IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Review Article
March 2022 Vol.:23, Issue:4

An Overview on High-Performance Liquid Chromatography

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
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Submitted: 23 February 2022
Accepted: 28 February 2022
Published: 30 March 2022

Keywords: Liquid chromatography, columns, detector, and elution.

ABSTRACT

The term chromatography is derived from the Greek words namely chroma (color) and graphein (to write). It is defined as a set of techniques used for the separation of constituents in a mixture. This involves 2 phases; they are stationary and mobile phases. The separation is based on the difference between the partition coefficients of the two phases. The instrumentation includes a solvent reservoir, HPLC pump, injector, HPLC column, detector, and data acquisition. It is used for purposes like identification of compounds, chemical separation, purification of compounds, etc. HPLC is mainly used in analytical chemistry. This review focus in detail on the HPLC technique, its principle, types, instrumentation, and applications.

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INTRODUCTION

In 1903 a Russian botanist succeeded in separating leaf pigment using a solid polar stationary phase. HPLC was derived from classical column chromatography and has found an important place in analytical techniques. The major advancement in HPLC was found by the use of efficient separators.[1]

It is a very popular technique and is mostly used analytically. Different types of chromatographic techniques are Paper Chromatography, Gas Chromatography (GC), Liquid Chromatography (LC), Thin Layer Chromatography (TLC), Ion exchange Chromatography, and lastly High-Performance Liquid Chromatography (HPLC).

High-Performance Liquid Chromatography is also known as High-Pressure Liquid Chromatography. HPLC is an advanced technique of column liquid chromatography. The solvent usually flows through the column with the help of gravity but in the HPLC technique, the solvent will be forced under high pressures up to 400 atmospheres so that sample can be separated into different constituents with the help of differences in relative affinities.

Here, pumps will be used to pass pressurized liquid solvent including the sample mixture to enter into a column filled with a solid adsorbent material. The interaction of each sample component is different which causes a difference in flow rates and finally leads to the separation of components of the column. Chromatography can be depicted as a mass exchange process including adsorption. It depends on pumps to pass a pressurized fluid and an example blend through a section loaded with adsorbent, prompting the partition of the specimen segments.

The pressurized fluid is commonly a blend of solvents and is known as the ‘mobile phase’. Its organization and temperature play an important part in the partition procedure. The separation of samples is based on the differences in the rates of migration through the column arising from the different partitions of the sample between the stationary and mobile phases. Depending upon the partition behavior of different components, elution takes place at different times. The sample compound with the greater affinity to the stationary layer will travel slower and less affinity which, travel faster.[2]

PROCESS

The sample blend to be isolated and dissected is presented, in a discrete little volume, in the stream of mobile phase permeating through the column. The sample travel through the segment at various speeds, which are a component of particular physical connections with the adsorbent. The velocity of every component relies on its compound nature, the composition of the mobile phase. Based on the time at which a particular analyte elutes out, various sorts of columns are available, which are loaded with adsorbents varying in molecule size, and in the nature of their surface. Sorbent particles might be hydrophobic or polar in nature. Basic mobile phases utilized incorporate any miscible mixture of water with a different natural solvent like acetonitrile and methanol. Some HPLC systems use without water mobile phases. The aqueous segment of the mobile phase may contain acids or salts to help with the separation of the sample components.[3]

Isocratic elution is normally successful in the partition of sample components that are not altogether different in their proclivity for the stationary stage. In gradient elution, the organization of the mobile phase is fluctuated ordinarily from low to high eluting quality. The eluting quality of the mobile phase is reflected by analyte maintenance times with high eluting quality delivering quick elution.

The selected structure of the mobile relies on the force of connections between different analytes and the stationary stage. The detachment of the solute from the column is called its retention time. The retention time measured under specific conditions is a distinguishing normal for a given analyte.[3] Procedure occurring in the sample is like what happens amid a liquid-liquid extraction however it is continuous and not step-wise. In this case, utilizing a more hydrophobic part will elute late, once the mobile stage gets more packed in acetonitrile.

TYPES OF HPLC TECHNIQUE

1 Based on mode of chromatography.

2 Based on elution technique.

3 Based on a scale of operation.

4 Based on the type of analysis.

BASED ON THE MODE OF CHROMATOGRAPHY

HPLC is divided into normal phase and reverse phase based on the mode of chromatography. In normal phase mode, the stationary phase is polar and the mobile phase is nonpolar. Here nonpolar compounds travel faster and get eluted first. This is due to the less affinity between solute and stationary phase. Polar compounds are retained for a longer time in the column due to higher affinity towards the stationary phase and take more time to elute. Since most of the drug molecule in the pharmaceutical industry is polar in nature It takes more time to get eluted and detected so this is not advantageous in pharmaceutical applications. Not widely used in pharmacy.

