ABSTRACT

Chromatography is the backbone of separation science and is being used in all research laboratories and pharmaceutical industries universally. The present review article focuses on the principle, types, instrumentation, process validation, application, advantages and disadvantages of HPLC. HPLC methods development and validation play important roles in new discovery, development, manufacture of pharmaceutical drugs and various other studies related to humans and animals. An analytical procedure is developed to test a defined characteristic of the drug substance or drug product against established acceptance criteria for that characteristic. Validation of HPLC method as per ICH Guidelines covers all the performance characteristics of validation, like Accuracy, precision, specificity, linearity, range and limit of detection, limit of quantification, robustness and system suitability testing.

Keywords: chromatography, validation, optimization, HPLC.

INTRODUCTION

Today, chromatography is the backbone of separation science and is being used in all research laboratories and pharmaceutical industries universally. The term ‘Chromatography’ covers those processes aimed at the separation of the various species of a mixture on the basis of their distribution characteristics between a stationary and a mobile phase. Mikhail S. Tswett, a Russian botanist in 1930 coined the name chromatography from the Greek words chroma meaning color, and graphein meaning to write. Today, liquid chromatography, in its various forms, has become one of the most powerful tools in analytical chemistry. Classical liquid chromatography has been around for quite a long time, and has been used in one form or another. In the original method an adsorbent, for instance alumina or silica, is packed into a column and is eluted with a suitable mobile phase combinations. A mixture to be separated is introduced at the top of the column and is passed through the column by the eluting liquid. If a component of the mixture (a solute) is adsorbed weakly onto the surface of the solid stationary phase it will travel down the column faster than another solute that is more strongly adsorbed. Thus separation of the solutes is possible if there are differences in their adsorption by the solid adsorbents. This method is called adsorption chromatography or liquid solid chromatography e.g.: column chromatography, thin layer chromatography, HPLC. Liquid-liquid chromatography uses a liquid stationary phase coated onto a finely divided inert solid support. Separation here is due to differences in the partition coefficients of solutes between the stationary liquid and the liquid mobile phase e.g.: paper partition chromatography, column partition chromatography. A powerful separation method must be able to resolve mixtures with a large number of similar analytes. High performance liquid chromatography (HPLC) is one of such a method, which is mostly used analytical technique. It is also known as high pressure liquid chromatography. The technique of high-performance liquid chromatography is so called because of its improved performance when compared to classical column chromatography.

The acronym HPLC, coined by the late Prof. Csaba Horváth for his 1970 Pittcon paper, originally indicated the fact that high pressure was used to generate the flow required for liquid chromatography in packed columns. In the beginning, pumps only had a pressure capability of 500 psi [35 bar] were used. This was called high pressure liquid chromatography, or HPLC. The early 1970s saw a tremendous leap in technology. These new HPLC instruments could develop up to 6,000 psi [400 bar ] of pressure, and incorporated improved injectors, detectors, and columns. HPLC really began to take hold in the mid-to late-1970s with continued advances in performance during this time [smaller particles, even higher pressure], the acronym HPLC remained the same, but the name was changed to high performance liquid chromatography.

Modes of chromatography are defined essentially according to the nature of the interactions between the solute and the stationary phase, which may arise from hydrogen bonding, Vander walls forces, electrostatic forces or hydrophobic forces or basing on the size of the particles (e.g. Size exclusion chromatography). Different modes of chromatography are as follows:
1. Normal Phase Chromatography
2. Reversed Phase Chromatography
3. Reversed Phase – ion pair Chromatography
4. Ion Chromatography
5. Ion-Exchange Chromatography
6. Affinity Chromatography
7. Size Exclusion Chromatography

Normal phase chromatography

Also known Normal phase HPLC (NP-HPLC), this method separates analytes based on polarity. NP-HPLC uses a polar stationary phase and a non-polar mobile phase.

Reversed phase chromatography

Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase.

High performance liquid chromatography is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster and it also allow a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules past through it and that allows a much better separation of the components of the mixture. The mobile phase mass transfer term is used in the van Deemter equation is dependent on both the square of the particle diameter of the stationary phase as well as the column diameter. Thus there is always a good reason to go to smaller stationary phase particles and smaller column length.

\[ H = \frac{A + B}{u} + Cu \]

A- Eddy’s diffusion
B- Longitudinal diffusion
C-Concentration u- Linear Velocity

All chromatographic separations operate under the same basic principle, separation of a component parts into its constituent parts due to the relative affinities of different molecules towards the mobile phase composition and stationary phase used for the separation. The other major improvement over column chromatography concerns the detection methods which are highly automated and extremely sensitive.

HPLC is distinguished from traditional ("low pressure") liquid chromatography because operational pressures are significantly higher (50–350 bar), while ordinary liquid chromatography typically relies on the force of gravity to pass the mobile phase through the column. Due to the small sample amount separated in analytical HPLC, typical column dimensions are 2.1–4.6 mm diameter and 30–250 mm length are normally used and these columns are made with smaller sorbent particles (2–50 μm in average particle size). This gives HPLC superior resolving power (the ability to distinguish between compounds) when separating mixtures, which makes it a popular chromatographic technique.

HPLC utilizes a liquid mobile phase to separate the components of a mixture. HPLC is the term used to describe liquid chromatography in which the liquid mobile phase is mechanically pumped through a column that contains the stationary phase. An HPLC instrument, therefore, consists of an injector, a pump, a column, and a detector. HPLC mainly utilizes a column that holds packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. In HPLC this partitioning is affected by the relative solute/stationary phase and solute/mobile phase interactions thus, unlike GC, changes in mobile phase composition can have an enormous impact on separation.

