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A REVIEW ON ANALYTICAL METHOD DEVELOPMENT AND METHOD VALIDATION

***T. Yugandharudu, M. Surendra, T. Viswasanthi**

Nirmala College of Pharmacy, Buddayapalle (P), Kadapa - 516002, Andhra Pradesh, India.

ABSTRACT

This article presents a review on chromatography and method validation applied to the determination of various pharmaceuticals is discussed. It describes about various Chromatographic techniques and its method validation. Also it briefly describes about the different types of chromatographic techniques and HPLC method validation parameters. The primary objective of validation is to form a basis for written procedure for production and process control which are designed to assure that the drug products have the identity, strength, quality and purity they purport or are represented to possess quality, safety and efficacy must be designed to build into the product. Each step of the manufacturing process must be controlled to maximize the probability that the finished products meet all quality and design specification.

Keywords: Chromatography, HPLC, Method Validation, Method development, ICH Guidelines.

INTRODUCTION

The term chromatography (Greek Kromatos-colour and Graphos-written) meaning colour writing. The study of chromatography started in eighteenth century when with a great interest the nature of inorganic compounds was studied on filter paper by Runge. He separated inorganic salts and observed that the inorganic salts travel to different extent producing attractive pattern.

A variety of methods are available for separation of components from the mixture and to analyzer them. They are physical methods, chemical methods. These methods are effective in purification, separation and identification of compounds. However difficulty, arises in case of compounds where individual components have very similar physical and chemical properties i.e. mixture of liquids having very close boiling points. Similarly,
In biological materials, these methods are not satisfactory. Chromatography method represent the most useful and powerful technique for these problems.
Advantages of chromatography [1]:

1) These methods are relatively gentle methods and decomposition of compounds does not occur. This is important particularly for labile type of substances and substances of biological origin.
2) These techniques are simple, rapid and require simple apparatus.
3) Very small quantity of mixture is required for analysis.
4) Complex mixtures can be handled with comparative ease.

Tswett defined chromatography as the method in which the components of a mixture are separated on an adsorbent column in a flowing system. Recently IUPAC has defined chromatography as a method used primarily for the separation of the components of a sample, in which the components are distributed between two phases, one of which is stationary while the other moves. The stationary phase may be solid or a liquid supported on a solid or gel, and may be packed in a column, spread as a layer or distributed as a film. The mobile phase may be gaseous or liquid.

Corresponding Author:- T. Yugandharudu Email:- yugandharbtpadu@gmail.com

TYPES OF CHROMATOGRAPHY

BASED UP ON THE NATURE OF STATIONARY AND MOBILE PHASE [2]

There are different types of chromatography based on the type of stationary and mobile phase used. They are

1. Gas – solid chromatography
2. Gas – liquid chromatography
3. solid – liquid chromatography
4. liquid – liquid chromatography

Examples for gas–solid and gas–liquid chromatography is gas chromatography, for solid–liquid chromatography are column chromatography, TLC, HPLC, for liquid – liquid chromatography are paper partition chromatography and column partition chromatography.

BASED ON PRINCIPLE OF SEPARATION

The principle of separation can be either adsorption or partition. Hence they can be called as adsorption chromatography or partition chromatography.

ADSORPTION CHROMATOGRAPHY

Adsorption chromatography is probably one of the oldest types of chromatography around. It utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. The equilibration between the mobile and stationary phase accounts for the separation of different solutes. The compound which has more affinity towards stationary phase travels slower and the compound which has lesser affinity towards stationary phase travels faster. Examples for adsorption chromatography are Gas–solid chromatography, Thin layer chromatography, Column chromatography and High performance liquid chromatography.

PARTITION CHROMATOGRAPHY

This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase. Solute equilibrates between the mobile phase and the stationary liquid. The stationary phase as such cannot be a liquid. Hence a solid support is used over which a thin film or coating of liquid is made which acts as a stationary phase. Examples for partition chromatography are Partition column chromatography, Paper chromatography, Thin layer chromatography, Gas-liquid chromatography, High performance liquid chromatography.

Counter current extraction works on similar principle. In this two immiscible solvents flow in opposite direction. The solute mixture is distributed between these solvents, depending upon their partition coefficient. The advantage is that fresh solvent comes in contact, therefore extraction is effective and thus solute mixture is separated into individual components.

BASED ON MODES OF CHROMATOGRAPHY

There are two types. They are based upon the polarity of stationary phase and mobile phase used.

NORMAL PHASE CHROMATOGRAPHY

In this stationary phase is polar and mobile phase is non-polar. This is not widely used in pharmacy.

REVERSE PHASE CHROMATOGRAPHY

In this the stationary phase is non-polar and the mobile phase is polar. This is most widely used in the pharmaceutical analysis.

BASED ON CHROMATOGRAPHIC BED SHAPE

COLUMN CHROMATOGRAPHY

Column chromatography is a separation technique in which the stationary bed is within a tube. The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole inside volume of the tube (packed column) or be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle part of the tube (open tubular column). Differences in rates of movement through the medium are calculated to different retention times of the sample.

In 1978, W. C. Still introduced a modified version of column chromatography called flash column chromatography (flash) [3]. The technique is very similar to the traditional column chromatography, except for that the solvent is driven through the column by applying positive pressure. This allowed most separations to be performed in less than 20 minutes, with improved separations compared to the old method. Modern flash chromatography systems are sold as pre-packed plastic cartridges, and the solvent is pumped through the cartridge. Systems may also be linked with detectors and fraction collectors providing automation. The introduction of gradient pumps resulted in quicker separations and less solvent usage.

In expanded bed adsorption, a fluidized bed is used, rather than a solid phase made by a packed bed. This allows omission of initial clearing steps such as centrifugation and filtration, for culture broths or slurries of broken cells.

PLANAR CHROMATOGRAPHY

Planar chromatography is a separation technique in which the stationary phase is present as or on a plane. The plane can be a paper, serving as such or impregnated by a substance as the stationary bed (paper chromatography) or a layer of solid particles spread on a support such as a glass plate (thin layer chromatography). Different compounds in the sample mixture travel different distances according to how strongly they interact with the stationary phase as compared to the mobile phase. The

specific Retardation factor (R_f) of each chemical can be used to aid in the identification of an unknown substance.

PAPER CHROMATOGRAPHY

Paper chromatography is a technique that involves placing a small dot or line of sample solution onto a strip of chromatography paper. The paper is placed in a jar containing a shallow layer of solvent and sealed. As the solvent rises through the paper, it meets the sample mixture which starts to travel up the paper with the solvent. This paper is made of cellulose, a polar substance, and the compounds within the mixture travel farther if they are non-polar. More polar substances bond with the cellulose paper more quickly, and therefore do not travel as far.

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is a widely employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose on a flat, inert substrate. Compared to paper, it has the advantage of faster runs, better separations, and the choice between different adsorbents. For even better resolution and to allow for quantification, high-performance TLC can be used.

