A REVIEW ARTICLE ON HPLC METHODS AND APPLICATIONS

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Abstract
Chromatography refers to a set of techniques used to separate constituents in a mixture. High-Pressure Liquid Chromatography (HPLC) is a specialized technique that uses columns and liquid chromatography to separate, characterize, and investigate the active components in a mixture. This review focuses on the principles, instrumentation, types, applications, advancements, and patents of the HPLC technique. HPLC is important for both quantitative and qualitative analysis and is used to evaluate biological and pharmaceutical samples.
Keywords: *HPLC, HPLC Method Development, Method Validation*

Introduction
Chromatography is known as the backbone of division science and is broadly connected to distinctive pharmaceutical businesses and inquire about organizations worldwide [1]. Chromatography is characterized as the partition of a combination of compounds into particular substances by utilizing two stages; one is mobile, and the other one is stationary [2,3]. This method was, to begin with, designed in 1903 by Mikhail Semyorivich Tswett, an Italian-born Russian botanist, and was afterward considered the 'Father of Chromatography' [4,5]. Chromatography combines two Greek words, i.e., chromo implies 'colour' and graphene implies 'to type in. By and large, the partition strategy in chromatography includes the foremost steps beginning from the maintenance or adsorption of a substance(s) in the stationary stage and at that point the division of the adsorbed substances with the offer assistance of the versatile stage. Taken after the recovery of the isolated substance by a persistent stream of the versatile stage called elation; gone before by quantitative and subjective examination of the eluted substances.[6]

Types of Chromatography
There are different types of chromatography based on the nature of the phases, modes of chromatography run, based on separation, based on elution technique.

Nature of the Mobile Phase and Stationary Phase
Various types of chromatographic techniques are available depending upon the type of phases used like Gas – Liquid Chromatography, Gas – Solid Chromatography, Liquid-Liquid Chromatography: further divided into column partition chromatography and paper partition chromatography, and Solid-Liquid Chromatography includes Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Column Chromatography [2,7,8].

High-performance liquid chromatography
High-Pressure Liquid Chromatography, also known as High-Performance Liquid Chromatography (HPLC), is a specialized technique that uses columns and liquid

chromatography to separate, characterize, quantify, and analyze the active components present in a mixture. Compared to traditional techniques, HPLC offers significantly improved performance [9]. The development of HPLC has evolved from conventional column chromatography, with its efficiency and resolution enhanced using a stationary phase composed of spherical particles ranging from 2 µm to 5 µm in diameter. Due to the small particle size, the high pressure in the system forces the mobile liquid or solvent to pass through the column, leading to its designation as high-pressure liquid chromatography [10,11]. HPLC is utilized for separating not only volatile compounds, but also ionic species, polymeric materials, labile natural products, macromolecules, and high molecular weight functional groups [12]. In HPLC, a pump is employed to allow a sample mixture dissolved in a liquid solvent to pass through a solid adsorbent material packed in the column. The individual components in the sample interact differently with the adsorbent material, resulting in diverse flow rates for different elements and separating components as they exit the column [2,13]. HPLC operates based on the adsorption-separation principle. In the HPLC column, compounds to be separated are introduced in a mixture, but different components travel at different rates based on their relative affinity toward the stationary phase [14]. Components with greater affinity for the stationary phase move at a slower rate, while those with less affinity move at a faster rate. The components get separated since no two compounds have the same affinity for the stationary phase [15].

HPLC Method Development:

Alternate methods are developed for new products when no official methods are available. An alternate method for existing (non-Pharmacopeial) products aims to reduce cost and time while improving precision and ruggedness. Comparative laboratory data including merits and demerits should be provided when proposing an alternate method to replace the existing procedure. The goal of the HPLC method is to separate and quantify the main active drug, any reaction impurities, all available synthetic intermediates, and any degradants [16]

METHOD DEVELOPMENT ON HPLC [16,17]

The steps involved in the method development of HPLC are as follows:

1 Understanding the Physicochemical properties of drug molecules.
2 Selection of chromatographic conditions.
3 Developing the approach of analysis.
4 Sample preparations
5 Method optimisation
6 Method validation

Understand the physicochemical properties of drug molecules.

The physicochemical properties of a drug molecule are crucial for method development. When developing a method, it is important to study the physical properties such as solubility, polarity, pKa, and pH of the drug molecule. The solubility of molecules can be explained based on their polarity. Polar solvents, like water, and nonpolar solvents, like benzene, do not mix. Generally, materials with similar polarity are soluble in each other. The selection of diluents is based on the solubility of the analyte. Acidity and basicity are typically defined by their pH value. pH and pKa play an important role in HPLC method development [18,19].

