High Pressure/Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is a form of column chromatography that pumps a sample mixture or analyte in a solvent (known as the mobile phase) at high pressure through a column with chromatographic packing material (stationary 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. That makes it much faster.

HPLC is used in a variety of industrial and scientific applications, such as pharmaceutical, environmental, forensics, and chemicals. Both qualitative and quantitative analysis can be done. Also, it is a repeatable method.

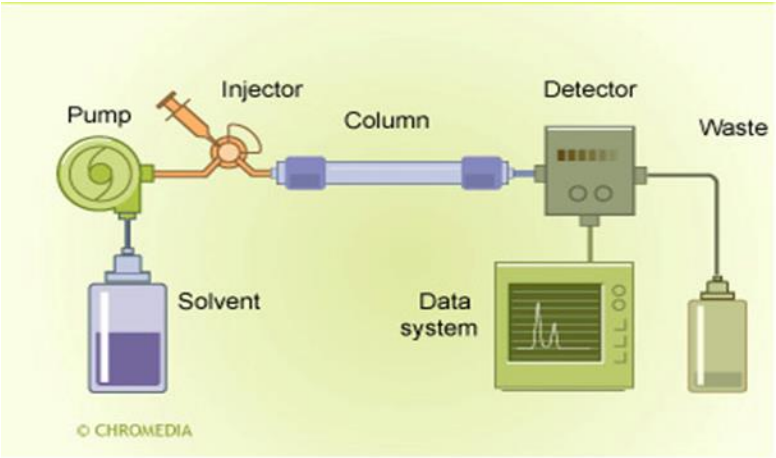
All chromatographic separations, including HPLC operate under the same basic principle; every compound interacts with other chemical species in a characteristic manner. Chromatography separates a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation.

Sample retention time will vary depending on the interaction between the stationary phase, the molecules being analyzed, and the solvent, or solvents used. As the sample passes through the column it interacts between the two phases at different rate, primarily due to different polarities in the analytes. Analytes that have the least amount of interaction with the stationary phase or the most amount of interaction with the mobile phase will exit the column faster.

Comparison of Gas Chromatography with HPLC	
GC There is no impressive feature of separating gas used in GC. At GC, analysis of gases and volatile substances can be provided.	HPLC The ability of solvents used as mobile phase in HPLC has an impressive property of separation. HPLC has a wider range of applications.

Instrumentation:

Main components in an HPLC system include the solvent reservoir, a high-pressure pump, a column, injector system and the detector.



- The reservoir holds the solvent, which is referred to as the mobile phase because it moves.

- A pump is used to generate a specified flow of the mobile phase.

The pumps are divided into two types

Constant pressure pumps provide consistent continuous flow rate through the column with the use of pressure from a gas cylinder.	**Constant flow pumps** Constant flow pumps are being used more often because of correction of pressure changes that might occur due to resistance in the column and viscosity of the mobile phase. • **Syringe Type Pumps** are suitable for small bore columns. • **Reciprocating Piston pumps**

During the chromatographic experiment, a pump can deliver a constant mobile phase composition (isocratic) or an increasing mobile phase composition (gradient).

- The injector, or auto sampler, introduces the solvent into a phase stream that carries the sample into the high pressure column. They should produce minimum band broadening and minimize possible flow disturbances.

The injectors are divided into two types

Valve type It is connected to the column by means of a pipe and consists of valves which can be opened and closed. it is used more often.	**Syringe type** It is directly connected to the column and the sample is delivered with the aid of a syringe. Column activity is better than valve type.

- The HPLC column consists of packing and material made of stainless steel or plastic that carries packing.

HPLC column sizes range from capillary to process scale (from 5cm to 150cm). The column diameter and size can affect the separation profile. Smaller diameters and sizes yield increased separation and detection sensitivity, reduce the retention time and thus the rapid analysis is carried out.

The diameters of the columns used in preparative work can be larger, a preparative column with a length of 30 cm and a diameter of 57 mm can separate the substance from 1-1000 g.

Columns can be packed with solids such as silica or alumina. Most common packing materials in HPLC columns is silica. The use of smaller-diameter beads generally results in improved separation sensitivity due to the increased surface area.