Since the compounds have different mobility’s, they exit the column at different times; i.e., they have different retention times, Rt. The retention time is the time between injection and detection or it is the time at which a specific analyte elutes. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used. The sample to be analyzed is introduced in small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase, which is mostly, used analytical technique. The amount of retardation depends on the nature of the analyte and composition of both stationary and mobile phase.

The components of the sample mixture are separated from each other due to their different degrees of interaction with the adsorbent particles. Common solvents used include any miscible combinations of water or organic liquids (the most common are methanol and acetonitrile). Its composition and temperature play a major role in the separation process by influencing the interactions taking place between sample components and adsorbent. These interactions are physical in nature, such as hydrophobic (dispersive), dipole–dipole and ionic, most often a combination of these is used.

Separation has been done by varying the mobile phase composition during the analysis; this is known as gradient elution. The choice of solvents, additives and gradient depend on the nature of the stationary phase and the analyte. In isocratic separation, the same mobile phase combination is used throughout the process of separation. The same polarity or elution strength is maintained throughout the process.

The schematic diagram of an HPLC instrument typically includes a pump, degasser, sampler injector, columns and a detector. The sampler brings the sample mixture into the mobile phase stream which carries it into the column.
The pumps deliver the desired flow and composition of the mobile phase through the column. Columns - usually stainless steel - can be PEEK (poly ether ether ketone), may cost from 20000-750000, packed column length is from 10-30 cm, with internal diameter 4-10 mm. Packing particles are of 3, 5, or 10 µm size. Most common column are 25 cm, 5 µ, 4.6 mm internal diameter, and cost around 40,000 to 60,000, normally packed under 6000 psi pressure at factory as a slurry.

Guard columns are normally used before the analytical column to protect & increase lifetime of column, operator usually use slurry or dry packs in short guard column with same or similar packing used in analytical column or can purchase guard systems, cartridges, etc.

The detector generates a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components. A digital microprocessor and user software control the HPLC instrument and provide data analysis. Some models of mechanical pumps in a HPLC instrument can mix multiple solvents together in ratios changing in time, generating a composition gradient in the mobile phase. Various detectors are in common use, such as UV/Vis, photodiode array (PDA) or based on mass spectrometry. Most HPLC instruments also have a column oven that allows for adjusting the temperature at which the separation is performed.

Thus HPLC is most often used when one is performing a target compound analysis, where one has a good idea of the compounds present in a mixture so reference standards can be used for determining retention times. For a sample of largely unknown composition qualitative identification can be determined by liquid chromatography-mass spectrometry. A mass spectrum of any or all peaks in the chromatogram is compared with spectra contained in spectral libraries on the system's computer.

It should be noted that LC-MS systems are very complex, expensive instruments which are not commonly found in an academic teaching environment. HPLC has over the past decade become the method of choice for the analysis of a wide variety of compounds and its main advantage over gas chromatography is that the analytes do not have to be volatile, so macromolecules are suitable for HPLC analysis.

New amendments in HPLC Technique include:
- Rapid Resolution Liquid chromatography (RRLC)
- Ultra Performance Liquid chromatography (UPLC)
- Ultra Fast Liquid chromatography (UFLC)
- Nano Liquid chromatography (Nano LC)

High performance liquid chromatography is used extensively in the pharmaceutical industry due to the availability of fully automated systems, excellent quantitative precision, accuracy, broad linear dynamic range, and availability of a wide variety of column stationary phases will help in important qualitative and quantitative technique and also it is the most versatile, safest, dependable and fastest chromatographic technique for the quality control of drug components. This article was prepared with an aim to review different aspects of HPLC, such as principle, types, instrumentation, validation process and application.

It is now one of the most powerful tools in analytical chemistry because it has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid. Today, compounds in trace concentrations as low as parts per trillion [ppt] may easily be identified. HPLC can be and has been applied to just about any sample, such as pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples, and industrial chemicals. Quantitative analysis is often accomplished with HPLC. An automatic injector providing reproducible
injection volumes is extremely beneficial, and is standard on modern commercial systems.

**General application includes**

- Separation of organic, inorganic, biological compounds, polymers, and thermally labile compounds
- Qualitative and quantitative methods
- Quantitative/qualitative analyses of amino acids, nucleic acids, proteins in physiological samples
- Measuring levels of active drugs, synthetic byproducts, degradation products in pharmaceuticals
- Measuring levels of hazardous compounds such as pesticides and insecticides
- Monitoring environmental samples
- Purifying compounds from mixtures

**Limitations**

- Qualitative analysis may be limited unless HPLC is interfaced with mass spectrometry
- Resolution is limited with very complex samples.

**Principle**

Chromatography involves the separation of the components of a mixture by virtue of differences in the equilibrium distribution (K) of the components between two phases: the mobile phase and the stationary phase. The principle of HPLC separation is the affinity between non-polar stationary phase and polar mobile phase. When a mixture of compound is introduced into the HPLC column, they travel according to their relative affinities towards the stationary phase. The compound which has less affinity towards the stationary phase travels faster. The compound which has more affinity towards the stationary phase travels slower. By this method, very smaller particles are used for column preparation which gives a much greater surface area for interaction between stationary phase and molecules flowing past it. This allows a much greater separation of components of mixture.