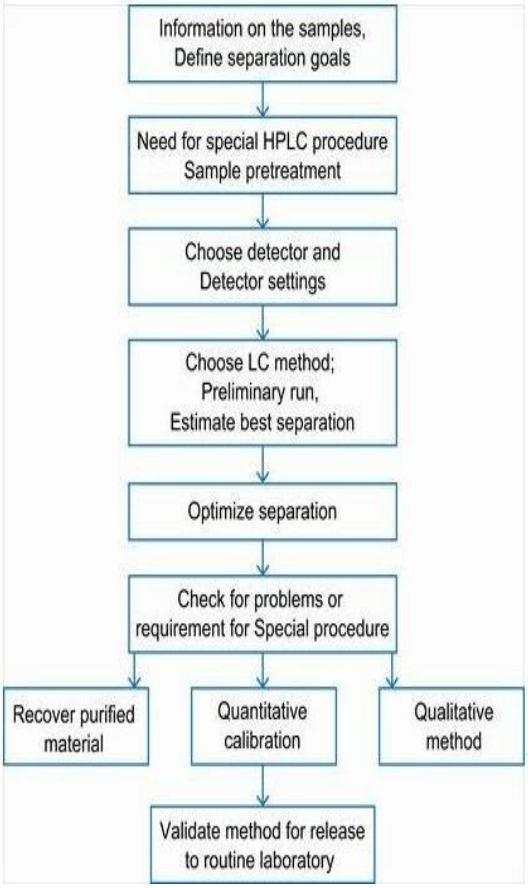


Fig.1: Steps involved in HPLC Method development

Selection of chromatographic conditions

In the initial stages of method development, a specific set of initial conditions (detector, column, mobile phase) is chosen to produce the first "scouting" chromatogramsof the sample. Typically, these conditions are based on reversed-phase separations using a C18 column with UV detection. At this stage, a determination should be made regarding whether to develop an isocratic or a gradient method [20].

Selection of Column:

The column is the first and most important component of a chromatograph. Selecting the right column can lead to effective chromatographic separation, yielding accurate and dependable analytical results. Conversely, an incorrectly used column can frequently produce confusing, insufficient, and poor separations, resulting in invalid or difficult-to-interpret results [21]. It is important to ensure that columns are stable and reproducible to avoid issues with sample retention during method development. A C8 or C18 column, made from specially purified, less acidic silica and designed specifically for separating basic compounds, is generally suitable for all samples and is strongly recommended.[17]. The main factors to consider are column dimensions, silica substrate properties, and bonded stationary phase characteristics. The use of silica-based packing is favored in most of the present HPLC columns due to several physical characteristics [22].

Selection of Chromatographic mode

The different chromatographic modes are determined by the molecular weight and polarity of the analyte. In our case studies, we will be concentrating on reversed-phase chromatography (RPC), which is the most widely used mode for small organic molecules. It's important to note that ionizable compounds, such as acids and bases, are often separated by RPC using buffered mobile phases to keep the analytes in a non-ionized state, or with the help of ion-pairing reagents [23].

Optimization of Mobile phase:

Buffer Selection:

The chromatographic performance of different buffers such as potassium phosphate, sodium phosphate, and acetate were tested for system suitability parameters. After several trials, it was found that potassium dihydrogen phosphate was the most effective in separating all peaks. Buffer concentrations of 0.02M, 0.05M, and 0.1M were tested, and it was observed that varying the concentration did

not significantly alter the elution pattern or resolution. However, the 0.05M concentration was found to increase the method's sensitivity [24].

Effect of pH:

If analytes are ionizable, the appropriate mobile phase pH must be chosen based on the analyte's pKa so that the target analyte is either ionized or neutral. The ability to change the pH of the mobile phase is one of the most powerful tools in the chromatographer's toolbox, allowing for simultaneous changes in retention and selectivity between critical pairs of components [24].

Effect of organic modifier:

In reverse-phase HPLC, choosing the type of organic modifier is quite straightforward. The most commonly used options are acetonitrile and methanol, with THF being used rarely. Gradient elution is typically employed for complex multicomponent samples because it might be impossible to elute all components using a single solvent strength between a retention factor (k) of 1 and 10 under isocratic conditions [24].