DISPLACEMENT CHROMATOGRAPHY

The basic principle of displacement chromatography is: A molecule with a high affinity for the chromatography matrix (the displacer) will compete effectively for binding sites, and thus displace all molecules with lesser affinities. There are distinct differences between displacement and elution chromatography. In elution mode, substances typically emerge from a column in narrow, Gaussian peaks. Wide separation of peaks, preferably to baseline, is desired in order to achieve maximum purification. The speed at which any component of a mixture travels down the column in elution mode depends on many factors. But for two substances to travel at different speeds, and thereby be resolved, there must be substantial differences in some interaction between the biomolecules and the chromatography matrix. Operating parameters are adjusted to maximize the effect of this difference. In many cases, baseline separation of the peaks can be achieved only with gradient elution and low column loadings. Thus, two drawbacks to elution mode chromatography, especially at the preparative scale, are operational complexity, due to gradient solvent pumping, and low throughput, due to low column loadings. Displacement chromatography has advantages over elution chromatography in that components are resolved into consecutive zones of pure substances rather than "peaks". Because the process takes advantage of the nonlinearity of the isotherms, a larger column feed can be separated on a given column with the purified components recovered at significantly higher concentrations.

OTHER TYPES OF CHROMATOGRAPHY [4]

ION EXCHANGE CHROMATOGRAPHY

In this type of chromatography, the use of a resin (the stationary solid phase) is used to covalently attach anions or cations onto it. Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces.

GEL PERMEATION CHROMATOGRAPHY

Gel permeation chromatography is a term used for when the separation technique size exclusion chromatography (SEC), that separates analytes on the basis of size, is applied to polymers in particular. It is also called as a Molecular Exclusion Chromatography (MEC) or gel filtration. This type of chromatography lacks an attractive interaction between the stationary phase and solute. The liquid or gaseous phase passes through a porous gel which separates the molecules according to its size. The pores are normally small and exclude the larger solute molecules, but allows smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones.

CHIRAL CHROMATOGRAPHY [4-6]

The biological activity of chiral substances often depends upon their stereochemistry, since the living body is a highly chiral environment. A large percentage of commercial and investigational pharmaceutical compounds are enantiomers, and many of them show significant enantioselective differences in their pharmacokinetics and pharmacodynamics. The importance of chirality of drugs has been increasingly recognized, and the consequence of using them as racemates or as enantiomers has been frequently discussed in the pharmaceutical literature during recent years. With increasing evidence of problems related to stereo selectivity in drug action, enantioselective analysis by chromatographic methods has become the focus of intensive research of separation scientists. Most of the pharmaceutical and pharmacological studies of stereoselectivity of chiral drugs before the mid eighties involved pre-column derivatization of the enantiomers with chiral reagents, forming diastereomers. The diastereomers were subsequently separated in the normal or reversed phase mode of chromatography.

AFFINITY CHROMATOGRAPHY

This is the most selective type of chromatography employed. It utilizes the specific interaction between one kind of solute molecule and a second molecule that is immobilized on a stationary phase. For example, the immobilized molecule may be an antibody to some specific protein. When solute containing a mixture of proteins are

passed by this molecule, only the specific protein is reacted to this antibody, binding it to the stationary phase. This protein is later extracted by changing the ionic strength or pH.

GAS CHROMATOGRAPHY

Gas chromatography makes use of a pressurized gas cylinder and a carrier gas, such as helium, to carry the solute through the column. The most common detectors used in this type of chromatography are thermal conductivity and flame ionization detectors. There are three types of gas chromatography that will be discussed here: gas adsorption, gas-liquid and capillary gas chromatography.

Gas adsorption chromatography involves a packed bed comprised of an adsorbent used as the stationary phase. Common adsorbents are zeolite, silica gel and activated alumina. This method is commonly used to separate mixtures of gases.

Gas-liquid chromatography is a more common type of analytical gas chromatography.

In this type of column, an inert porous solid is coated with a viscous liquid which acts as the stationary phase. Diatomaceous earth is the most common solid used. Solutes in the feed stream dissolve into the liquid phase and eventually vaporize. The separation is thus based on relative volatilities.

Capillary gas chromatography is the most common analytical method. Glass or fused silica comprises the capillary walls which are coated with an absorbent or other solvent. Because of the small amount of stationary phase, the column can contain only a limited capacity. However, this method also yields rapid separation of mixtures.

LIQUID CHROMATOGRAPHY

There are a variety of types of liquid chromatography. There is liquid adsorption chromatography in which an adsorbent is used. This method is used in large-scale applications since adsorbents are relatively inexpensive. There is also liquid- liquid chromatography which is analogous to gas-liquid chromatography. The three types that will be considered here fall under the category of modern liquid chromatography. They are reverse phase, high performance and size exclusion liquid chromatography, along with supercritical fluid chromatography.

Reverse phase chromatography is a powerful analytical tool and involves a hydrophobic, low polarity stationary phase which is chemically bonded to an inert solid such as silica. The separation is essentially an extraction operation and is useful for separating non-volatile components.

High performance liquid chromatography (HPLC) is similar to reverse phase, only in this method, the process is conducted at a high velocity and pressure drop. The column is shorter and has a small diameter, but it is equivalent to possessing a large number of equilibrium stages.

Size exclusion chromatography, also known as gel permeation or filtration chromatography does not involve any adsorption and is extremely fast. The packing is a porous gel, and is capable of separating large molecules from smaller ones. The larger molecules elute first since they cannot penetrate the pores. This method is common in protein separation and purification.

Supercritical fluid chromatography is a relatively new analytical tool. In this method, the carrier is a supercritical fluid, such as carbon dioxide mixed with a modifier. Compared to liquids, supercritical fluids have solubilities and densities have as large, and they have diffusivities and viscosities quite a bit larger. This type of chromatography has not yet been implemented on a large scale.

SEPARATION TECHNIQUES [1]

Chromatographic separation can be carried out by following techniques:

ELUTION ANALYSIS

It is a very common method used in column chromatography. In this method a small volume of mixture to be separated is added on the top of column and mobile phase is allowed to flow through the column. As mobile phase moves down the column, the mixture introduced on the column gets separated into zones as the components of mixture are adsorbed to the column material to different extent. On further passage of mobile phase, each component of mixture is eluted out as separated component. This method is called elution analysis.

FRONTAL ANALYSIS

In this method, the solution of sample mixture is added continuously on the column. No mobile phase (solvent) is used for development of column. A mixture containing A, B and C is added on the column. If component A is least adsorbed, component B to intermediate extent and component C most strongly to the column material, then as the mixture flows through the column, the least adsorbed A runs the column fast, component B to intermediate extent while C is retained at the top of column. When more sample mixture passes through the column, first few fractions of eluate will contain A, subsequent fractions will contain A+B and then A+B+C. Only a partial separation of A from B and C occurs. A complete separation of A, B and C cannot be

achieved unless the fractions are again developed on another column.