In the HPLC column, the components of the sample separate based on their differing interactions with the column packing. If a species interacts more strongly with the stationary phase in the column, it will spend more time adsorbed to the column's adsorbent and will therefore have a greater retention time.

Two additional columns are used to extend the life of the analytical columns.

- Pre-column: A pre-column connect between pump and injector, and it is packed with silica to saturate an alkaline mobile phase, so it won't attack a silica-based analytical column. If your mobile phase is properly filtered, you shouldn't ever need to replace the pre-column (until a significant portion has dissolved away).

- Guard column: A guard column connect between injector and analytical column, and it is packed with the same packing used for the analytical column, so anything that would gum up the packing is caught on the guard column first. It is used to protect the analytical column from impurities and adsorbing compounds in sample.

- A chromatography detector is a device used in high performance liquid chromatography (HPLC) to detect components of the mixture being eluted off the chromatography column. The detector senses the presence of the individual components as they leave (elute) the column. A detector is needed to see the separated compound bands as they elute from the high pressure column. The HPLC detector, located at the end of the column, must register the presence of various components of the sample, but must not detect the solvent. The information is sent from the detector to a computer which generates the chromatogram. The mobile phase exits the detector and is either sent to a waste, or collected, as desired.

The detectors used in HPLC are of majorly two types:

- Selective detectors (solute property) : These detectors respond to a particular physical or chemical property of the solute, being ideally independent of the mobile phase. They are as follow: Fluorescence detectors, Electrochemical detectors, UV absorption detectors
- Universal detectors (bulk property): measure the difference in some physical property of the solute in the mobile phase compared to the mobile phase alone. They are generally universal in application but tend to have poor sensitivity and limited range. Such detectors are usually affected by even small changes in the mobile-phase composition which precludes the use of techniques such as gradient elution. Most used: Refractive index detectors

Fluorescence Detector

By using a specific wavelength, analyte atoms are excited and then emit light signal (fluorescence). The intensity of this emitted light is monitored to quantify the analyte concentration. Some pharmaceuticals, natural products, clinical samples, and petroleum products have fluorescent absorbance. For some compounds which do not have fluorescence absorbance or low absorbance, they can be treated with fluorescence derivatives.

Electro Chemical Detector

Compounds capable of electrochemically altering under controlled potential conditions can undergo electrolytic oxidation or reduction at the surface of an electrode. In order to use these detectors, it is necessarly to mobile phase be able to transmit electricity. This conductivity is provided by the addition of tetraalkylammonium perchlorate for mobile phases, addition of potassium nitrate for aqueous phases. These detectors can be used for the determination of many drugs by oxidative pathways (eg morphine, paracetamol, phenothiazines, tricyclic antidepressants, haloperidol, salicylic acid etc.). Reductive analysis is more difficult (eg, chloramphenicol, benzodiazepines, etc.) because it requires removal of oxygen dissolved in the mobile phase,

UV, VIS Absorbance Detector

During the analysis, sample goes through a clear color-less glass cell, called flow cell. When UV light is irradiated on the flow cell, sample absorbs a part of UV light. Thus, the intensity of UV light observed for the mobile phase (without sample) and the eluent containing sample will differ. By measuring this difference, the amount of sample can be determined. Two types of HPLC UV detectors are single and variable wavelength detectors.

Single wavelength detectors measure the samples absorption of a single wavelength (Most commonly used is 254 nm), while variable wavelength detectors measure absorption of multiple wavelengths and are