**Liquid Chromatographic Separation Modes**

**Normal-Phase Chromatography**

The principle of normal-phase chromatography is known from classical column and thin-layer chromatography. A relatively polar material with a high specific surface area is used as the stationary phase, silica being the most popular, but alumina and magnesium oxide are also often used. The mobile phase is relatively nonpolar (heptane to tetrahydrofuran). The different extents to which the various types of molecules in the mixture are adsorbed on the stationary phase provide the separation effect. In this technique, non polar compounds travel faster and are eluted first. This is because of the lower affinity between non-polar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore, take more time to elute. Normal phase of separation is therefore, not generally used for pharmaceutical applications because most of the drugs are in polar nature thus require longer time to elute.

**Reversed-Phase Chromatography**

It is the most popular mode of analytical and preparative separation of interest in pharmaceutical, biological and other fields. Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. RPC operates on the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar analyte, and the non-polar stationary phase. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand in the aqueous eluent. Here the polar solvents get eluted first and nonpolar solvents are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer periods and get eluted faster. The different columns used are Octa Decyl Silane (ODS) or C18, C8, C4 (in the increasing order of polarity of stationary phase).

**Based on principle of separation**

1. Adsorption chromatography
2. Ion exchange chromatography
3. Ion pair chromatography
4. Size exclusion or Gel permeation chromatography
5. Affinity chromatography
6. Chiral phase chromatography
7. Aqueous normal-phase chromatography

**Adsorption chromatography**

The principle of separation is adsorption. Separation of components takes place due to difference in the affinity between nonpolar stationary phase and polar mobile
phase. This principle is seen in both normal phase and reverse phase mode, where adsorption takes place.

**Ion exchange chromatography**

The principle of separation is ion exchange, which is reversible exchange of functional groups. In ion exchange chromatography, an ion exchange resin is used to separate a mixture of similar charged ions. The retention is based on the attraction between solute ions and charged ions bound to the stationary phase. Cationic and anionic exchange resins are used where similar ions are excluded and opposite charge ions are retained. Thus, this is used for separation of charged molecule only. pH and ionic strength are used to control elution time.

**Ion pair chromatography**

This technique is also called as RP Ion Pair Chromatography or Soap Chromatography. A reverse phase column is converted temporarily into a ion exchange column by using ion pairing agents like pentane or hexane or heptanes or octane sulphonic acid sodium salt, tetra methyl or tetraethyl ammonium hydroxide, etc. Strong acidic and basic compounds can be separated by this method by forming ion pairs with suitable counter ions.

**Size exclusion chromatography**

Size exclusion chromatography (SEC), also called as gel permeation chromatography or gel filtration chromatography mainly separates particles on the basis of size using gels. The column is filled with material having precisely controlled pore sizes, and the particles are separated according to its their molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later. Soft gels like dextrone, agarose or polyacrylamide are used. Semi rigid gels like polystyrene, alkyd dextrin in aqueous medium are also used. The mechanism of separation is by stearic and diffusion effects. It is also useful for determining the tertiary structure and quaternary structure of proteins and amino acids.

**Affinity chromatography**

Affinity chromatography uses the affinity of sample with specific stationary phases involving highly specific biochemical interaction for separation. This technique is mostly used in the field of Biotechnology, microbiology, Biochemistry, etc. It can be used to isolate proteins, enzymes, even antibodies from complex mixtures.

Bio-affinity chromatography: This chromatographic process relies on the property of biologically active substances to form stable, specific, and reversible complexes. The separation is based on specific reversible interaction of proteins with ligands, which are covalently attached to solid support on a bio-affinity matrix. It retains proteins by the interaction to the column-bound ligands. The formation of these complexes involves the participation of common molecular forces such as the Van der Waals interaction, electrostatic interaction, dipole-dipole interaction, hydrophobic interaction, and the hydrogen bond.

Proteins bound to a bioaffinity column can be eluted in two ways:

- **Biospecific elution:** it includes inclusion of free ligand in elution buffer which competes with column bound ligand.
- **Aspecific elution:** the change in pH, salt, etc. which weakens interaction of protein with column-bound substrate.

**Chiral phase chromatography**

It involves separation of enantiomers, mainly optical isomers using chiral HPLC column that is packed with a chiral stationary phase. Different principles operate for different types of stationary phases and different samples. Enantiomers are separated based on the number and type of each interaction that occurs during their exposure to the chiral stationary phase.

**Aqueous normal-phase chromatography**

Aqueous normal-phase chromatography (ANP) is a chromatographic technique which encompasses the mobile phase region between reversed-phase chromatography (RP) and organic normal phase chromatography (ONP). This technique is used to achieve unique selectivity for hydrophilic compounds, showing normal phase elution using reversed-phase solvents.

**Based on elution technique**

**Isocratic elution**

A separation in which the mobile phase composition remains constant throughout the process is termed isocratic (meaning constant composition). In isocratic elution, peak width increases with retention time, linearly so peaks get very flat and broad. The same polarity or elution strength is used throughout the process.

**Gradient elution**

A separation in which the mobile phase composition is changed during the separation process is described as a gradient elution. Gradient elution decreases the retention of the later-eluting components so that they elute faster, giving narrower (and taller) peaks for most components. In this technique a mobile phase combination of lower polarity or elution strength are used followed by gradually increasing the polarity or elution strength.

**Based on scale of operation**

**Analytical HPLC**

Where only the analysis of the samples are done. Recovery of the samples is not done because only very low quantity samples used.
Preparative HPLC

Where the individual fractions of pure compound can be collected using fraction collector. The collected samples are reused.

Based on type of analysis

Qualitative analysis

This is used to identify the compound, detect the presence of impurities, to find out the number of components etc. This is done by retention time values.

Quantitative analysis

This is done to determine the quantity of individual or several components in a mixture. This is done by comparing the peak area of standard and sample.