Selection of detector and wavelength

After the chromatographic separation, the analyte of interest is detected using suitable detectors. Some commercial detectors used in LC include ultraviolet (UV) detectors, fluorescence detectors, electrochemical detectors, refractive index (RI) detectors, and mass spectrometry (MS) detectors. Choosing the right detector is crucial as it depends on the sample and the specific purpose of the analysis. In the case of multicomponent analysis, the absorption spectra may have shifted to longer or shorter wavelengths compared to the parent compound. Therefore, the UV spectra of the target analyte and impurities must be taken and overlaid with each other, and the spectra should be normalized due to the different amounts present in the mixture. A wavelength must be chosen such that an adequate response for most of the analytes can be obtained [25,26].

Developing the approach of analysis

When developing an analytical method for Reverse Phase High-Performance Liquid Chromatography (RP-HPLC), the first step is to select various chromatographic parameters such as the mobile phase, column, mobile phase flow rate, and mobile phase pH. Each characteristic is optimized through trials and then compared against system suitability parameters. These parameters include a retention time of more than five minutes, a theoretical plate count exceeding 2000, a tailing factor less than two, a resolution greater than five, and a percent Relative Standard Deviation (R.S.D.) of the area of analyte peaks in standard chromatograms not exceeding two percent. These parameters ensure the reliability and precision of the RP-HPLC method. In the simultaneous estimation of two components, the detection wavelength is typically chosen at an isosbestic point [27,28].

Sample preparation

Sample preparation is the crucial step in the HPLC analysis. Its purpose is to create a processed sample that produces better analytical results compared to the initial sample. The prepared sample should be an aliquot that is largely free from interferences, compatible with the HPLC method, and safe for the column. Common sample preparation techniques include Liquid-Liquid Extraction (LLE) and Solid-Phase Extraction (SPE). In these methods, the analyte of interest is separated from the sample matrix, minimizing potentially

interfering species carried through to the analytical separation stage [26].

Method optimisation

The method is optimized to improve sensitivity after separation. The compositions of the mobile phase and stationary phase need to be taken into account. Mobile phase parameters are optimized first as it is easier and more convenient than stationary phase optimization. To minimize the number of trial chromatograms, only examine parameters likely to significantly affect selectivity during optimization. The primary control variables (factors) in the optimization of liquid chromatography (LC) methods are the different components of the mobile phase determining acidity, solvent strength, gradient, flow rate, temperature, sample amounts, injection volume, and diluent solvent type [29]. These are used to find the desired balance between resolution and analysis time after achieving satisfactory selectivity. The parameters involved include column dimensions, column-packing particle size, and flow rate. These parameters may be changed without affecting capacity factors or selectivity [30].

Method Validation

The validation of an analytical procedure involves conducting laboratory studies to ensure that the procedure meets the necessary performance criteria for its intended use. The process begins with the systematic collection of validation data by the applicant to support the analytical procedure. All analytical methods intended for analysing clinical samples must undergo validation. The validation of analytical methods is carried out according to ICH guidelines [31,32]

Types of Analytical Procedures to be validated

The discussion focuses on the validation of analytical procedures, covering the four most common types.:

- Identification tests;
- Quantitative tests for impurities' content;
- Limit tests for the control of impurities;
-Quantitative analysis of the active ingredient in drug substance or drug product samples, as well as other specific components in the drug product.

• Before they are put into routine use; • When the conditions for which the method has been validated change; • When the method is changed,

The following are typical parameters recommended by the FDA, USP, and ICH [24].

1. Specificity
2. Linearity
3. Precision

I Method precision (Repeatability)
II Intermediate precision (Reproducibility)

4. Accuracy (Recovery)
5. Solution stability
6. Limit of Detection (LOD)
7. Limit of Quantification (LOQ)
8. Robustness
9. Range
10. System suitability

1. Specificity

The selectivity of an analytical method refers to its ability to accurately measure a specific substance even when other interfering substances are present, such as synthetic precursors, excipients, enantiomers, and known or potential degradation products that may be found in the sample matrix [20].

2. Linearity

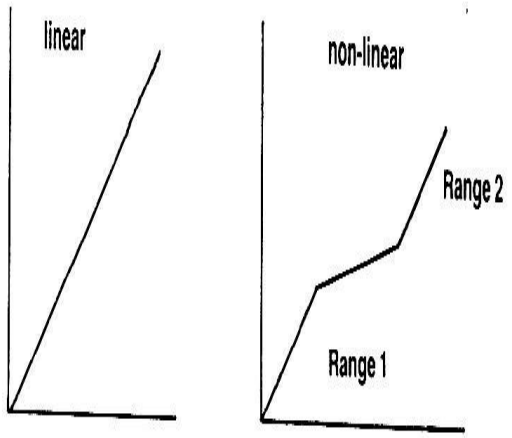
Linearity refers to the ability of an analytical method to produce a response that is directly proportional to the amount of the substance being analyzed in the sample. When a method is linear, the test results show a consistent mathematical relationship to the concentration of the substance within a specific range. Linearity is often described by the confidence limit around the slope of the regression line [33].

