CHRISTIAN EMINENT COLLEGE, INDORE

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On

"HIGH PERFORMANCE LIQUID CHROMATOGRAPHY"

By:

Dr. Shiv Om Pratap

Professor and Head
Department of Bioscience
Christian Eminent College, Indore

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Introduction

High-performance liquid chromatography (**HPLC**; formerly referred to as **high-pressure liquid chromatography**), is a technique in analytical chemistry/Biochemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column.

Principle of Chromatography:

Chromatography is based on the concept of **separating molecules in a mixture added to the ground or solid and liquid stationary state (stable phase) when travelling with the aid of a mobile phase**.

The separation principle of HPLC is based on the distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase (packing material of the column). Depending on the chemical structure of the analyte, the molecules are retarded while passing the stationary phase.

High-performance liquid chromatography or commonly known as HPLC, is an analytical technique used to separate, identify or quantify each component in a mixture.

The mixture is separated using the basic principle of column chromatography and then identified and quantified by spectroscopy.

In the 1960s, the column chromatography LC with its low-pressure suitable glass columns was further developed to the HPLC with its high-pressure adapted metal columns.

HPLC is thus basically a highly improved form of column liquid 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.

- The purification takes place in a separation column between a stationary and a mobile phase.
- The stationary phase is a granular material with very small porous particles in a separation column.
- The mobile phase, on the other hand, is a solvent or solvent mixture which is forced at high pressure through the separation column.
- Via a valve with a connected sample loop, i.e. a small tube or a capillary made of stainless steel, the sample is injected into the mobile phase flow from the pump to the separation column using a syringe.
- Subsequently, the individual components of the sample migrate through the column at different rates because they are retained to a varying degree by interactions with the stationary phase.
- After leaving the column, the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer.
- At the end of this operation/run, a chromatogram in the HPLC software on the computer is obtained.
- The chromatogram allows the identification and quantification of the different substances.

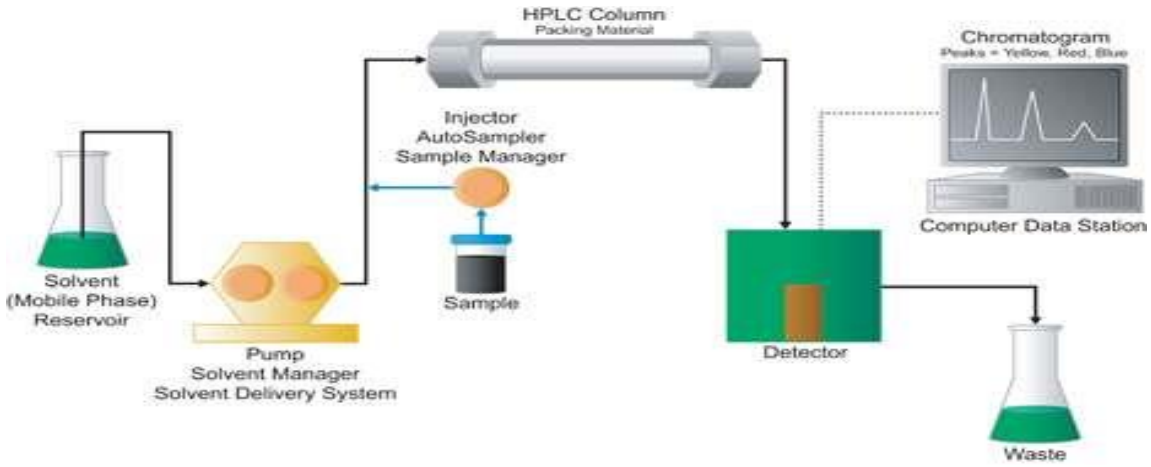
Mechanism of HPLC:

- HPLC relies on pumps to pass a pressurized liquid and a sample mixture through a column filled with an adsorbent, leading to the separation of the sample components. The active component of the column, the adsorbent, is typically a granular material made of solid particles (e.g. silica, polymers, etc.), by having a pore size of 2–50 μm in size.
- The components of the sample mixture are separated from each other due to their different **degrees of interaction** with the adsorbent particles.
- The pressurized liquid is typically a mixture of solvents (e.g. water, aceto-nitrile and methanol) and is referred to as a **"Mobile Phase".** 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.

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.

- Also HPLC columns are made with smaller adsorbent 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.
- The schematic of a HPLC instrument typically includes a degasser, sampler, pumps, 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.
- 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.



- A reservoir holds the solvent [called the mobile phase, because it moves
- A high-pressure pump is used to generate and meter a specified flow rate of mobile phase, typically milliliters per minute.
- An injector is able to introduce [inject] the sample into the continuously flowing mobile phase stream that carries the sample into the HPLC column.
- The column contains the chromatographic packing material needed to effect the separation.
- This packing material is called the stationary phase because it is held in place by the column hardware.
- A detector is needed to *see* the separated compound bands as they elute from the HPLC column [most compounds have no color, so we cannot see them with our eyes].
- The mobile phase exits the detector and can be sent to waste, or collected, as desired.
- When the mobile phase contains a separated compound band, HPLC provides the ability to collect this fraction of the eluate containing that purified compound for further study. This is called preparative chromatography [discussed in the section on HPLC Scale].

Note that high-pressure tubing and fittings are used to interconnect the pump, injector, column, and detector components to form the conduit for the mobile phase, sample, and separated compound bands.

Applications of HPLC:

HPLC has been used for the identification and confirmation of Industrial products; pharmaceutical

and biological products, researches, and medical purposes which are as follows-

A. Manufacturing:

HPLC can produce extremely high quality (pure) products, it is not always the primary method used in the production of bulk drug materials. It is a common technique used in pharmaceutical development, as it is a dependable way to obtain and ensure product purity.

B. Legal

This technique is also used for detection of illicit drugs in urine. The most common method of drug detection is an immunoassay.

C. Researches:

Similar assays can be performed for research purposes, detecting concentrations of potential clinical candidates like anti-fungal and asthma drugs. This technique is obviously useful in observing multiple species in collected samples, as well, but requires the use of standard solutions when information about species identity is sought out. It is used as a method to confirm results of synthesis reactions, as purity is essential in this type of research. However, mass spectrometry is still the more reliable way to identify species.

D. Medical Purposes:

Medical use of HPLC can include drug analysis, but falls more closely under the category of nutrient analysis. While urine is the most common medium for analyzing drug concentrations, blood serum is the sample collected for most medical analyses with HPLC. Other methods of detection of molecules that are useful for clinical studies have been tested against HPLC, namely immunoassays. Useful for diagnosing vitamin D deficiencies in children, it was found that sensitivity and specificity of this CPBA reached only 40% and 60%, respectively, of the capacity of HPLC