should be analyzed at the limit to validate the level.

4. Robustness

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. The robustness of a method is evaluated by varying method parameters such as percentage of organic matter, pH range, ionic strength, temperature, etc., and determining the effect (if any) on the results of the method. As per ICH guidelines, robustness should be considered early in the development of a method and in case they are susceptible to variations in method parameters, these parameters should be adequately controlled and precautionary statement must be included in the method documentation.

	Nominal Concentration (ng/mL)			
	LLOQ QC	LQC	MQC	HQC
	3.004	8.010	250.313	500.626
Day 1	3.587	7.929	240.300	493.613
	3.550	8.008	240.631	520.701
	3.460	8.029	251.169	510.304
	3.542	7.409	245.788	474.765
	2.851	7.432	242.889	498.441
		7.787	236.065	475.781
Day 2	3.236	8.415	239.702	522.111
	2.795	8.422	243.142	494.885
	3.210	7.588	251.804	474.529
	3.492	7.546	255.853	493.617
	3.268	7.131	239.100	488.781
	2.817	8.030	225.795	486.451
	2.825	7.766	230.738	471.394
Day 3	2.785	7.314	241.884	483.234
	2.621	7.912	232.014	444.095
		8.269	252.213	523.325
	3.470	7.702	243.032	512.529
	2.866	7.726	239.625	529.290
	2.836	7.779	235.726	486.646
Day 4	2.856	7.518	243.768	510.760
	3.248	8.383	258.177	488.902
	2.970	7.964	235.011	517.146
	3.217	8.679	242.844	490.112
	3.146	8.300	237.597	491.985
		8.046	233.876	521.030
Mean	3.1204	7.8834	241.5497	496.1771
S.D. (+/-)	0.30728	0.38821	7.84341	20.46255
C.V. (%)	9.85	4.92	3.25	4.12
% Nominal	103.87	98.42	96.50	99.11
N	22	25	25	25

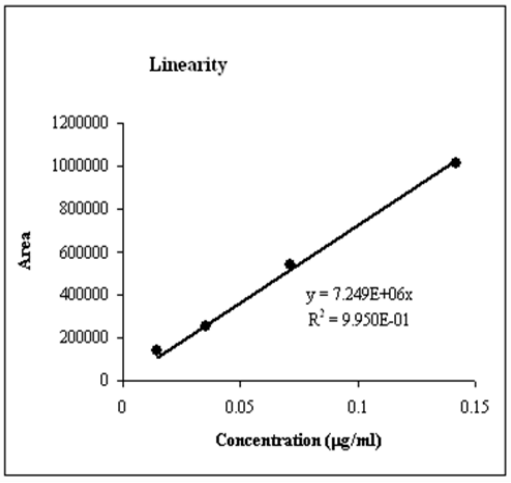
5. Limit of Quantitation

The Limit of Quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. Signal-to-noise ratio of ten-to-one is used to determine LOQ. As the LOQ concentration level decreases, the precision increases. If better precision is required, a higher concentration must be reported for LOQ. As per ICH guidelines ten-to-one as signal-to-noise ratio as is typically required. Also LOQ can be calculated from standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula: LOQ = 10(SD/S). Standard deviation is of the blank, on the residual standard deviation of the regression line, or the standard deviation of y-intercepts of regression lines. The analytical method should be validated before the start of obtaining clinical samples. Each analytical run should contain sufficient QC samples at the beginning, middle and end at at least 3 levels (LQC, MQC and HQC). Acceptance or rejection of a run should be predefined before the starting of actual analysis of the clinical samples.

6. Specificity

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix. Specificity is measured and documented in a separation by the resolution, plate count (efficiency), and tailing factor. ICH also uses the term specificity, and divides it into two separate categories: identification and assay/impurity tests (Detailed calculation –refer Module 5)

For identification purpose, specificity is demonstrated by the ability to discriminate between compounds of closely related structures, or by comparison to known reference materials.