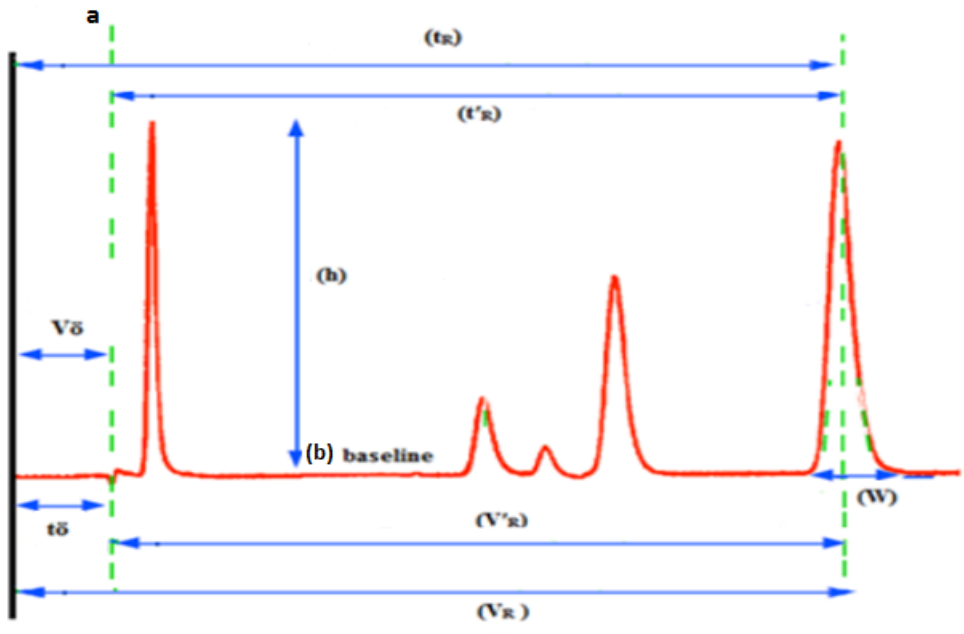
therefore more sensitive (180-400nm deuterium lamp or 400-700nm tungsten lamp). When deciding on a HPLC UV detector, consider whether a single wavelength will be sufficient or if a variable wavelength detector is needed. The wavelength range that can be used and the sensitivity of the detector should also be factored into the decision.

Refractive-Index Detector

RI detector measures change in reflex index. By measuring this change, the presence of components can be observed. RI detector has lower sensitivity compared to UV detector, and that's the main reason why RI is not as commonly used as UV. However there are some advantages over UV detector. It is suitable for detecting all components. For an example, samples which do not have UV absorption, such as sugar, alcohol, or inorganic ions obviously cannot be measured by a UV detector. In contrast, change in reflective index occurs for all analyte, thus a RI detector can be used to measure all analyte.

Theoretical Basics

The signal from the detector is converted to a concentration / time graph in the form of a Gaussian curve. This graph is called the chromatogram. Chromatogram is a graphical display in real time of peaks generated as the separated components pass through the detector. A chromatogram is a representation of the separation that has chemically [chromatographically] occurred in the HPLC system. A series of peaks rising from a baseline is drawn on a time axis. Each peak represents the detector response for a different compound.



b: Base line: It is the baseline of the chromatogram that occurs only when the mobile phase passes through the column

a: Dead point: The dead point is the position of the peak-maximum of an unretained solute.

tö: Dead time: The dead time (to) is the time elapsed between the injection point and the dead point.

Vö: Dead volume: The dead volume (Vo) is the volume of mobile phase passed through the column between the injection point and the dead point.

t_R: Retention time: The retention time (tr) is the time elapsed between the injection point and the peak maximum. Each solute has a characteristic retention time.

V_R: Retention volume: The retention volume (Vr) is the volume of mobile phase passed through the column between the injection point and the peak maximum.

t'_R: Corrected retention time: The corrected retention time (t'r) is the time elapsed between the dead point and the peak maximum.

V'_R: Corrected retention volume: The corrected retention volume (V'r) is the volume of mobile phase passed through the column between the dead point and the peak maximum. It will also be the retention volume minus the dead volume.

h: Peak height: The distance between the maximum height of the peak and the base line.

W: Peak width

AU: Area of Under

Column efficiency

It is a parameter that controls the peak width and shows how well the column is filled.

The peak width is an indication of peak sharpness and, in general, an indication of the column efficiency . However, the peak width and column efficieny are dependent on a number of parameters They are column length, flow rate, particle size.

A narrow chromatographic band, in other words, the sharpness of the peak is an indication of how good, or efficient a column is.

Column efficiency is expressed as N. (N is the number of theoretical plates.) It can be measured directly from the chromatogram.