3. Precision

The precision of an analytical procedure indicates how close a series of measurements are to each other when taken from multiple samples of the same homogeneous sample under specified conditions. Precision can be evaluated at three levels: repeatability, intermediate precision, and reproducibility. It is best to assess precision using homogeneous, authentic samples. If obtaining a homogeneous sample is not possible, artificially prepared samples or a sample solution can be used. Precision is typically expressed as the variance, standard deviation, or coefficient of variation of a series of measurements [34].

4. Accuracy (Recovery)

Accuracy refers to how close a measured value is to the true or accepted value.. It indicates the deviation between the mean value found and the true value. Accuracy is determined by applying the method to samples to which known amounts of analyte have been added. These samples should be analyzed against standard and blank solutions to ensure that no interference exists. Accuracy is calculated from the test results as the percentage of the analyte recovered during the assay. It is often expressed as the recovery of known, added amounts of the analyte in the assay.[35].



Solution stability

During validation, standards and samples are evaluated under normal and sometimes instrument-specific storage conditions to determine the need for special storage requirements such as refrigeration or protection from light [36].

6. Limit of Detection (LOD)

The limit of detection (LOD) of an individual procedure is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated as an exact value. In analytical procedures showing baseline noise, the LOD may be determined based on a signal-to-noise (S/N) ratio (3:1), typically expressed as the analyte concentration in the sample. The signal-to-noise ratio is determined by the formula: S = H/h, where H is the height of the peak corresponding to the component and h is the absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution [20,37].

7. Limit of Quantification (LOQ)

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be accurately and precisely determined. This limit serves as a parameter in quantitative assays for detecting low levels of compounds within sample matrices, making it particularly valuable for identifying impurities and degradation products. [34].

8. Robustness

The robustness of an analytical method refers to its ability to produce consistent results despite variations in operational parameters. This property is important for determining the suitability of the method for its intended purpose. Robustness can be evaluated using statistical experimental design. In a robustness test, chromatographic factors such as resolution, efficiency, capacity factor, and peak asymmetry can be considered as responses. It's worth noting that system suitability limits can be defined based on the evaluation of robustness, as it explores extreme variations in factors that may occur. This approach helps avoid ambiguous situations, as it is rare to find a universally satisfactory solution [38].

9. Range

The method's range is the interval between the upper and lower levels of an analyte that have been established with acceptable precision, accuracy, and linearity. It is determined on either a linear or nonlinear response curve (i.e., where more than one range is involved, as shown below) and is usually expressed in the same units as the test results [39].

10. System suitability

Liquid chromatographic methods typically incorporate system suitability tests as a standard procedure. These tests are used to ensure that the chromatographic system's detection sensitivity, resolution, and reproducibility are adequate for the analysis. The tests are based on the idea that the equipment, electronics, analytical operations, and samples to be analysed are all part of a whole that can be evaluated as a whole. To determine the suitability of the method used factors such as peak resolution, the number of theoretical plates, peak tailing, and capacity are measured [40].

Instrumentation of HPLC [41]

Components of the HPLC system are shown in Fig. (8):

A. Solvent reservoir, mixing system, and degassing system
B. High-pressure pump
C. Sample injector
D. Column
E. Detector
F. Data recording system

Solvent reservoir

The mobile phases and wash solvents used in the pump system are typically stored in sealed, clean, and inert containers ranging from a few hundred milliliters to several litters, depending on the specific application. These reservoirs help maintain the purity of the solvents by preventing dust from entering, ensuring that leachable contaminants are not introduced, and minimizing the evaporation of volatile components. It is recommended to use spill-proof, shatterproof (e.g., polymer-coated glass), and fire-resistant containers for safety. While HPLC-grade solvents and prefiltered solutions generally do not contain solid matter, a final filter is used to protect the system and column from any solids adhered to the reservoir or tubing, as well as from insoluble substances that may form from buffered mobile phases over

time. It is crucial to use a high-quality and inert frit to avoid introducing contamination into the mobile phase; some lower-quality stainless steel frits may cause discoloration of mobile phases over time [42].