DISPLACEMENT ANALYSIS

This method is encountered in adsorption column chromatography. In this method, a small volume of mixture is added to the column and elution is carried out by a solvent containing a solute which has high adsorptivity for column material. The adsorbed constituents of mixture are displaced by the solute from mobile phase. Each solute in the mixture in turn displaces another solute which is less firmly adsorbed. The least adsorbed constituent is pushed out of column. The substance used in mobile phase is called as displacer and the technique as "displacement analysis". This technique is mainly used in preparative work and is not suitable for analysis.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY [7]

It is known that the resolving power of a chromatographic column increases with column length and the number of theoretical plates per unit length. As the number of theoretical plates is related to the surface area of the stationary phase it follows that the smaller the particle size of the stationary phase, the better the resolution. Unfortunately the smaller the particle size, the greater the resistance to eluant flow. All of the forms of column chromatography rely on gravity or low pressure pumping systems for the supply of eluant to the column. The consequences of this is that the flow rates achieved are relatively low and this gives greater time for band broadening by simple diffusion phenomena. The use of faster flow rates is not possible because it creates a back-pressure which is sufficient to damage the matrix structure of the stationary phase, thereby actually reducing the eluant flow and impairing resolution. In the past decade there has been a dramatic development in the column chromatography technology which has resulted in the availability of new particle size stationary phases which can withstand these pressures and of pumping systems which can give reliable flow rates. These developments, which have occurred in adsorption, partition, ion-exchange, exclusion and affinity chromatography, have resulted in faster and better resolution and thus HPLC has emerged as the most popular, powerful and versatile form of chromatography.

Originally, HPLC was referred to as high pressure liquid chromatography but now the term high performance liquid chromatography is preferred since it better describes the characteristics of the chromatography and avoids creating the impression that high pressures are an inevitable pre-requisite for high performance. This is now known not to be the case and the term *medium pressure liquid chromatography* (MPLC) has been coined for some separations.

INSTRUMENTATION

In order to attain reasonable high flow rates and yet keep particle size of packing
very low (3-10μm), pumping pressures of several hundred atmospheres (2000-8000 psi) are required. Thus the equipment for HPLC is quite elaborate though simple.

MOBILE PHASE RESERVOIR AND SOLVENT TREATMENT SYSTEMS [1]

A modern HPLC apparatus is equipped with one or more glass or stainless steel reservoirs, each of which contain 500 ml or more solvent. The reservoirs are often equipped with a means of removing dissolved gases usually oxygen and nitrogen that interfere by forming bubbles in the columns and detector systems. These bubbles cause band spreading, in addition they interfere with the performance of the detector.

Degassers may consist of: 1) a vacuum pumping system or, 2) a distillation system or, 3) devices for heating and stirring the solvents or, 4) device for sparging in which the dissolved gases are swept out of solution by fine bubbles of an inert gas of low solubility. Often there is a filter for removing dust and particulate matter from solvents. In Analytical HPLC the mobile phase is pumped through the column at a flow rate of 1-5 ml/min.

In HPLC the mobile phase can be aqueous organic mixture, mixture of organic solvents or buffer solution, depending on the chromatographic method and on the detector used. A separation that employs a single solvent of constant composition is termed an ISOCRATIC elution. Frequently separation efficiency is greatly enhanced by GRADIENT elution. Here two and sometimes more solvent systems that differ significantly in polarity are employed. Modern HPLC equipment is often equipped with devices that introduce solvents from two or more reservoirs into a mixing chamber at rates that vary continuously; the proportioning values that are provided alter the volume ratio of the solvents linearly or exponentially with time.

PUMPS

The pumps are used to pass mobile phase through the column at high pressures and controlled flow rate. In addition to this the pumps used in HPLC should have the following features:

a) The generation of pressures up to 6000 psi. b) Flow rates ranging from 0.1 to 10 ml/min. c) Flow control and flow reproducibility of (+/-) 0.5. d) It should be composition resistant and give a pulse free output. e) It should be easy to change from one mobile phase to another. f) The pump should be easy to dismantle and repair.

These pumps are necessary to force the liquid (mobile phase) through the column with finally packed particles. It should be noted that the high pressures generated by the pumps should not lead to an explosion hazard as liquid are not very compressible. The pumps are categorized into

A) constant displacement pumps or syringe pumps
B) Reprocating pumps
C) Constant pressure or pneumatic pumps

DISPLACEMENT PUMPS

It consists of a large, syringe like chamber equipped with a plunger that is activated by a screw driven mechanism powdered by a stepping motor. The advantages of the pumps are 1) flow is independent of viscosity and column back pressure 2) the flow is pulse free. The disadvantage is that they have limited solvent capacity (200-500 ml) and it is not easy to change solvent for purposes of gradient elution

RECIPROCATING PUMPS

It consists of a small chamber in which the solvent is pushed back and forth with the help of a motor driven piston or pressure may be transmitted by a diaphragm which is hydraulically pumped by a reciprocating piston. Advantages of this mechanism and pump are 1) small internal volume (35-400 µl) 2) high output pressure (upto 10000 psi) 3) their ready adaptability to gradient elution and 4) their constant flow rates which are independent of column back pressure and solvent viscosity. Disadvantage is that they give a pulsed flow which must be damped as they produce a base line noise on the chromatogram.

PNEUMATIC PUMPS

In this the mobile phase is contained in a collapsible container housed in a vessel that can be pressurized by a compressed gas. Advantages of this pump are 1) flow is pulse free and 2) equipment is cheap. Disadvantages are 1) limited capacity 2) pressure output and flow rate depend on solvent viscosity and column back pressure 3) gradient elution not possible with this pump and 4) pressure output is less than 2000 psi.

PRECOLUMN

Some HPLC instruments are equipped with a precolumn, which contains a packing chemically identical to that in the analytical column. Particle size is large hence the pressure drop across the precolumn is negligible with respect to the analytical column. The precolumn is mainly used to remove the impurities from the solvent and thus prevent contamination of the analytical column.

SAMPLE INJECTORS

Often the limiting factors in the precision of liquid chromatographic measurement lies in the reproducibility with which samples can be introduced into the column packing. It must be noted that the overloading of the sample causes band broadening. Therefore minimum amount of sample must be introduced. It is convenient to introduce the sample with out depressurizing the system.

The sample is usually injected at the head of the column with minimum disturbance of the column material. The sample injectors are of the following types:

SYRINGE INJECTION

This is the earliest and simplest technique. Hence the sample is injected through a self sealing elastomeric septum and the syringes are designed to withstand pressures upto 1500 psi. The advantage is that the reproducibility is poor.

STOP FLOW INJECTION

This too is a syringe injection but here the solvent flow is stopped momentarily. A fitting at the column head is removed and sample is injected directly on to the head of the column packing at atmospheric pressure. Then the fitting is replaced and the system is again depressurized. This technique is extremely simple and as diffusion in liquids is small resolution is not affected.

SOLVENT FLOWING

Here sampling valves or loops are used. These types of injectors are usually used for injecting sample volumes more than 10µl. This is now a days used in all automatic system. In the fill position the sample loop is filled at atmospheric pressure. When the valve is actuated the sample in the loop is also activated. Samples in the range of 1-9 ml can be handled without affecting column efficiency and hence system is very popular.

LIQUID CHROMATOGRAPHIC COLUMN

They are usually constructed from smooth bore stainless steel or heavy walled glass tubing. If prepared from heavy walled glass tubing, but their, use is very limited. The columns are of three types

- Analytical columns whose length varies from 25 to 100 cm with internal diameter 2 to 6 mm.
- Preparative columns whose length varies from 25 to 100 cm with internal diameter 6 mm or more.
- Micropore columns.

The main advantage of these columns is speed and minimum consumption. These columns must also be able to withstand high pressure and they must also be provided with a system for temperature control.