Parameters

Precise analysis of a compound involves some parameters which are used as a standard for a particular compound. These are used to know how well HPLC separates a mixture into two or more components that are detected as peaks (bands) on a chromatogram. Change in the parameters may significantly alter results to a great extent. The most commonly used parameters are internal diameter, particle size, pore size, pump pressure. For different compounds the parameters can be tainted according to their nature and chemical properties.

Internal diameter

The internal diameter (ID) of an HPLC column is a critical 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 generally seen in industrial applications mainly for the purification of a drug product for later use. Low ID columns have improved sensitivity and lower the solvent consumption at the expense of loading capacity. Analytical scale columns (4.6 mm) are used in traditional quantitative analysis of samples and often use a UV-Vis absorbance detector.

Particle size

Most traditional HPLC is done with the stationary phase attached to the outside of small spherical silica particles (very small beads). Smaller particles usually provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter squared. Larger particles are used in preparative HPLC and for non-HPLC applications such as solid-phase extraction.

Pore size

Pore size defines an ability of the analyte molecules to penetrate inside the particle and interact with its inner surface. 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. This is especially important because the ratio of the outer particle surface to its inner one is about 1:1000. The surface molecular interaction chiefly occurs on the inner particle surface.

Pump pressure

Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible flow rate. Pressure may reach as high as 60 MPa (6000 lbf/in²), or about 600 atmospheres. Modern HPLC systems have been improved to work at much higher pressures, and therefore be able to use much smaller particle sizes in the columns (< 2 μm).

"Ultra High Performance Liquid Chromatography" systems or UHPLCs can work at up to 120 MPa (17,405 lbf/in²), or about 1200 atmospheres.

Some of the important chromatographic parameters include

Retention time is the difference in time between the time of injection of a solute and time of elution of the peak maximum of that solute. It is the time taken for a particular compound to travel through the column to the detector.

Retention volume is the volume of mobile phase required to elute 50% of the component from the column.

Retention factor (kappa prime) measures how long a component of the mixture stuck to the column, measured by the area under the curve of its peak in a chromatogram (since HPLC chromatograms are a function of time).

A theoretical plate is an imaginary or hypothetical unit of column where distribution of solute and stationary phase and mobile phase has attained equilibrium.

The efficiency of a chromatographic column is defined in terms of the number of theoretical plates (N). It measures the band spreading of a peak. When band spread in smaller, the number of theoretical plates is higher.

HETP (Height Equivalent to a Theoretical Plate) it is the height, which decides the efficiency of separation. If HETP is less the column is more efficient. If HETP is more, the column is less efficient. The height equivalent to a theoretical plate (HETP) is given by

\[
HETP = \frac{\text{Length of column}}{n}
\]

Separation factor is the ratio of partition coefficient of the two components to be separated.

Resolution is a measure of the extent of separation of two components and the baseline separation achieved.

Instrumentation

Solvent delivery system

Pump

Solvent degassing system

Sample injector
Solvent delivery system

The mobile phase is pumped under pressure at a high pressure at about 1000 to 3000 psi; from one or several reservoirs and flows through the column at a constant rate. With micro particulate packing, there is a high-pressure drop across a chromatography column. Eluting power of the mobile phase is determined by its overall polarity, the polarity of the stationary phase and the nature of the sample components. For normal phase separations eluting power increases with increasing polarity of the solvent but for reversed phase separations, eluting power decreases with increasing solvent polarity. Some other properties of the solvents, which need to be considered for a successful separation, are boiling point, viscosity, detector compatibility, flammability and toxicity. Optimum separating conditions can be achieved by making use of mixture of two solvents.

Pump

The most important component of HPLC in solvent delivery system is the pump, because its performance directly effects the retention time, reproducibility and detector sensitivity. The particle size of the stationary phase is 5-10 μm. So resistance to flow is observed. This is the reason that high pressure is required and this is provided by using pumps. The different types of pumps include:

Constant pressure pump which uses a constant pressure to the mobile phase; the flow rate through the column is determined by the flow resistance of the column and any other restrictions between the pump and the detector outlet.

Constant flow pump generates a given flow of liquid, so that the pressure developed depends on the flow resistance.

Constant pressure pumps

The simplest type of constant pressure pump is the pressurized coil pump or gas displacement pump. consisting of some form of pneumatic device for the direct pressurization of the mobile phase with an inert gas, give a reliable pulse-free flow and have the advantage of low cost and simplicity. They are however not as accurate as constant volume pumps but can be used where flow accuracy and reproducibility are less critical. But this is now only of historical interest.

Constant flow/volume (Constant Displacement) Pumps

If a constant flow pump is used, changes in the permeability of the system, caused by settling or swelling of the packing, or viscosity changes in the mobile phase (due to temperature fluctuations or composition changes) are compensated for by pressure changes and the flow rate remains constant. Since flow changes cause non-reproducible retention times, adversely affect resolution, and give unstable base-lines, the constant volume pump provides a more precise analysis. It is particularly useful when gradient elution is used. There are two main types of constant volume pumps: single stroke (syringe type) pumps, and reciprocating pumps having either a diaphragm or a piston. The syringe type pump consists of a syringe the plunger of which is driven by a stepping motor through a gear box. The rate of delivery from the syringe is controlled by varying the voltage on the motor. The main advantage of this type of pump is that it is capable of providing a pulse-free flow at high pressure and the flow rate is independent of the operating pressure, if the compressibility of the liquid is ignored. Its main disadvantage is that it has a finite solvent capacity. Dual syringe systems found in some instruments are of course expensive, but with suitable gradient formers they also provide the gradient elution capacity. Due to its high cost and lack of flexibility this type of pump is little used in today’s commercial instruments. Reciprocating pumps
fall into two types: diaphragm and piston pumps. Among the several solvent delivery systems (direct gas pressure, pneumatic intensifier, reciprocating etc.) reciprocating pump with twin or triple pistons is widely used, as this system gives less baseline noise, good flow rate reproducibility etc.