In reverse phase mode, the stationary phase is nonpolar and the mobile phase is polar. Polar components eluted first and nonpolar are retained for a longer time. Since most of the drugs are polar in nature, they are not retained for a longer time and eluted faster, viz advantageous.

BASED ON THE ELUTION TECHNIQUE

Based on the technique of elution they are divided as isocratic and gradient separation. In isocratic separation, the combination of the mobile phase is used throughout the process of separation. Same polarity or elution strength is maintained. The concentration of the mobile phase is also the same. It influences the retention of the analyte. It is used in order to maximize the loading capacity.

In gradient separation, the mobile phase combination of lower polarity or elution strength is used followed by gradually increasing polarity or elution strength. Their selectivity depends on the dimensions of the column used. The advantages of gradient elution are that it enhances the peak resolution, faster analysis time, and better detectability.

BASED ON THE SCALE OF OPERATION

In analytical HPLC only analysis of the sample is done. Recovery of sample is not done during analytical high-performance liquid chromatography. Reusing is also not done for analytical purposes as the sample quantity retained is very low after the process. For this technique analytical columns are used.

By using preparative HPLC individual fractions of pure compounds can be collected using the fractional collector. In this HPLC the retained samples can be reused hence wastage is

low. Specialized preparative columns are used for this method. Preparation of pure compounds can be done using HPLC.

BASED ON THE TYPE OF ANALYSIS

Qualitative analysis helps in identifying the compounds from a mixture. This is done by comparing the retention time of the sample compound with that of the standard compound. Detection of impurities is also possible by the method of qualitative analysis.

Quantitative analysis is used in the determination of the quantity of individual or several components, it is the main function of the quantitative analysis in HPLC. Here the analysis is based on the comparison with the measurement of peak height from a sample with the unknown concentration. This method should be subjected to validation before starting the analysis.

INSTRUMENTATION

The HPLC instrumentation involves a pump, injector, column, detector, integrator, and display system. It is illustrated in fig. no. 1.

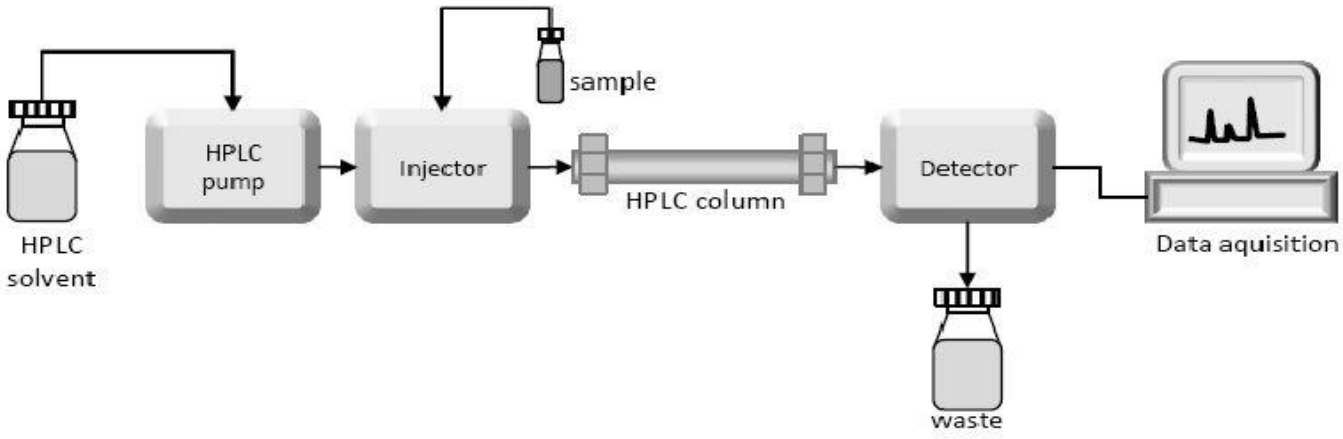

FIG. NO. 1: INSTRUMENTATION OF HPLC

SOLVENT RESERVOIR

FIG. NO. 2: SOLVENT RESERVOIR IN HPLC

The contents of the mobile phase are present in a glass container called solvent reservoir as shown in fig. no. 2. In high-performance liquid chromatography, the mobile phase or the solvent is a mixture of polar and non-polar liquid components. Depending on the composition of the sample, the polar and non-polar solvents will be varied. The solvent is used to carry the sample through the system. From the solvent reservoir, the pump collects the solvent and then passes it to the injector.