COLUMN PACKING MATERIALS

Three basic types of packing's have been used in HPLC, they are

- Pellicular: It consists of non porous, spherical glass or polymer beads with a diameter of 30 to 40 µm. A thin porous layer of silica or alumina or ion exchange resin is deposited over it. Sometimes a liquid stationary phase is held by adsorption. Alternatively the beads may be treated

chemically to give an organic surface layer example silica beads which are esterified with alcohols (CORASIL)

- Porous particle: These particles have sizes ranging from 3 to 10 μm and are composed of silica or alumina or ion exchange resin. These particles are often wetted with a thin organic film which is physically or chemically bonded to the surface. Eg- PORASIL, PORAGEL/STYRAGEL. ZIPAX particles are often coated with liquid stationary phase.
- Bonded phases where the stationary phase is chemically bonded on to an inert support [7].

Now a days analytical HPLC is done with microparticulate column packings which are small porous particles usually spherical or irregular silica with diameter 3, 5 or 10 μm. They have high efficiency as well as a large surface area. Microparticulates are generally packed into columns using slurry of the material in a suitable solvent and under considerable pressure.

DETECTORS

A detector is required to sense the presence and the amount of the sample component in the column effluent. The output of the detector is an electrical signal that is proportional to some property of the mobile phase and/or the solute. A detector that measures the property which is possessed by the both mobile phase and solute is called bulk property detector eg: Refractive index detector. Alternatively if the property is possessed essentially by the solute eg: absorption of uv or visible light of electrochemical property, the detector is called a solute property detector.

The ideal characters of a detector include

1)It should respond to all components of the mixture in a wide range of mobile phase.

2) It should not respond to mobile phase.

3) It should be unaffected by changes in temperature and flow rate.

4) It should have high sensitivity that is larger detector signal for smaller amount of solute. Low noise and a wide linear response to solutes present.

5) It should not constitute to zone spreading.

6) Non –destructive, cheap, reliable and easy to use.

Generally two types of detectors are used:

REFRACTIVE INDEX MONITOR DETECTORS

Since every compound has its own refractive index this property becomes a universal indicator. A differential refractometer continuously monitors the difference in R.I between the pure mobile phase (reference stream) on the column effluent. The disadvantages of these detectors are

1) They respond to nearly all solutes

2) They are reliable and unaffected to flow rate

3) They do not require any double bond or aromaticity to be present in the structure for elucidating a response while disadvantage is that there must be a difference between the refractive index of the solutes and of the mobile phase and this is not a very sensitive detector.

UV-VISIBLE ABSORPTION DETECTOR

These are most popular detectors in HPLC. The principle is that the mobile phase from the column is passed through a small flow cell in the radiation beam of the uv or visible spectrophotometer. These detectors are selective, in that they detect only those solutes that absorb uv/visible radiation eg: alkenes, aromatic compounds and compounds having multiple bonds between C and O, N or S. This is thousand times more sensitive than R.I detectors. A low pressure mercury lamp acts as a source. Alternatively a deuterium lamp or a tungsten filament with intermediate filters can also act as a source.

Both fixed and variable wavelengths uv/visible detectors are available, the later can operate between 190 to 700 mm and will have number of absorbance ranges. Fixed wavelength detector can operate at 254 nm, 280 nm or other wavelength where most organic compounds, double bonds/aromatic groups cause absorption.

OTHER TYPES OF DETECTORS INCLUDE [8]

- IR detectors
- Fluorescence detectors
- Mass spectrometric detectors
- Electro chemical detectors
- Differential refractometers
- Christiansen Effect detector
- Conductivity detector

RECORDERS

The signals from a detector are recorded as deviation base line. Two open recorders are used with instruments having two detectors. The peak position along the curve relative to the starting point denotes the particular component. With proper calibration, the height or area of the peak is a measure of amount of component in a sample.

APPLICATIONS OF HPLC

NATURAL PRODUCTS

HPLC is an ideal method for the separation of various components in plant extract which resemble in structure and thus demand a specific and very sensitive method e.g. analysis of digitalis, cinchona, liquorice, ergot extracts.

STABILITY STUDIES

HPLC is now used for ascertaining the stability of various pharmaceuticals. With HPLC the analysis of various degradation products can be done and thus stability indicating HPLC systems have been developed e.g. stability studies of atropine.

BIOASSAYS AND ITS COMPLEMENTATION

Complex molecules as antibiotics and peptide hormones are mainly analysed by bioassay which suffer from high cost, necessity of replicates, poor precision and length of time required. Also bioassays give an overall estimate of potency and give no guidance about the composition. Thus HPLC can be used to complement bioassays and give an activity profile. It has been used for analysis of chloramphenicol, penicillins, cotrimoxazole, sulfas and peptide hormones.

SOLVENTS USED IN HPLC [8]

Solvent is selected normally by matching the relative polarity of the solvent to that of
the sample components. As a first approximation, a solvent is chosen to match the most polar functional group in the sample. For e.g. alcohols for hydroxyl group and ketones or acetates for the carbonyl group. From this first chromatogram, the separation can be refined. If the sample elutes too rapidly then a less polar solvent is substituted. If the sample does not elute in a reasonable time then a solvent with high polarity is selected. Two solvents whose solvent strength parameters are, respectively too small and too large may be blended together in various proportions to allow continuous variation in solvent strength between that of each pure solvent. Hence greater retention and the need to select a stronger solvent. The various solvents used in HPLC in increasing order of their polarity are as follows pentane, hexane, cyclohexane, carbon disulfide, carbon tetrachloride, 1-chlorobutane, diisopropyl ether, 2-chloropropane, benzene, diethyl ether, chloroform, methylene dichloride, acetone, ethyl acetate, acetonitrile, propanol, methanol and water.

Following points are considered for the selection of a mobile phase.

- Viscosity.
- Compressibility.
- Refractive index.
- UV cutoff.
- Polarity.
- Vapour pressure.
- Flash point.

The viscosity generally increases with the number of carbons in the solvent. Straight chain alcohols show a very pronounced relationship of this nature. For example, to achieve 1ml/min flow rate in a 4.6 ×250mm column packed with 5 µm Octadecyl Silane material, a pressure of 1500 psi is required with methanol. Solvents of low viscosity are needed to be compatible with the limitations of the pump. Also as viscosity increases, the efficiency of the system, as measured by the number of theoretical plates decreases. The sensitivity of the detection is related to the difference between the respective refractive indices, i.e. the greater the difference, greater is the sensitivity. The UV cutoff is defined as the wavelength below which the solvent will absorb more than 1.0 absorbance unit.

The polarity of the solvent is a measure of the dielectric constant or the ability to elute a particular type of compound. The vapour pressure of a solvent plays an important role in mobile phase selection. Solvent reservoir could easily change in composition due to the evaporation of one of the more volatile constituents. The flammability of the mobile phase is a safety consideration. Careful attention should be paid to adequate ventilation and waste solvent disposition.

FACTORS INFLUENCING HPLC

The various factors that effect HPLC are as follows

- Internal diameter
- Particle size
- Pore size
- Pump pressure
- Temperature
- Column length
- Viscosity

INTERNAL DIAMETER

The internal diameter (ID) of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution. It also determines the quantity of analyte that can be loaded onto the column. Larger columns are usually seen in industrial applications, such as the purification of a drug product for later use. Low-ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity.

- Larger ID columns (over 10 mm) are used to purify usable amounts of material because of their large loading capacity.
- Analytical scale columns (4.6 mm) have been the most common type of columns,

though smaller columns are rapidly gaining in popularity. They are used in traditional
quantitative analysis of samples and often use a UV-Vis absorbance detector.