Check valves
These are present to control the flow rate of solvent and back pressure.

Pulse dampeners
These are used to dampen the pulse observed from the wavy baseline caused by the pumps.

Solvent degassing system
The constituents of the mobile phase should be degassed and filtered before use because several gases are soluble in organic solvents. When solvents are pumped at high pressure, gas bubbles are formed which will interfere with the separation process. Numerous methods are employed to remove the dissolved gases in the mobile phase. They include heating and stirring, vacuum degassing with an aspirator, filtration through 0.45 filters, vacuum degassing with an air-soluble membrane, helium purging, ultra-sonication or purging or combination of these methods. HPLC systems are also provided an online degassing system, which continuously removes the dissolved gases from the mobile phase.

Sample injector
Two means for analyte introduction on the column are injection in to a flowing stream and a stop flow injection. Several devices are available either for manual or auto injection.

a. Septum injectors- for injecting the sample through a rubber septum.

b. Stop flow- in which the flow of the mobile phase is stopped for a while and the sample is injected through a valve device.

c. Rheodyne injector (loop valve type) - it is the most popular type. This has a fixed volume loop like 20-50 μl or more. The injector has two modes, i.e., load position when the sample is loaded in the loop and the inject mode, when the sample is injected.

The injector can be a solitary infusion or a computerized infusion framework. An injector for a HPLC framework should give infusion of the fluid specimen inside the scope of 0.1 mL to 100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).

Automatic injector is a microprocessor-controlled version of the manual universal injector. Usually, up to 100 samples is capable of being loaded on to the auto injector tray. The system parameters such as flow rates, volume to be injected, gradient, run time, etc. are selected, stored in memory and sequentially executed on consecutive injections.

Guard column
Guard column has very small quantity of adsorbent and improves the life of the analytical column. It also acts as a prefilter to remove particulate matter, if any, and other material. Guard column has the same material as that of analytical column. It does not contribute to any separation but is necessarily used before the analytical column to protect & increase lifetime of column. Operator usually slurry or dry packs short guard column regularly with same or similar packing used in analytical column (old column material) are used for guard columns. However, the particle size of the packing material is bigger in size than analytical column in order to avoid the pressure drop in the HPLC system.

Analytical column
Columns are typically made of polished stainless steel, glass, and polyethylene or poly ether ether ketone (latest). Column length is around 50 mm to 300 mm and has an internal diameter across of somewhere around 2mm to5 mm. They are generally loaded with a stationary phase with a particle size of 3 μm to 10 μm. 1gm of stationary phase provides surface area ranging from 100-860 sq.m, with an average of 400sq.m. Columns with inner diameters of <2 mm are regularly normally suggested to as microbore segments. Rather the temperature of mobile phase and the column should be kept consistent during investigation. The functional group present in stationary phase depends on the type of chromatographic separation. In normal phase mode it contains the silanol groups (hydroxyl group). In the reverse phase mode C18 (Octa Decyl Silane), C8, C4, CN, NH2 columns are used.
Detector

Several ways of detecting are used when a substance has passed through the column. A detector used depends upon the property of the compound to be separated.

UV detector

This is the most commonly used type of detector as it can be rather sensitive, has a wide linear range, is relatively unaffected by temperature fluctuations and is also suitable for gradient elution. It records compounds that absorb ultraviolet or visible light. Many organic compounds absorb UV light of various wavelengths. The amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time. Absorption takes place at a wavelength above 200nm, provided that the molecule has at least:

(a) A double bond adjacent to an atom with a lone electron pair
(b) Bromine, iodine or sulfur;
(c) A carbonyl group or a nitro group
(d) Two conjugated double bonds
(e) An aromatic ring;

The mobile phase we use, on the other hand, should absorb little or no radiation.

Absorption of radiation by solutes as a function of concentration, c, is described by the Beer-Lambert law:

\[ A = E c \text{%} \]

Where \( A \) = absorbance = path length of the cell and \( E \) = molar absorptivity, which is a constant for a given solute and wavelength.

Fluorescence detectors

Compounds that have fluorescence or of with fluorescing derivatives can be obtained are picked up with high sensitivity and specificity by this detector. The sensitivity may be up to 1000 times greater than with UV detection. Light of a suitable wavelength is passed through the cell and the higher wavelength radiation emitted is detected in a right-angled direction. The light intensity and hence the sensitivity are increased by using a relatively large cell (20ml or greater). Simple units have affixed excitation wavelength for which band width must not be too narrow and a fixed wavelength range for fluorescent light detection. The excitation wavelength can be selected in the more expensive models and the most advanced equipment has a monochromator for excitation and fluorescent light, providing a highly specific (but less sensitive) level of detection.

Electrochemical (Amperometric) detectors

Electrochemistry provides a useful means of detecting traces of readily oxidizable or reducible organic compounds with great selectivity. The detection limit can be extraordinarily low and the detectors are both simple and inexpensive. The potential between the working and reference electrodes may be selected. The working electrode is made up of glassy carbon, carbon paste or amalgamated gold. Frequently a silver/silver chloride electrode is used as the reference. The diffusion current recorded is proportional to the concentration of the compound eluted. This is applicable only when compounds have functional group which can be either oxidized or reduced.