The larger the N value, the narrower the peak is obtained. The narrower the peak, the better the column efficiency.

$N = a (V_R / W)^2$

N: Number of theoretical plates

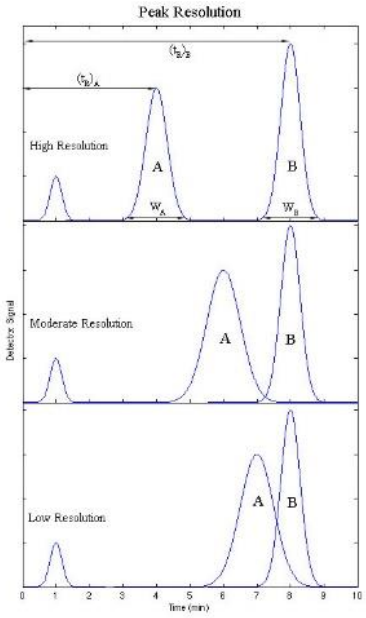
W: Peak width

V_R: Retention Volume

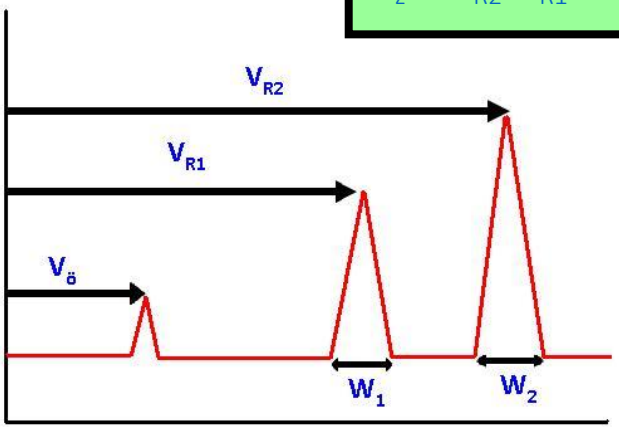
a: Constant value

Resolution (R_s)

The **resolution** of a elution is a quantitative measure of how well two elution peaks can be differentiated in a chromatographic separation. It is defined as the difference in retention times between the two peaks, divided by the combined widths of the elution peaks. If the resolution is greater than one, the peaks can usually be differentiated successfully.



$$R_z = (V_{R2} - V_{R1}) / [(w_1 + w_2)/2]$$



Normal Phase vs. Reverse Phase

The key to an effective and efficient separation is to determine the appropriate ratio between polar and non-polar components in the mobile phase. The goal is for all the compounds to elute in as short a time as possible, while still allowing for the resolution of individual peaks.

If the stationary phase is more polar than the mobile phase, the separation is deemed **normal phase**. If the stationary phase is less polar than the mobile phase, the separation is **reverse phase**. In other words, Normal-phase HPLC columns have polar stationary phase (silica gel, cyanopropyl, aminopropyl-bonded silica) and nonpolar mobil phase (hexane, chloroform, ethylacetate etc.). So that, the apolar compounds are first removed from the column. It is used in the separation of water-sensitive compounds, geometric isomers, chiral compounds. Reversed-phase HPLC columns have nonpolar stationary phase (C18, C8, C3 etc.) and polar mobil phase (acetonitrile, water, methanol, etc.). So that, the retention time of a compound increases with decreasing polarity of the particular species. The polar compunds are first removed from the column. With this method, polar, apolar, ionizable and ionic compounds are separated.

Typical columns for normal phase separation are packed with alumina or silica.

HPLC and Pharmacy

HPLC play an important and critical role in the field of pharmaceutical industries and analysis, since it is used to test the products and to detect the raw ingredient used to make them i.e., qualitative and quantitative analysis. The analysis of drug substances can be done quickly, reliably and sensitively. The most important benefits gain from the uses of HPLC technique in the industrial and analytical field that it is help in structure elucidation and quantitative determination of impurities and degradation products in bulk drug materials and pharmaceutical formulations and enantiomeric separation. HPLC is also used for the identification of drugs in biological samples, determination of drug level in the plasma, dose adjustment.