- Narrow-bore columns (1-2 mm) are used for applications when more sensitivity is desired either with special UV-vis detectors, fluorescence detection or with other detection methods like liquid chromatography-mass spectrometry
- Capillary columns (under 0.3 mm) are used almost exclusively with alternative

detection means such as mass spectrometry. They are usually made from fused silica capillaries, rather than the stainless steel tubing that larger columns employ.

PARTICLE SIZE

Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). These particles come in a variety of sizes with 5 μm beads being the most common. Smaller particles generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter squared [9,10].

This means that changing to particles that are half as big, keeping the size of the column the same, will double the performance, but increase the required pressure by a factor of four. Larger particles are used in preparative HPLC (column diameters 5 cm up to >30 cm) and for non-HPLC applications such as solid-phase extraction.

PORE SIZE [11]

Many stationary phases are porous to provide greater surface area. Small pores provide greater surface area while larger pore size has better kinetics, especially for larger analytes. For example, a protein which is only slightly smaller than a pore might enter the pore but does not easily leave once inside.

PUMP PRESSURE

Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible flow rate. Pressure may reach as high as 40 MPa (6000 lbf/in^2), or about 400 atmospheres. Modern HPLC systems have been improved to work at much higher pressures, and therefore are able to use much smaller particle sizes in the columns (<2 μm). These "Ultra High Pressure Liquid Chromatography" systems or RSLC/UHPLCs can work at up to 100 MPa (15,000 lbf/in²), or about 1000 atmospheres. The term "UPLC" is a trademark of Waters Corporation and designates the first commercial, holistically designed system that not only operates at high pressure but also has reduced system dispersion and system volume required to take full advantage of sub-2-micron-diameter particle columns.

TEMPERATURE [8]

Variations in temperature can cause significant changes in retention times, making
qualitative analysis difficult and affecting the precision of quantitative measurements. Elevated temperatures are advantageous because decreased mobile-phase viscosity, increased mass transfer, and increased sample solubility result in either better resolution or faster analysis.

COLUMN LENGTH

Finer particles are usually paired with shorter columns to achieve a given separation. This necessitates the use of higher inlet pressures to move the mobile phase through the column at the optimum velocity. Both effects eventually result in serious technical difficulties.

VISCOSITY

A solvent with low viscosity is always preferred in HPLC. While maintaining constant the pressure drop across the column, an increase in the viscosity of the solvent always decreases the flow rate of the mobile phase. The diffusion coefficient of the solutes is also affected by the viscosity of the mobile phase.

REVERSE PHASE HPLC (RP HPLC)

Reversed-phase chromatography (RPC) includes any chromatographic method that uses a non-polar stationary phase. Most liquid chromatography was done on non-modified silica or alumina with a hydrophilic surface chemistry and a stronger affinity for polar compounds - hence it was considered "normal". The introduction of alkyl chains bonded covalently to the support surface reversed the elution order (I. Molnar and C. Horvarth, 1976). Now polar compounds are eluted first while non-polar compounds are retained - hence "reversed phase". Since most of the drugs and pharmaceuticals are polar in nature; they are not retained for a longer time and eluted faster, which is advantageous.

STATIONARY PHASES

SILICA BASED STATIONARY PHASES

Any inert non-polar substance that achieves sufficient packing can be used for
reversed-phase chromatography. The most popular column is a octadecyl carbon chain (C18) bonded silica (USP classification L1) with 297 columns commercially available. This is followed by C8 bonded silica (L7 - 166 columns), pure silica (L3 - 88 columns), cyano bonded silica (L10 - 73 columns) and phenyl bonded silica (L11 - 72 columns). Note that C18, C8 and phenyl are dedicated reversed phase packings while cyano columns can be used in a reversed phase mode depending on analyte and mobile phase conditions. It should be noted at this point that not all C18 columns have identical retention properties. Surface functionalization of silica can be performed in a monomeric or a polymeric reaction with different short-chain organosilanes used in a second step to cover remaining silanol groups (end-capping). While the overall retention mechanism remains the same subtle differences in the surface chemistries of different stationary phases will lead to changes in selectivity.

MOBILE PHASE CONSIDERATIONS

Mixtures of water or aqueous buffers and organic solvents are used to elute
analytes from a reversed phase column. The solvents have to be miscible with water and the most common organic solvents used are acetonitrile, methanol or tetrahydrofuran

(THF). Other solvents can be used such as ethanol, 2-propanol (iso-propyl alcohol). Elution can be performed isocratic (the water-solvent composition does not change during the separation process) or by using a gradient (the water-solvent composition does change during the separation process). The pH of the mobile phase can have an important role on the retention of an analyte and can change the selectivity of certain analytes. Charged analytes can be separated on a reversed phase column by the use of ion-pairing (also called ion-interaction). This technique is known as reversed phase ion-pairing chromatography.

VALIDATION [12,13]

Validation is a key process for effective Quality Assurance (QA). "Validation" is establishing documented evidence, which provides a high degree of assurance that a specific process or equipment will consistently produce a product or result meeting its predetermined specification and quality attributes.

OBJECTIVE OF VALIDATION

The primary objective of validation is to form a basis for written procedure for production and process control which are designed to assure that the drug products have the identity, strength, quality and purity they purport or are represented to possess quality, safety and efficacy must be designed to build into the product. Each step of the manufacturing process must be controlled to maximize the probability that the finished products meet all quality and design specification.

BENEFITS OF VALIDATION

- Produce quality products.
- Helps in process improvement technology transfer, related product validation, failure investigation, and increased employee awareness.
- Cost reduction by increasing efficacy, few reject, longer equipment life, production of cost effective products.
- Help in optimization of process or method. Regulatory affairs-produces approved products and increased ability to export.

VALIDATION AS DEFINED BY DIFFERENT AGENCIES

USFDA

According to this "validation is the process establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product of predetermined specifications and quantity attributes".

WHO

Defines validation as on action of providing any procedure, process, equipment, material, activity or system actually leads to the expected results.

EUROPEAN COMMITEE

Define validation as "action of proving in accordance with the principle of Good manufacturing practice (GMP), that any material, activity or system actually lead to expected result".

ANALYTICAL METHOD VALIDATION

Establishing an accurate assay procedure for each ingredient of complex dosage formulation containing several therapeutically compatible drugs with very similar chemical nature is a critical process. The presence of recipients, additives and decomposition products further complicates the analysis. Therefore analytical development is done for few drugs where no compendial methods are available.

Method development is done for

- New drug products.
- Already existing products.

Methods are developed for new products when no official methods are available and for already existing products to reduce the cost and time for better precision and ruggedness.

STEPS OF METHOD DEVELOPMENT

It starts with the documentation of the developed studies. All the data related to these studies are established and recorded in laboratory notebook.

ANALYTICAL STANDARD CHARACTERIZATION

- All the known information about the drug or analyte and its structure is collected such as its physical and chemical properties, toxicity, purity, hygroscopic nature solubility and stability.
- The standard analyte is obtained. Necessary arrangement is made for proper storage in refrigerator, dissector, and freezer.
- When multiple compounds are to be analyzed in the sample matrix the number of components is noted, data is assembled and the availability of standards for each one is determined.
- Special attention to be taken when sample is in less quality.
- Only the methods, which are compatible with sample stability, are conceded.