Conductivity detectors

This is the classical ion chromatography detector and measures the eluate conductivity, which is proportional to ionic sample concentration (provided that the cell is suitably constructed). Its sensitivity decreases as the specific conductivity of the mobile phase increases. The active cell volume of 2ml is very small. Good conductivity detectors have automatic temperature compensation (conductivity is highly temperature-dependent) and electronic background conductivity suppression.

Light scattering detectors

The evaporative light-scattering detector (ELSD) is an instrument for the nonselective detection of nonvolatile analytes. The column eluate is nebulized in a stream of inert gas. The liquid droplets are then evaporated, thus producing solid particles which are passed through a laser, LED, or polychromatic light beam. The resulting scattered light is registered by a photodiode or photomultiplier. Volatile buffers can be prepared with formic, acetic and tri fluoro acetic acid; all these compounds must be of high purity. “Gradient grade” is not necessarily pure enough for ELSD detection. The
nebulizer gas is usually nitrogen, helium, or compressed air. The detector response is a complex function of the injected amount of analyte and not of its chemical composition or of the presence of certain functional groups.

**Photo diode array detectors (PDA) detectors**

A photodiode array detector is a lined array of discrete photodiodes on an integrated circuit (IC) chip for spectroscopy. It is placed at the image plane of a spectrometer to allow a range of wave length to be sensed concomitantly. PDA can be programmed for any wavelength range and all the compounds that absorb at this range can be identified in a single range. It can also analyze peak purity by matching spectra within a peak. The resulting spectra are 3-D plot of Response Vs Time Vs Wavelength.

**Other detectors**

**Photoconductivity Detectors**

These are sensitive, selective detectors for organic halogen and nitrogen compounds. The eluate is split up as it leaves the column. One half passes through the reference cell of a conductivity detector and the other half is irradiated with 214 or 254nm UV light whereupon suitable sample molecules become dissociated into ionic fragments. The ensuing high level of conductivity is recorded in the measuring cell.

**Infrared Detectors**

Every organic molecule absorbs infrared light at one wavelength or another. When an IR detector is used, the mobile phase chosen must not be self-absorbent at the required wavelength. Hexane, dichloromethane and acetonitrile are suitable mobile phases for ester detection whereas ethyl acetate is not. The sensitivity is no greater than that of refractive index detectors.

**Radioactivity Detectors**

These are used especially for detecting the b-emitters H3, C14, P32, S35 and I131. The scintillator required for this relatively weak radiation is either added as a liquid between the column and the detector or is contained as a solid in the cell.

Hyphenated HPLC detectors

LC-MS using thermospray—new popularity (pharmaceuticals)

Evaporative light scattering -polymers

LC-FTIR

LC-plasma emission or ICP-MS

**Recorders and Integrator**

Recorders: the signals from the detector after amplification (if necessary) are recorded as a series of peaks, each one representing a compound in the mixture. Baseline and the peaks are recorded with respect to time. Retention time for all the peaks can be found from the recordings. The area under the peak is proportional to the amount of substance passed through detector, and this area can be calculated automatically by the computer linked to the display.

Integrator: improved version of recorders by which signals from the detector are gathered on graph recorders or electronic integrators that fluctuate in many-sided quality and in their capacity to process, store and reprocess chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret.

**Derivatization**

In HPLC derivitization is used to enhance the sensitivity and selectivity of detection when available detectors are not adequate for the underivatized compounds. Both ultra violet absorbing and fluorescence derivatives have been extensively used. Ultra violet derivitization reagents include N-succinimidyl p-nitro phenyl acetate, phenyl hydrazine and 3, 5-dinitro benzyl chlorides, while fluorescent derivatives are formed with reagents such as dansyl chloride, 4-bromo methyl-7-methoxy-coumarin and fluorescamine. Derivative formation can be carried out before the sample is injected on to the column or by online chemical reactions between the column out let and the detector. Derivative preparation carried out before separation is called precolumn derivatisation and afterwards is called post column derivatisation. This allows optimization of the separation process.

**Method Development and Validation**

**Method Development**

Methods are developed for new products when there are no official methods available. Alternate methods for existing (Non Pharmacopoeial) products are used to reduce the cost and time for obtain better precision and ruggedness. When alternate methods proposed is intended to replace the existing procedure comparative laboratory data including merit/demerits are made available. The goal of the HPLC-method is to try & separate, quantify the main active drug, any reaction impurities, all available synthetic inter-mediates and any degradants. Steps involved in Method development are

Understanding the Physicochemical properties of drug molecule.

Selection of chromatographic conditions.

Developing the approach of analysis.

Sample preparation

Method optimization

Method validation
Method Validation

Validation of an analytical method is the process by which it is established by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application. Validation is required for any new or amended method to ensure that it is capable of giving reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories. The type of validation program required depends entirely on the particular method and its proposed applications.

Scope of process validation

Validation is one of the wide and most difficult areas because it engages in all levels of product manufacturing. The scope of validation in different fields is follows:

Analytical
Instrument Calibration
Process Utility services
Raw materials
Packaging materials
Equipment
Facilities
Manufacturing operations
Product Design
Cleaning
Operators

Importance of validation

Assurance of quality
Time bound
Process optimization
Reduction in rejections
Increased output
Avoidance of capital expenditures
Fewer complaints about process related failures
Reduced testing in process and in finished goods
More rapid and reliable start-up of new equipments
Easier scale-up for development work.

Basic concept of process validation

Calibration, verification and maintenance of process equipment.
Prequalification or revalidation.
Establishing specifications and performance characteristics.