7. Linearity and Range

Linearity is the ability of the method to elicit test results that are directly proportional to analyte

concentration within a given range. Linearity is generally reported as the variance of the slope of the regression line. Range is the interval between the upper and lower levels of analyte (inclusive) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. The range is normally expressed in the same units as the test results obtained by the method. The ICH guidelines specify a minimum of five concentration levels, along with certain minimum specified ranges. For assay, the minimum specified range is from 80-120% of the target concentration. For an impurity test, the minimum range is from the reporting level of each impurity, to 120% of the specification.

8. Ruggedness

As per USP the degree of reproducibility of the results obtained under a variety of conditions, expressed as % RSD. These conditions include different laboratories, analysts, instruments, reagents, days, etc. But ICH did not address ruggedness specifically.

Preparation of biological samples for HPLC

Preparation of biological samples for the quantification by LC-MS/MS is a very important as well as skilled procedure. By sample preparation techniques extraneous materials are removed before injection onto the column, which leads to reduction in run time and a fast isocratic separation instead of the gradient. Advantage of sample preparation is often trace enrichment, increase in sample concentration with a corresponding increase in sensitivity and reduction of ion suppression.

Types of Biological Samples

1) Whole Blood
2) Plasma
3) Urine
4) Vitreous humour/Aqueous humour
5) Tissues

Processing of Biological Samples

The biological fluids should never be injected directly to the HPLC. These samples have to be deproteinized before analytical injection into the column. Proteins also can interact with bonded – phase columns through non polar partitioning. Chemical denaturation of proteins is a wise decision to remove proteins before analysis. This can be achieved by mixing equal volume of acetonitrile or methanol, vortex and centrifuge to settle the denatured proteins. Inject the clear supernatant for analysis. This method is quick and quite efficient to remove 95% proteins from the matrix. During the toxicological emergencies this method would be less time consuming and effective enough to purify and analyze the biological sample.

For quantification, again the similar de-proteinization method should be used in the drug free plasma (normal plasma/ serum) spiked with known amount of the same compound. Using an external calibration curve plotted with spiked concentration will take care of matrix effect in releasing the unbound drug.

In reverse- phase separation, plasma sample is injected that is an irreversible binding of protein to the column. When proteins are removed polar peaks are found, which overload the early part of the chromatogram and tail into the compounds of interest. The non-polar components get adhere to the column and must be washed off before the next injection. To ensure polar elution before our target compounds and non-polar removal afterwards, solvent gradients need to be run.

Matrix Effect

The combined effect of all components of the sample other than the analyte on the measurement of the quantity is known as matrix effect. Therefore, every time when an analyst is trying to design or develop or validate a method one must document the matrix interference. Drugs or chemicals or toxins, binds to various components of the blood when blood is used as a sample for analysis. Briefly, plasma, serum or whole blood or ultrafiltrate of plasma are used as a matrix for most of the drug analysis. Plasma contributes to 50-60% of blood depending upon hematocrit status. Typically, plasma contains albumin, globulin, and lipids. Albumin is a 57 KD protein known to bind acidic drugs and chemicals, whereas basic drugs binds to alpha acid glycoprotein. Steroidal hormones are known to bind to steroid binding globulins in the plasma. Apart from this many lipid soluble drugs are known to bind to RBCs, in this case analyzing only plasma will not predict the real values of the drugs. For example, cyclosporine is known to bind to RBC that is why it is estimated in whole blood rather than plasma or serum.

Plasma would be a much better matrix for drug estimation since it contains fibrinogen, therefore even the nonspecific binding of chemical or drug to fibrinogen can be accounted for estimation. While subjecting it for removal by allowing the blood to clot while on standing the formed fibrin takes all matrix and bound drugs entangled in fibrin mesh and separates out on centrifugation. However, as serum would be good and simple matrix for sample preparation whereas may not indicate the exact drug or chemical levels. According to our experience plasma is an excellent solvent for the solubilization of any drug for intravenous administration when performing preclinical pharmacokinetic studies. Due to its nature and composition it can dissolve almost all the drugs that are having considerable partition coefficient Log P less than 5. As far as the solubility is concerned, it is purely dependant upon its individual concentration. Expected plasma to dissolve the amounts of drug equal to oral dose may not be possible since the volume used would be much low.