METHOD REQUIRMENTS

The objectives of method are defined. The required detection limits, linearity, range, accuracy and precision are defined.

LITERATURE SEARCH AND RESEARCH METHODOLOGY

The literature survey for all types of information to the analyte was carried out. Literature is done for synthesis physico-chemical properties, solubility and

relevant analytical methods. Books, periodicals, chemical manufacturers and regulatory agency compendia such as USP/NF, AOAC publications are reviewed along with chemical abstract service (CAS) automated computerized literature searches.

CHOOSING A METHOD

- If any reported methods from the literature are adaptable to the current laboratory setting and future needs is determined.
- Using information in the literature and prints, methodology is adapted. The methods are modified wherever necessary; acquire additional existing methods for in house analyte and sample.
- If there are no prior methods for the analyte in the literature, from analogy, the compounds that are similar and chemical properties are investigated and are worked out.

INSTRUMENTAL SET UP AND INITIAL STUDIES

a) The required instrument is set up. Installation, operational and performance of instrumentation using laboratory standard operating procedure are reviewed.

b) Always new consumables (solvents, filter and gases) are used.

c) The analyte standard in a suitable injection/introduction solution and in known concentration and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix. If the sample is extremely close to the standard (example: - bulk drug) than it is possible to start work with the actual sample.

d) Feasibility of method with regards to the analytical figures of merit obtained is evaluated.

OPTIMIZATION

During optimization one parameter is changed at a time and set of conditions are isolated rather than using a trial and error approach. Work has been done from an organized methodological plan and every step is documented in case of dead ends.

DOCUMENTATION OF METHOD DEVELOPMENT WITH ACTUAL SAMPLE

The sample solution should lead to absolute identification of the peak of interest apart
from all other matrix components.

EVALUATION OF METHOD DEVELOPMENT WITH ACTUAL SAMPLE

The sample solution should lead to absolute identification of the peak of interest apart from all other matrix components.

DETERMINATION OF PERCENT RECOVERY OF ACTUAL SAMPLE AND DEMONSTRATION OF QUANTITATIVE SAMPLE ANALYSIS

Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery from sample to sample and whether recovery has been optimized has been shown. It is not necessary to obtain 100% recovery as long as the results are reproducible and known with high degree of certainly.

METHOD VALIDATION [13-16]

This process consists of establishments of the performance characteristics and the limitation of the method.

METHOD PERFORMANCE PARAMETERS ARE DETERMINED USING EQUIPMENT THAT IS

- Within specification
- Working correctly
- Adequately calibrated

METHOD VALIDATION IS REQUIRED WHEN

- A new method is been developed
- Revision established method
- When established method are used in different laboratories and different analysts etc.
- Comparison of methods
- When quality control indicates method changes.

PERFORMANCE CHARACTERISTICS EXAMINED WHEN CARRYING OUT METHOD VALIDATION ARE

- Accuracy / precision
- Repeatability / Reproducibility
- Linearity / Range
- Limit of detection (LOD) / Limit of quantification (LOQ)
- Selectivity / Specificity
- Robustness / Ruggedness

ACCURACY

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

DETERMINATION OF ACCURACY

The accuracy may be determined by application of analytical method to an analyte of known purity (example: reference standard) and also by comparing the results of the method with those obtained using an alternate procedure that has been already validated.

Accuracy is calculated as the percentage of recovery by the assay of the known added amount of the analyte in the sample or the difference between the mean and accepted true value together with confidence intervals.

The ICH guidelines recommend to take minimum of 3 concentration levels covering the specified rang and 3 replicates of each concentration are analyzed (totally 3*3=9 determination.)

PRECISION

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision of an analytical procedure is usually expressed the variance, standard deviation or coefficient of variation of a series of measurements.

SYSTEM PRECISION

A system precision was evaluated by measuring the peak response of drug for six replicate injection of the standard solution prepared as per the proposed method.

METHOD PRECISION

The method precision was determined by preparing the sample of a single batch of Aripiprazole formulation six times and analyzing as the proposed method.

DETERMINATION OF PRECISION

The procedure is applied repeatedly to separate identical sample drawn from the homogeneous batch of material and measured by the scatter of individual results from the mean and expressed as the standard deviation or as the coefficient of variation (relative standard deviation).

SPECIFICITY

ICH document divides specificity into two categories.

- Identification tests
- Assay / impurity tests

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures.

IDENTIFICATION TEST

It is demonstrated by the ability to discriminate between compounds of closely related structures or by comparison to known reference materials.
Use of positive and negative control is recommended.

ASSAY IMPURITY TEST

It is demonstrated by resolution of the two closest eluting compounds. If impurities are available it has to be shown that the assay is unaffected by the presence of spiked material.

If impurities are not available the test results are compared to a second well-characterized method.

DETERMINATION OF SPECIFICITY

When chromatographic procedures are used representative chromatograms should be presented to demonstrate the degree of selectivity.
Samples generated by stress testing of the drug substances using acid and base hydrolysis, temperature, photolysis and mass spectrometry nay be useful to show that the chromatographic peak is not attributable to more than one component.

SELECTIVITY

It is a procedure to detect qualitatively the analyte in the presence of compounds that may
be expected to be present in the sample matrix or the ability of a seperative method to resolve different compounds. It is the measure of the relative method location or two peaks.

DETERMINATION OF SELECTIVITY

Is determined be comparing the test results obtained on the analyte with and without addition of potentially interfering material. When such components are either unidentified or unavailable a measure of selectivity can be obtained by determining the recovery of a standard addition of pure analyte to a material containing a constant level of the other compounds.

SENSITIVITY

Sensitivity is the capacity of the test procedure to record small variation in concentration. It is the slope of the calibration curve.

LIMIT OF DETECTION

The limit of detection is the lowest concentration of the analyte in a sample that can be detected but not necessarily determined in quantitatively using a specific method under the required experiment conditions. Such a limit is expressed in terms of a concentration of analyte (example: -µg/ml) in the sample.

MEASUREMENT IS BASED ON

- Signal to noise ratio
- Visual evaluation (relevant chromatogram acceptable)
- The standard deviation of the response and the slope.

$$LOD = \frac{3.3\sigma}{S}$$

Where

σ = The standard deviation of the response

S = the slope of the calibration curve (of the analyte)

LIMIT OF QUANTIFICATION

The LOQ is the lowest concentration of analyte in a sample, which can be quantitatively determined and might be measured with an acceptable level of accuracy and precision under the stated operational conditions of the method. LOQ can very with the type of method employed and the nature of the sample. Based on the standard deviation of the response and the quantitation limit may expressed as

$$LOD = \frac{10\sigma}{S}$$

Where

σ = The standard deviation of the response

S = the slope of the calibration curve (of the analyte)

MEASUREMENT

For instrumental and non-instrumental methods the quantitation limit is generally determination by the analysis of the samples with known concentration of the analyte and by establishing the minimum level at which the analyte can be determined with acceptable accuracy and precision.

In case of instrumental methods that exhibit background noise the ICH document describes to compare measured signals from samples with known concentration of analyte with those of blank samples.

A typically acceptable signal to noise ratio is 10:1.

LINEARITY AND RANGE

LINEARITY

Linearity is the ability of the method to obtained tests that are directly proportional to the analyte concentration within a given range.