Selection of methods, process and equipment to ensure the product meets specifications.
Qualification or validation of process and equipment.
Testing the final product, using validated analytical methods, in order to meet specifications.

Significance of process validation

Process validation is defined as “establishing documented evidence which provides a high degree of assurance that a specific system, related equipment and process consistently meet the approved specifications and produce products meeting predetermined quality attributes.

Process validation is a basic factor for drug product safety and quality and thus a fundamental component of the quality assurance system used by pharmaceutical manufacturers. The basic principle of Quality Assurance is that a drug should be produced that is fit for its intended use. Effective Process Validation contributes significantly to assure the drug quality; this principle incorporates the understanding that the following conditions exist:

Quality, safety, and efficacy are designed or built into the product.
Quality cannot be adequately assured merely by in-process and finished-product inspection or testing.
Each step of a manufacturing process is controlled to assure that the finished product meets all design characteristics and quality attributes including specifications.

Types of process validation

1. Prospective validation
2. Concurrent validation
3. Retrospective validation
4. Revalidation

Prospective validation

Prospective validation is defined as the establishment of documented evidence that a system does what it purports to do based on a pre planned protocol. The objective of prospective validation is to prove or demonstrate that the process will work in accordance with a validation master plan or protocol prepared for pilot product trails. It is not limited to

List of analytical methods, as appropriate.
Proposed in-process controls with acceptance criteria
Additional testing to be carried out, with acceptance criteria and analytical validation, as appropriate.
Sampling plan.
Methods for recording and evaluating results.
Functions and responsibilities.
Retrospective validation

Validation of such processes should be based on historical data. The steps involved require the preparation of a specific protocol and the reporting of the results of the data review, leading to a conclusion and a recommendation. The basis for retrospective validation is “Valid in-process specifications for such characteristics shall be consistent with drug product final specifications and shall be derived from previous acceptable process average and process variability estimates where possible and determined by the application of suitable statistical procedures where appropriate.”

Some of the essential elements are;
Batch size/strength/manufacturer/year/period.
Master manufacturing/packaging documents.
Current specifications for active materials/finished products.
List of process deviations, corrective actions and changes to manufacturing

Concurrent Validation

In-process monitoring of critical processing steps and end-product testing of current production can provide documented evidence to show that the manufacturing process is in a state of control. It is similar to the prospective, except the operating firm will sell the product during the qualification runs, to the public as its market price

Revalidation

It is the repetition of a validation process or a part of it. Some of the changes in revalidation are
Changes in the source of active raw material manufacturer.
Changes in packaging material
Changes in the process
Changes in the plant/facility.

Order of priority in process validation

The order priority is always dependent upon how much the product needs quality assurance, accuracy, precision, robustness etc. The parenteral products need high degree of quality comparing with others. The order priority is

A. Sterile Products and Their Processes
1. Large-volume parenterals (LVPs)
2. Small-volume parenterals (SVPs)
3. Ophthalmic, other sterile products, and medical devices.

B. Nonsterile Products and Their Processes
1. Low-dose/high-potency tablets and capsules/transdermal delivery
2. Drugs with stability problems
3. Other tablets and capsules
4. Oral liquids, topicals, and diagnostic aid

Various approaches in process validation

1) Process design
2) Process qualification
3) Continued process verification

Process Design

The goal of this stage is to design a process suitable for routine commercial manufacturing that can consistently deliver a product that meets its quality attributes

Process Qualification

This stage has two elements:
1. Design of the facility and qualification of the equipment and utilities.

Continued Process Verification

The goal of the third validation stage is continual assurance that the process remains in a state of control (the validated state) during commercial manufacture.

Three stages of lifecycle approaches are
1. Product/process design and development
2. Qualification of the commercial manufacturing equipment
3. Maintenance of the process in a state of control during production

Strategy for method validation

The preparation and execution should follow a validation protocol written in a step by step as follows:
Develop a protocol or operating procedure
Define the application purpose and scope of method
Define the performance parameters and acceptance criteria
Define validation experiments
Verify relevant performance characteristics of the equipment
Select quality materials, e.g. standards and reagents
Perform pre-validation experiments
Adjust method parameters and/or acceptance criteria, if necessary
Perform full internal and external validation experiments
Validation protocols

It is a written form that explains how the validation should conduct. Which is documented and used for the testing of various products, process, etc?

Protocol as following:

Objective.

Background/revalidation.

Summary of development and technical transfer (form R & D or another site activity to justify in process testing and controls

Any previous validations.

List of equipments and their qualification status.

Facilities qualification.

Process flow chart.

Manufacturing procedure narrative.

Application

HPLC finds application in the fields of pharmacy, environmental, clinical, and forensic and also in food industry. The information that can be obtained by HPLC includes resolution, identification and quantification of a compound. It also aids in chemical separation, molecular weight determination and purification of mixture of compounds.

Chemical Separations is based on the fact that certain compounds have different migration rates for a particular column and mobile phase. Thus separations of individual components are achieved.

Purification is defined as the process of separating or extracting the target compound from a mixture of compounds or contaminants. Each compound shows a characteristic peak under certain chromatographic conditions such that the pure desired compound can be collected or extracted without incurring any other undesired compound.

Molecular weight determination allows determining the molecular weight of chemical substance, pharmaceutical substances, proteins, etc.

Identification usually is the assay of compounds that are carried using HPLC. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels so that pure compounds are identified easily.

Other applications of HPLC includes

Pharmaceutical applications

Identification of active pharmaceutical ingredients of dosage forms.

Assay of pharmaceutical formulation and analyzing impurities.

Quality control.

Research and development.