When we use blood as a matrix, the disrupted RBC (hemolysed blood) might rebind the drug to a higher extent. As compared to blood or plasma, urine samples are much simpler matrix for the estimation, however this matrix have very high amount of dissolved salts, urobilin and urobilinogens. While trying to remove the salts by using ion exchange resins, there are chances that the drugs or metabolites in ionized form will also be removed. Alternatively if we prefer to go for liquid-liquid extraction, we might land up extracting urobilin and other metabolites.

Extraction Recovery

For extraction recovery, drug free matrix is spiked with known amount of drug (LLOQ, MLOQ & HLOQ) and extraction procedure is followed and the extracted matrix is subjected for HPLC estimation. Also drug free matrix is subjected to extraction as per protocol and the extracted matrix is spiked with known amount of drug (LLOQ, MLOQ & HLOQ) and subjected for HPLC estimation. The comparison of these two responses gives extraction recovery. For a given analyte, the extent of ionization dictates its ability to compete with the matrix components present. As the amount of ionizable matrix components increases, the ability of an analyte to ionize, hence its apparent concentration, is diminished. This is called as ionization suppression or ion suppression. It can severely distort the determination of assay recovery.

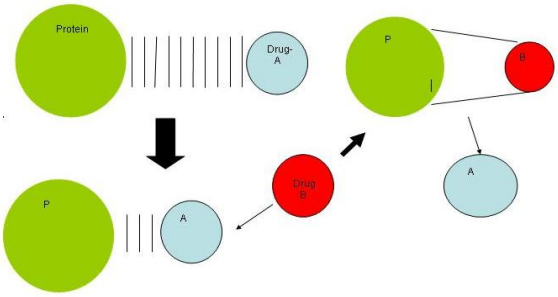
Plasma Protein Binding of Drugs

Drug distribution in body is significantly altered by its ability to bind with proteins in plasma. The protein usually responsible for binding is albumin, although globulins may participate in such protein–drug interactions, especially in relation to the binding of some hormonal agents and drug. Drug distribution by transport in blood bound to plasma albumin. Bound fraction is inactive

reservoir of drug and drug candidates having higher affinity for the binding site replaces the former one from the binding site.

The importance or consequence of protein drug binding lies in several areas:

1. Tissue distribution of the drug may be strongly modified.
2. In combination drug therapy there is a competition between drugs for the binding site, drugs with higher affinities displacing those with lower ones. If this situation occurs the free (unbound) fraction and pharmacological effect of the displaced drug will be increased. The concentration of the active form may be doubled by as small as absolute change of 3% in binding equilibrium.



3. Only the free fraction of the drug is in equilibrium with the extravascular compartments, which are the site of the bio-phase. Hence this free concentration is available for distribution into the tissues, only at the site of action.
4. Binding may be modified in disease state due to changes in nature and amount of protein, dehydration or pH alteration.
5. Drug excretion may be delayed by prevention of tubular secretion or of glomerular filtration.
6. Since intestinal absorption of drug is a diffusion process, its rate is dependent on the concentration gradient on both sides of epithelium. Free drug absorption is enhanced since plasma protein binding of the drug increases the free drug's concentration gradient.
7. The relative inherent potency of drugs is often determined by plasma binding.
8. Accumulation is more likely to occur with a drug that is strongly bound to plasma proteins.
9. The bound drug elution from the binding site is required to analyze the concentration of the total drug in plasma (bound + unbound).
10. In order to achieve this, confirmation of the binding site need to be disturbed to release the contents or for easy extraction with more non-polar solvents.
11. Boiling, adding acid are the ways of denaturing the proteins, however at times they cannot be employed to avoid destabilization of drug.

Removal of Proteins, Lipids, Salts

<u>By Direct De-Proteination</u>

In serum, mostly proteins are present which interact with bonded-phase columns through nonpolar partitioning. Proteins can be removed by ultrafiltration through a very fine membrane filter. Ultracentrifugation at high speeds can also be used to separate proteins from smaller molecules based on their size differences. The most commonly used protein removal technique for LC-MS/MS involves protein denaturation. Heating denatures most proteins. If the compounds to be analyzed are temperature resistant, blood cells can be spun down and the crude mixture remaining can be boiled and then filtered and centrifuged. Particulates and denatured proteins are removed together.