Mixing system

In mixing systems, individual pressure pumps are typically employed to introduce different liquids. The outlet of each pump is connected to mixing connectors, which consist of one outlet line and two inlets, leading to a mixing chamber. This setup is utilized to blend the solvents in varying proportions [43].

Degassing system

Solvent reservoir systems are designed to remove dissolved gases like O2 and N2 from the solvent because these gases can interfere with the separation process. They form bubbles in the column or detector system, which can block the column. Therefore, degassing is necessary [6].

Different method is used to remove dissolved gases,

Ultrafiltration

Ultrasonication

Helium Sparging

Refluxing

High-pressure pump

The role of a pump is to force a liquid and give a specific flow rate. The flow rate is expressed in milliliters per minute (ml/min). Normal flow rate is 1-2 ml/min. The pump pressure range is 6000-9000 psi (400-600 bar) [44].

Types of HPLC pump

Three common types of pumps include constant pressure pumps, syringe pumps, and reciprocating piston pumps.

Constant Pressure Pumps

As the name suggests, they provide a constant flow rate in the column thanks to constant pressure. They utilize the low pressure of gas stored in a cylinder to generate the necessary high pressure for the mobile phase.

Fig.2: Constant Pressure Pumps

Syringe Type Pumps

They utilize a motorized syringe to ensure a consistent delivery of the solvent. By adjusting the motor voltage, various flow rates can be achieved.

Fig.3: Syringe Pumps

Reciprocating Piston Pumps

They consist of a hydraulic chamber where a reciprocating piston is placed. This way, the solvent enters the pump when the piston moves back and is pushed into the column when the piston moves forward.

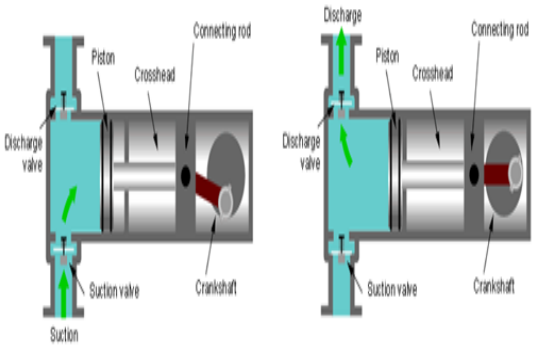


Fig.4: Reciprocating Piston Pumps

Sample injector

The injection component of an HPLC has the challenging task of introducing a sample from atmospheric pressure into the high-pressure fluid stream between the pump and the column. It needs to be accurate, precise, and rapid, cause minimal disruption to the flow stream, and deliver a sharp, symmetrical zone. Sample introduction often contributes the most to analysis imprecision. A six-port, two-position switching valve is commonly used for injections. In its simplest operation, the valve initially passes the mobile phase from the pump to the column while in the "load" position, directly passing the mobile phase from the pump to the column [42].

Several Devices Used in Sample Injector System

Septum Injector
Stopped Flow (online) Injector
Rheodyne Injector or Loop Valve Injector

Column

Column is a place where the actual separation of components takes place. The column is made up of stainless steel. It is 5-25 cm long and 2-4.6 cm internal diameter [44].

Types of Columns used in HPLC

Normal-phase columns
Reverse-phase columns
Ion-exchange columns
Size-exclusion columns
Chiral columns

Detector

Detectors are devices used to sense the presence of a solute in the eluent are known as detectors. There are different types of detectors available, and their selection is crucial for effectively detecting the solute. These detectors are designed and used based on the nature of the components to be separated. The detecting element should be highly sensitive and stable because the amount of material applied to the column is minimal. The detector used for HPLC should have properties such as high sensitivity, stability, reproducibility, linearity in response to the solute, affordability, non-destructiveness, reliability, ease of operation, low baseline noise, and a temperature range from 25°C to at least 400°C. The response of the HPLC detector should be independent of the mobile phase composition and stable at all temperatures and flow rates. Additionally, there should be minimal extra column band broadening, and the detector should provide both quantitative and qualitative information on the detected peaks with a fast response time [6].

There are several types of detectors,
UV/Visible Detector
Photodiode Array Detector
Fluorescence Detector
Chemiluminescence detector
Electrochemical detector
Chemiluminescence detector
Radio HPLC detector
Evaporative light scattering detection (ELSD)

Fig.6: UV Visible Detector

Data recording system

The output is captured as a series of peaks, and the computer linked to the display can automatically calculate the area under each peak [45].