RANGE

Range of analytical procedure is the interval between the upper and lower concentration of analyte in the sample (including concentrations) for which it has been demonstrate that the analytical procedure has a suitable level of precision, accuracy, and linearity.

MEASUREMENT

A range of standards should be prepared containing at least 5 different concentrations of analyte, which are approximately evenly spaced, and span 50-150% of the label claim.

At least 6 replicates per concentration to be studied. Plot a graph of concentration (on x – axis) Vs mean response (on y – axis). Calculate the regression equation, y- intercept and correlation coefficient. Plot another graph of concentration (on x – axis) Vs response ratio (replicate response divided by concentration, (on y – axis).

The range of the method is validated by verifying that the analytical method provides acceptable precision, accuracy and linearity when applied to samples containing analyte at the extreme of the range as well as within the range.

RUGGEDNESS

Ruggedness is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of test conditions such as different laboratories, analysis, instruments, reagent lots, elapsed assay times, temperature, days etc.

It can be expressed as lack of influence of the operation and environmental variable on the test results of the analytical method.

DETERMINATION

By analysis of aliquots from homogeneous lot in different laboratories by different analysis using different operational and environmental condition that may differ but are still within the specified parameter.

ROBUSTNESS

It is measure of capacity of an assay to remain unaffected by small but deliberate variation in method parameters and provide an indication of its reliability in normal usage degradation and variation in chromatography columns, mobile phases and inadequate method development are common causes of lack of robustness.

DETERMINATION OF ROBUSTNESS

Method characteristics are assessed when one or more operating is varied by following certain designs.

In case of liquid chromatography, examples of typical variation are,

- Influence of variation of pH in a mobile phase.
- Influence of variation in mobile phase composition.
- Influence of variation in nm.
- Different columns [different lots and suppliers].
- Temperature.
- Flow rate.

SYSTEM SUITABILITY SPECIFICATION AND TESTS

It is essential for the assurance of quality performance of chromatographic system. The accuracy and the precision of HPLC data collected, which begins with a well-behaved chromatographic system. The system suitability parameter and tests are the parameters that help in achieving this purpose. Suitability parameters are:

1. Capacity factor
2. Precision / Injection repeatability
3. Relative retention
4. Resolution

5. Tailing factor
6. Theoretical plate number

CAPACITY FACTOR

This reflects the location of interest with respect to the void volume i.e., elution time of the unretained components.

$$K_1 = \frac{(t_r - t_0)}{t_0}$$

t_r --- Retention time of analyte.

t_0 --- Retention time of void or unretained component

REPEATABILITY AND REPRODUCIBILITY

Repeatability is the results of the method operating over a short interval of time under the same condition. Also known as "within days" or "within run" precision.

Repeatability is assessed by means of inter laboratory trial.

Example: In case of standardization of an analytical procedure for incursion in pharmacopoeia. Also known as "inter-laboratory" and between run" precision.

6.1. RESOLUTION (R)

$$R = \frac{2\,(t_2 - t_1)}{W_2 + W_1}$$

This gives the resolution between the measured peaks on the chromatogram.

Where

t_2 and t_1 - retention times of the two components.

w2 and w1 - widths of the two components at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

ASYMMETRY (OR) TAILING FACTOR (T)

$$T = \frac{W_{0.05}}{2f}$$

The assessment of peak shape is in terms of asymmetry factor.

$W_{0.05}$ - Width of peak at 5% height

f - Distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline (or) width of front half of the peak at 5% height.

THEORETICAL PLATE NUMBER (N)

$$N = 5.54 \left[\frac{t}{W_{h/2}} \right]^2$$

The assessment of performance of efficiency of a column is in terms of the number of theoretical plates.

$W_{h/2}$ - width of peak at half height

Theoretical plate number is a measure of efficiency i.e. how many peaks can be located per unit run-time of the chromatogram.

"H" or 'HETP" the height equivalent to the Theoretical plate, measures the column efficiency per unit length of the column. Parameters, which can "N" or "H", include peak position, particle size in column, flow rate of mobile phase, and molecular weight of the analyte.

METHODS USED FOR THE EXAMINATION OF PHARMACEUTICAL MATERIAL MAY BE BROADLY CLASSIFIED AS

CLASS A: Tests designed to establish identity, whether of bulk drug substances or have a particular ingredient in a finished dosage form.

CLASS B: methods designed to detect and quantitate impurities in a bulk drug substance or finished dosage form.

CLASS C: Methods used to determine quantitatively the concentration of bulk drug substance or of a major ingredient in a finished dosage form.

CLASS D: Methods used assess the characteristics of finished dosage forms such as dissolution profile and content uniformity.

Table 1. Characteristics for different types of Analytical procedure (As per WHO guidelines)

S.No	Parameter	Class A	Class B		Class C	*Class D*
			Quantitative Tests	Limit Tests		
1.	Accuracy	--	Yes	--	Yes	Yes
2.	Precisions	--	Yes	--	Yes	Yes
3.	Robustness		Yes	Yes	Yes	Yes
4.	Linearity and range	--	Yes	--	Yes	Yes
5.	Selectivity	Yes	Yes	Yes	Yes	Yes
6.	Limit of detection	Yes	--	Yes	--	--
7.	Limit of quantification	--	Yes	--	--	