- Chemical denaturation of proteins is more reliable method that has less harmful effect on sensitive compounds.

- To obtain maximum removal of proteins it is acidified with trichloracetic acid (TCA) (5% in final solution) then centrifuge it to remove protein and finally neutralize it with sodium hydroxide.
- Perchloracetic acid is also used for protein precipitation. After protein precipitation excess perchloracetic acid is precipitated as $KCLO_4$ by neutralization with potassium hydroxide.
- TCA absorbs strongly below 230 nm and the perchloracetic acid treatment leaves large amount of salt in solution, which can precipitate with organic solvents.
- Acetonitrile or methanol which are well known common HPLC mobile phases known to precipitate protein at >50% of their final content (they are also transparent at 190nm). Mixing and centrifugation of an equal volume of sample solution and acetonitrile will lead to precipitation of about 95% of the proteins. However, non-polar proteins, such as the albumin fraction, remain in the sample.
- Therefore, supernatant can be injected directly if a guard column is used to remove the last 5% of the protein.
- The guard column need to be inverted and washed into a beaker with 70% acetonitrile / water containing 0.1% trifluoroacetic acid periodically to prevent protein breakthrough to the main column.
- Apart from the above sulphosalicylic acid 20%, phosphotungstic 1% acid are also used for deproteination.
- The combination containing zinc sulphate and acetonitrile (1:1) is effective for extraction of plasma for analysis by LC-MS/MS.

By Liquid-Liquid Extraction

Liquid- liquid extraction involves partitioning of the sample between two immiscible phases and is used for separating analytes from interfering matrix. The distribution of a solute between two immiscible phases is an equilibrium condition described by partition theory. Out of the two phases, one is usually aqueous and the other is organic.

More hydrophilic compounds prefer the polar aqueous phase, whereas more hydrophobic compounds will be found mainly in the organic solvent. Analytes extracted into the organic phase are easily recovered by evaporation of the solvent, while analytes extracted into the aqueous phase are often injected directly into a reverse phase HPLC column.

Extraction and Concentration

To obtain compounds free of interferences, extraction and concentration need to be done. Non-polar compounds are extracted using equal volumes of sample and Folsch mixture (2:1, chloroform/ Methanol) that gives a very broad polarity cut. Everything from steroids to triglycerides is pulled down into the chloroform rich bottle layer. But extraction done with methylene chloride from a sample acidified with sulphuric acid is more accurate in pulling steroids, phospholipids, fat-soluble vitamins and free fatty acids. The triglyceride fraction can be extracted using isopropyl alcohol/hexane (1:9) with little emulsification.

After extraction, these fractions should be dried to remove water. After drying the solvent should be removed by evaporation and the sample is reconstituted with a solvent or mobile phase before injection. Care must be taken that these evaporated samples go completely back into solution. Sonicating the sample with the mobile phase is usually sufficient. However when the extraction is performed for the first time, it is always good to sonicate the dry down tube with a strong solvent and inject this wash as a check that everything has redissolved. For gradient work, the stronger of

the two mobile phase solutions is an excellent choice for this second sonication solvent. Particulate matter must be removed from these sonicates or for that matter from any sample. Centrifugation or filtration should be done as a last step before injection as it protects the column filter from plugging and the system from the pressure build up. (McMaster MC et al 1994)

Solid Phase Extraction (SPE)

Brief Introduction about SPE:

Solid- phase extraction (SPE) is an extraction process, which comprises a solid and a liquid phase. The components of interest and the matrix interferences are in the liquid phase. This technique replaces the classical liquid-liquid extraction. SPE is based on the principle that the components of interest, mainly organic molecules, are retained on a special sorbent placed in a disposable extraction minicolumn. The goals of the technique are the removal of the matrix interfering components, selective enrichment of sample by factors of 100 to 5,000 and isolation of the traces of the components of interest.