Environmental applications

Detection of phenolic compounds in drinking water.

Identification of diphenhydramine in sedimanted samples.

Bio-monitoring of pollutant.

Forensics

Quantification of the drug in biological samples.

Identification of anabolic steroids in serum, urine, sweat, and hair.

Determination of presence of cocaine and metabolites in blood.

Forensic analysis in textile industry.

Clinical

Quantification of ions in the human urine.

Analysis of antibiotics in the blood plasma.

Estimation of bilirubin and bilivirdin in blood plasma in case of hepatic disorders.

Detection of endogenous neuropeptides in extracellular fluids.

Food and Flavor

Ensuring the quality of soft drink and drinking water.

Analysis of alcohol and its derivatives.

Sugar analysis in fruit juices.

Analysis of polycyclic compounds in vegetables.

Trace analysis of military high explosives in agricultural crops.

Screening for pesticides and insecticides in fruits.

Advantages

HPLC has many advantages over other methods of chromatography. It has made significant contribution to the growth of analytical science and its diverse application in pharmaceuticals, environmental, forensics, foods, polymers and plastics, clinical fields etc.

HPLC provides a highly specific, reasonably precise, and fairly rapid analytical method for a plethora of complicated samples.

HPLC is capable of tackling macromolecules.
It is profoundly suitable for most ‘pharmaceutical drug substances’.

It offers an efficient means of analysis pertaining to ‘labile natural products’.

HPLC allows the dependable analysis of a good number of products including biochemical, metabolic products, non-volatile substance, polar compounds etc.

Preparation and introduction of sample is easy and simple in HPLC.

Resolution of compounds and speed of separation is high.

HPLC software is capable of reporting precise and accurate results.

Sensitivity of detectors used is high.

A large number of stationary phases and columns can be used to suit different ranges of application.

Recording and storage of information is easy.

The columns operated carefully under controlled conditions without overloading can be reused for significant period of time.

HPLC coupled with mass spectrophotometers and FT-IR system have improved efficacy.

Along with hyphenated techniques HPLC have been used to analyse impurities in pharmaceutical formulations.

Disadvantages

HPLC is considered one of the most important techniques of the last decade of the 20th century. Despite of the several advantages there are certain limitations also. Limitations include price of columns, solvents and a lack of long term reproducibility due to proprietary nature of column packing. Others include:

Complexity of separation of certain antibodies specific to the protein.

The cost of developing an HPLC apparatus for assay or method of separation of individual components is tremendous.

Due to the speed of the HPLC and its reliance on the different polarities; two compounds with similar structure and polarities can exit the chromatographic apparatus at the same time (co-elution). This is difficult in detecting compounds.

Low sensitivity of some compounds towards the stationary phase in the columns is difficult.

Certain compounds get absorbed or react with the chemicals present in the packing materials of the column.

Sometimes the pressure may get too high or low that the column cannot withstand or separation may not takes place.

Qualitative analysis may be limited unless HPLC is interfaced with mass spectrometry.

Resolution is limited with very complex samples.

Newer trends with better efficacy have been established.

CONCLUSION

The literature review of HPLC was done vigilantly and it was found to be one of the most widely used system among the chromatographic techniques. The choice of detection approach is critical to guarantee that all the components are detected accurately. Chromatography is a separation technique used to separate the individual compound from a mixture using a stationary and mobile phase. The discovery of chromatography is a milestone event in biomedical research. Chromatographic separation is based on the principles of adsorption, partition, ion exchange, molecular exclusion, affinity and Chirality. HPLC is a highly assertive analytical technique which uses sophisticated technologies that have been extensively practiced from decades. Modernizations such as ultrahigh-pressure liquid chromatography, nano liquid chromatography, liquid chromatography-mass spectrometry, chiral phase separations, core-shell columns, and novel stationary phases have helped HPLC to acquire higher performance levels; in diverse factors, yielding faster speed, higher resolution, greater sensitivity, and increased precision. The practice of HPLC is restricted to analyzers, but is now widely performed by students, chemists, biologists, production workers, and other novices in academia, research, and quality control laboratories.

The review focuses on the principle, types, instrumentation, process validation, application, advantages and disadvantages of HPLC. The pump delivers the mobile phase from a reservoir and on to a column packing material that typically consists of 3-5 μm silica particles. Sample solutions are injected using a pressure and leak resistant injector onto the mobile phase just before the column. It follows isocratic or gradient elution techniques and the substance eluted from the column are detected using one or more detectors. The UV detectors are the standard detector in pharmaceutical quality and control. Thus, HPLC is used in the determination of drug substances and their metabolites in biological material. It is used for screening of compounds in pharmaceutical preparation and detecting the impurities present. Also used in the identification of active ingredients. Using HPLC one can produce extremely pure compounds. It can be used in both laboratory and clinical science. Most of the drugs in multi component dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in the same. HPLC methods development and validation play important roles in new discovery, development, manufacture of pharmaceutical drugs and various other studies related to humans and animals. An analytical procedure is developed to test a defined characteristic of the drug substance or drug product against established acceptance criteria for that characteristic. Validation of
HPLC method as per ICH Guidelines covers all the performance characteristics of validation, like Accuracy, precision, specificity, linearity, range and limit of detection, limit of quantification, robustness and system suitability testing. In conclusion, the main advantages of this relatively new approach of chromatography are: increased sensitivity, faster analysis through the use of a novel separation material of very fine particle size, reduced operation cost, less solvent consumption and increased sample throughput. However, the negative aspect of HPLC has been overcome by the discovery of hyphenated technologies but only disadvantage is their high cost.

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