Fig 1. Adsorption Chromatography

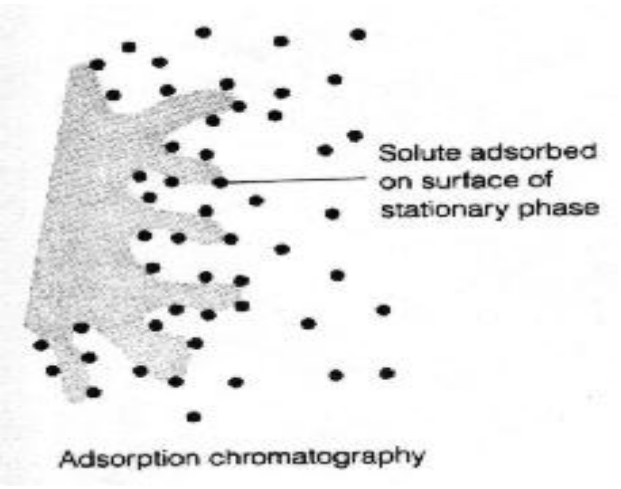


Fig 2. Partition chromatography

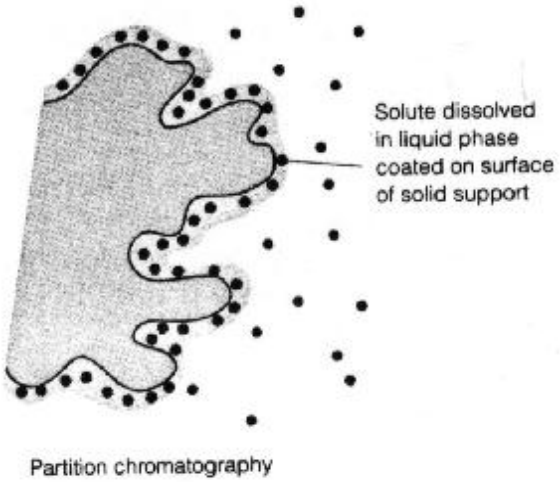


Fig 3. Ion exchange chromatography

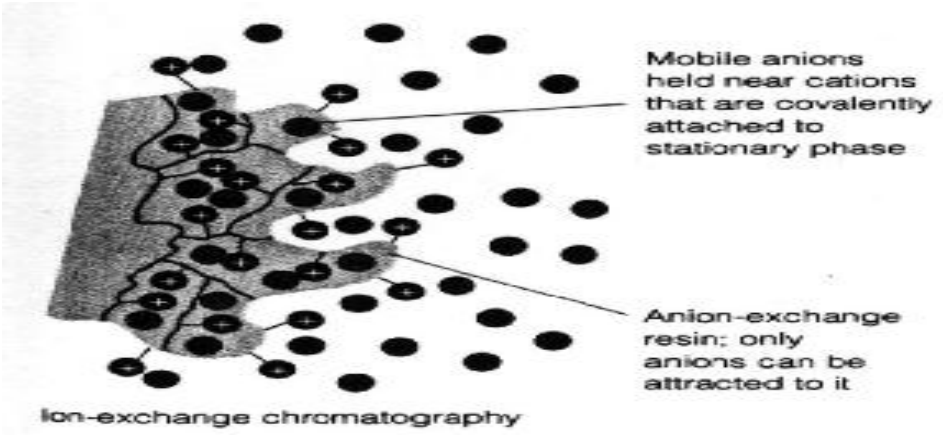


Fig 4. Molecular exclusion chromatography

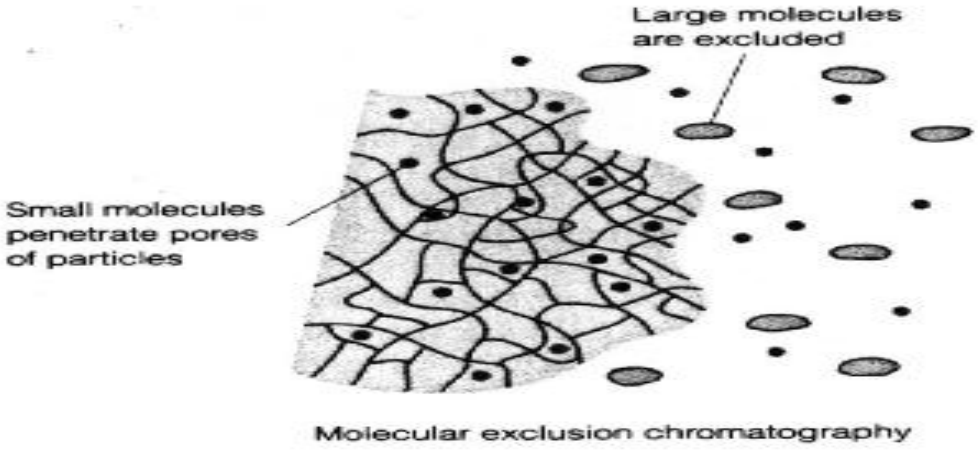


Fig 5. Affinity chromatography

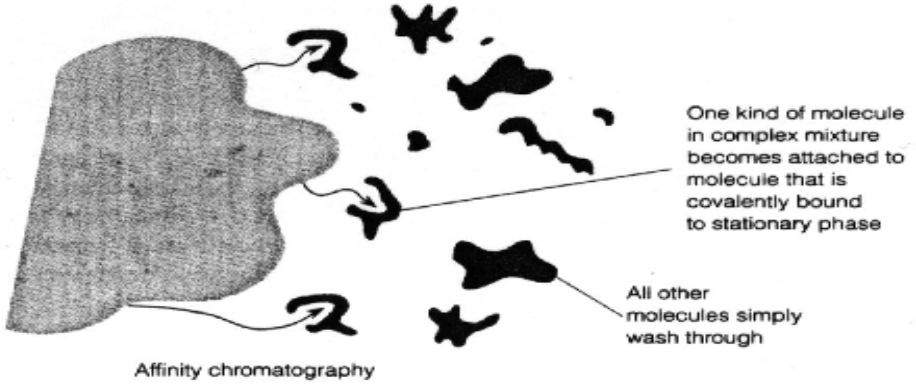
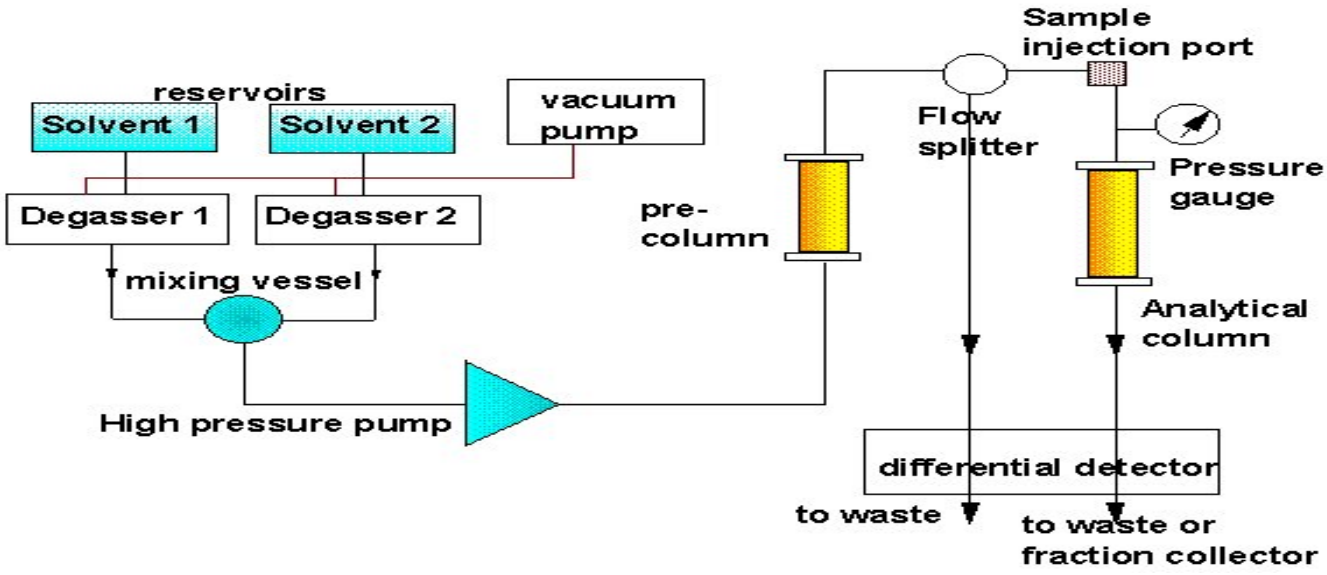


Fig 6. High pressure liquid chromatography

Fig 7. A LINE DIAGRAM OF HPLC UNIT



CONCLUSION

HPLC is used for chemistry and biochemistry research analyzing complex mixtures, purifying chemical compounds, developing processes for synthesizing chemical compounds, isolating natural products, or predicting physical properties. It is also used in quality control to ensure the purity of raw materials, to control and improve process yields, to quantify assays of final products, or to evaluate product stability and monitor degradation. In addition, it is used for analyzing air and water pollutants, for monitoring materials that may jeopardize occupational safety or health, and for monitoring pesticide levels in the environment. Federal and state regulatory agencies use HPLC to survey food and drug products, for identifying confiscated narcotics or to check for adherence to label claims.

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