SPE is safe and fast means of successful sample preparation. The great interest in this sample and easy-to-use technique led to the marketing of disposable minicolumns. These columns are packed with sorbents of different particle sizes. The particle size permits the use of low pressure to force the sample and wash solutions through the column. The sorbents include modified silica, unmodified silica, alumina polymers etc.

SPE for sample preparation involves the following sorbents: Reversed phase high hydrophobic octadecyl (C_{18}), octyl (C_8), ethyl (C_2), cyclohexyl, phenyl; Wide pore reversed phase, butyl (C_4); Normal phase, silica modified by cyano (-CN), amino (-NH_2), diols (-COHCOH); Adsorption, silica gel (-SiOH), florisil (Mg_2 SiO_3), alumina (Al_2O_3); Ion-exchangers, amino (-NH_2), quaternary amine (N^+), carboxylic acid (-COOH), aromatic sulfonic acid ($ArSO_2OH$); Wide pore ion exchanger, carboxylic acid (-COOH), polyethyleneimine [-($CH_2CH_2NH)_n$ -].

Methods to carry out SPE:

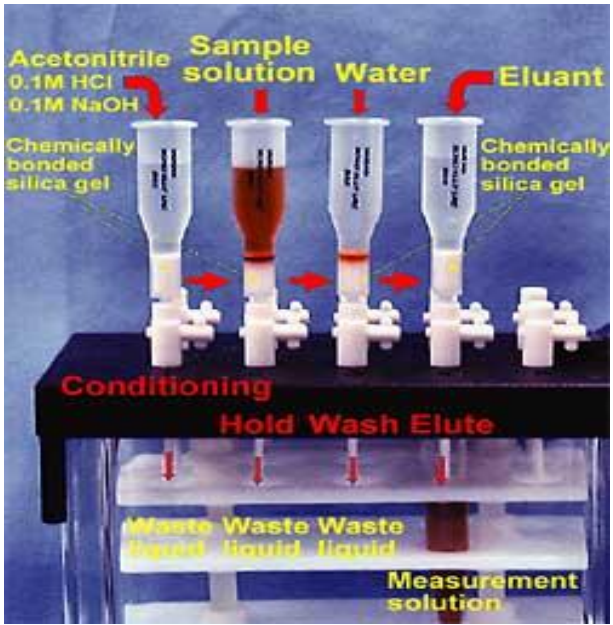
It consists of four steps:

1) **Conditioning of the sorbent**: Conditioning means the preparation of the SPE cartridge to interact with sample.

2) **Sample injection**: The sample solution is forced through the sorbent of the cartridge. The component of interest and some undesired compounds are adsorbed by the sorbent.

3) **Rinsing**: Rinse the cartridges to remove undesired matrix components.

4) **Elution**: Selectively desorbing the compounds of interest from the sorbent and collecting the cartridge effluent.



SPE can be applied either to standard solution or to real sample (i.e. biological fluids, pharmaceuticals, environmental samples etc). For both methods the SPE cartridges are conditioned prior to sample application with 3ml of methanol and 3ml of water under a low vacuum. The samples are slowly passed through the cartridges without using a vacuum, followed by washing steps with 3ml of water. The analytes are finally eluted with methanol or acidified methanol. The eluent can be injected directly to HPLC or after evaporating it to low temperatures 40-45ºC under a stream of nitrogen or using vacuum concentrator. In case if they are concentrated by drying under low temperature then the residues should be reconstituted with 100µl (or a suitable amount) of mobile phase. While using SPE for sample preparation, utilization of internal standard is necessary to calculate recovery absolutely. Moreover, care to be taken that the internal standard need to have approximately same Log-P value that of the analyte for its equal elution profile.