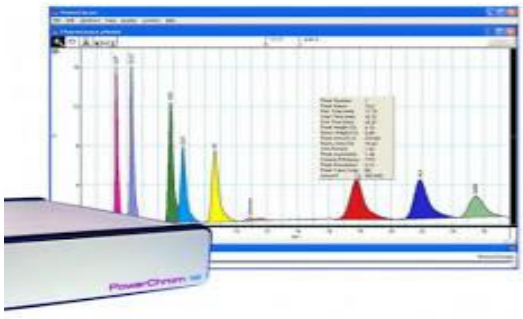


Fig.7: Data Recorder

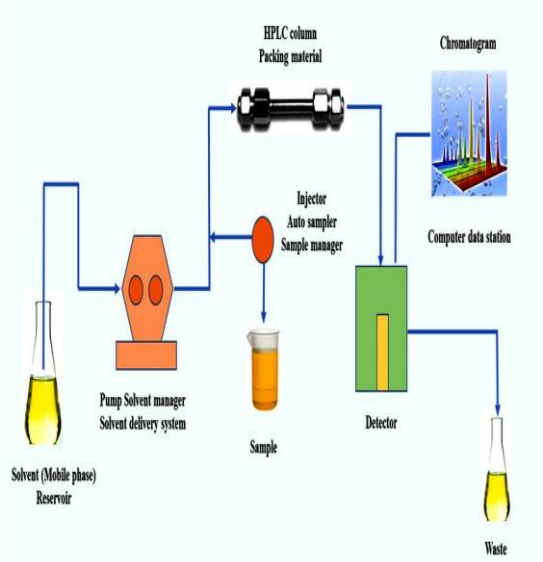


Fig.8: Schematic Representation of High-Performance Liquid Chromatography [6]

Advantages Of HPLC [46,47]

High-performance liquid chromatography (HPLC) is a rapid, automated, and highly accurate method used to detect specific chemical components in a sample.

It provides quick and accurate quantitative analysis.

Some methods involve using a gradient solvent system.

Unlike gas chromatography (GC), there is no need to vaporize the sample."

Disadvantages Of HPLC [46]

HPLC can be a costly technique due to the need for expensive organic solvents, a reliable power source, and ongoing maintenance.

Troubleshooting problems or developing new methods for HPLC can be complex.

The reliability of the HPLC pump process depends on the cleanliness of the sample, the mobile phase, and the correct application of procedures.

Applications of HPLC [6,48]

HPLC has several applications which includes,

1. Forensic Sciences
2. Clinical and Health Related Disorders
3. Food and Flavour
4. Environmental Sciences
5. Pharmaceutical Industry
6. Recent Applicatio

Forensic Sciences

- It helps to determine the presence of cocaine, heroin, and other abused drugs presence in blood.
- It also helps in the forensic analysis of textile dye.
- It also helps to determine unknown elements present in the compound.

Clinical and Health Related Disorders

- It helps to analyze the urine and blood samples.
- It helps in the analysis of bilirubin and biliverdin in hepatic disorders.
- Detection of endogenous neuropeptides in extracellular fluids of the brain.

Food and Flavour

- HPLC is used in the food industry to determine the presence of sugar in fruit juice.
- It helps to test the excellence of water and soft drinks.
- Analysis of polycyclic compounds in vegetables.

Environmental Sciences

- It helps to detect phenol compounds in drinking water.
- Bio-monitoring of pollutants.
- Rapid separation and identification of carbonyl compounds by HPLC.
- Identification of diphenhydramine in sedimented samples.

Pharmaceutical Industry

- HPLC helps to detect impurities present in the pharmaceutical product.
- It helps to control drug stability, and shelf-life determination.
- It helps to identify the active ingredients.
- It helps pharmaceutical quality control.

Recent Application

- It is used in inorganic chemistry for separating anions & cations
- It also used to determining antioxidants and preservatives present in the food.
- It is used for separating various components of plant products with bear structural resemblance.
- It is used in the agrichemical industry for the separation of herbicides.
- It is used to separate coal and oil products from their crude sources.

Conclusion:

High-performance liquid chromatography (HPLC) is a commonly utilized analytical method. This method allows for the production of very pure compounds. HPLC is valuable in both laboratory and clinical settings, providing precise and accurate results with increased specificity. It is the preferred separation technique for quantitative trace analysis of toxic chemicals, impurities, manufacturing high-purity products, medicinal uses, and research purposes.

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