Extracting Encapsulated Compounds

Previously membrane- bound or encapsulated compounds that cause problem in extraction are removed by adding detergent so that membrane breaks, everything is pulled into solution and then the compound of interest is extracted out. But detergent contaminates chromatography. A better method is to add an equal volume of dimethyl sulfoxide (DMSO) or dimethyl form amide (DMF) to the aqueous sample. This breaks membranes and pulls both polar and nonpolar material into solution. Then dilute the sample with 10 volumes of water. At this point, nonpolar compound can be removed by solvent extraction or with a C_{18} SPE column. Charged molecules can be recovered with pH-controlled extraction or with ion pairing reagents. The DMSO or DMF stays with the water. Simultaneously quantitative recovery of fat-soluble and water-soluble vitamins can be recovered from encapsulated mixtures. Vitamins are encapsulated to protect potency from air-oxidation. Water-soluble vitamins have non-polar encapsulation; fat-soluble vitamins have a polar encapsulation.

By this technique cell extractions can be done which are lipid/protein membranes –encapsulated mixtures of polar and non-polar compounds. Protein might get denatured in 50% DMSO and precipitate so they could be filtered off or might denature and refold on 10 dilution in water and stay in solution.

Tissue Preparation:

Usually the preparation of tissues extract is done with liquid extraction procedure. Most of the drug like substances are soluble in methanol. Even though, methanol is a good deproteinating agent, therefore, homogenizing tissues along with chilled methanol with the help of tissue homogenizer can also extract most of the drugs. Conventionally it is extracted with an organic solvent like

chloroform or diethylether or mixed with 3 volumes of methanol. The homogenate is vortexed and centrifuged at more than 6000 g for 15 min at 4°C. The supernatant is subjected for concentration step either by evaporation in the water bath for thermostable compounds or by using nitrogen flushing for thermolabile compounds. Alternatively, vacuum concentration would be an alternative option to gradually remove the solvent without the loss due to bumping, bubbling, and spitting in the above process.

The important problem in extraction with organic solvents is that it fetches lipids from the tissues. Biological membrane is made up of lecithin and cholesterol, therefore, the extract would be having all above components, due to which during reconstitution cycle better to re-extract it with mobile phase with strong vortexing followed by high-speed centrifugation. In certain estimation it is usual to wash the reconstituted extract with mobile phase with n-heptane to remove hydrophobic substances. This would decrease the problems associated with the change in peak shape (tailing) and column clogging, while running the HPLC. However, care must be taken while washing with n-heptane the percentage of loss of analyte should be determined that it is within the limit or not (Papadoyannis I.N.1993).

Summary

Chromatography is a separation science wherein the separation of the analyte along with time in the given mobile phase are governed by some basic principles. Based on the principle of separation, chromatography can be classified into adsorption (TLC, gas/column chromatography), partition (paper chromatography), ion-exchange and size-exclusion chromatography. Based on the column chemistry, there are affinity, normal phase and reverse phase chromatography. HPLC and UHPLC are the two types of chromatography classified on the basis of the pressure. An HPLC system consists of six basic units, the mobile phase supply system, the pump and programmer, the sample valve, the column, the detector and finally a means of presenting and processing the results. In an isocratic mobile phase ratio, constant solvent system ratio is used throughout, while in gradient system, the ratio of mobile phase composition changes with respect to time. Buffers are used to control the ionization of one or more molecules in the mobile phase and to provide separate sharp bands, based on pKa and pH. Most of the modern day HPLCs are fitted with Rheodyne ^{TM} (Cotati, California, US) injector. HPLC columns are packed with very fine particles (usually a few microns in diameter), required to attain the low dispersion that give the high plate counts expected of modern HPLC. The stationary phase is generally made up of hydrophobic alkyl chains (-CH2-CH2-CH2-CH3) that interact with the analyte. . As adsorption and partition principles are employed in the separation in bonded phase silica columns, appropriate combination of solvents must be selected based on their miscibility index and viscosity. In a well resolved chromatographic peak the peak area/height increases with respect to the increase in concentration and is then analyzed. Method validation is performed to ensure that any analytical methodology is accurate, specific, reproducible and rugged over the specified range. Regulated laboratories need to perform method validation in order to be in compliance with FDA regulations. The correct use of an HPLC column is extremely important for the life time of a column and therefore for the benefit of HPLC analysis.