PUMP

FIG. NO. 3: PUMP SYSTEM IN HPLC

The pump suctions (FIG. NO. 3) the mobile phase from the solvent reservoir and forces it to column and then passes to the detector. 42000 KPa is the operating pressure of the pump. This operating pressure depends on column dimensions, particle size, flow rate, and composition of the mobile phase. Some of the different types of pumps are discussed.

Direct gas pressure systems

This system consists of a cylinder gas pressure, which is applied directly to the eluent in a holding coil. Advantages of this pump are that it is reliable and economical although solvent changing is found to be tedious.

Syringe type pumps

In these pumps, an electrically driven lead-screw moves a piston, which is able to pressurize a finite volume of solvent, and thus delivers a pulseless constant flow of solvent to the system. These pumps are found to be reliable although they are expensive, solvent changing is tedious and they have a finite capacity.

Pneumatic intensifier

Pneumatic intensifier pumps are operated via gas pressure. A large area piston drives a small area piston when acted on by pressure from a gas line. The gas pressure is thus amplified in the ratio of the areas of the forces of the pistons and a high-pressure liquid at constant pressure is introduced into the system. If a partial blockage occurs in this system a drop-in flow rate occurs but the pressure remains constant.

Reciprocating pumps

A reciprocating pump is the most generally used, as it is economical and allows a wide range of flow rates. With this pump, there is no limit on the reservoir size or operating time as is commonly found with other pumps. This type of pump is electrically driven by a motor, which moves back and forth within a hydraulic chamber.

SAMPLE INJECTOR

The injector can be a solitary infusion or a computerized infusion framework. An injector for an HPLC framework should give an 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) in

the load position a sample loop is filled with the sample while the system is equilibrating. The flow delivered by the pump flows through the loop and feeds the sample onto the column.

COLUMNS

FIG. NO. 4: COLUMNS USED IN HPLC

Columns as shown in figure 4 are typically made of cleaned stainless steel, are somewhere around 50 mm and 300 mm long, and have an inward distance across somewhere around 2 and 5 mm. They are generally loaded with a stationary phase with a molecule size of 3 μm to 10 μm. Columns with inner diameters of <2 mm are regularly alluded to as microbore segments. Preferably the temperature of the mobile phase and the column should be kept consistent during the investigation.

Separation Columns

There are various columns that are secondary to the separating column or stationary phase. They are guard, derivatizing, capillary, fast, and preparatory columns.

Guard Columns

They are placed anterior to the separating column. This serves as a protective factor that prolongs the life and usefulness of the separation column. They are dependable columns designed to filter or remove.

- Particles that clog the separation column.

- Compounds and ions could ultimately cause "baseline drift", decreased resolution, decreased sensitivity, and create false peaks.

- Compounds that may cause precipitation upon contact with the stationary or mobile phase.

- Compounds that might co-elute and cause extraneous peaks and interfere with detection and/or quantification.

These columns must be changed on a regular basis in order to optimize their protective function. The size of the packing varies with the type of protection needed.

Derivatizing Columns

Pre or post-primary column derivatization can be an important aspect of the sample analysis. Reducing or altering the parent compound to a chemically related daughter molecule or fragment elicits potentially tangible data which may complement other results or prior analysis. In a few cases, the derivatization step can serve to cause data to become questionable, which is one reason why HPLC was advantageous over gas chromatography, or GC. Because GC requires volatile, thermally stable, or nonpolar analytes derivatization was usually required for those samples which did not contain these properties. Acetylation silylation is concentrated acid hydrolysis are few derivatization techniques.

Capillary columns

Advances in HPLC led to smaller analytical columns. Also known as microcolumns, capillary columns have a diameter much less than a millimeter and there are three types:

- open-tubular
- partially packed
- tightly packed

They allow the user to work with nanoliter sample volumes, decreased flow rate, and decreased solvent volume usage which may lead to cost-effectiveness. However, most conditions and instrumentation must be miniaturized, the flow rate can be difficult to reproduce, gradient elution is not efficient, and care must be taken when loading minute

sample volumes. Microbore and small-bore columns are also used for analytical and small volumes assay.

Fast column

One of the primary reasons for using these columns is to obtain improved sample throughput (amount of compound per unit time). For many columns increasing the flow or migration rate through the stationary phase will adversely affect the resolution and separation. Therefore, fast columns are designed to decrease the time of the chromatographic analysis without forsaking significant deviations in results. These columns have the same internal diameter but much shorter length than most other columns, and they are packed with smaller particles that are typically 3 um in diameter.

Preparatory columns

These columns are utilized when the objective is to prepare bulk (milligrams) of samples for laboratory preparatory applications. A preparatory column usually has a large column diameter which is designed to facilitate large volume injections into the HPLC system. Accessories important to mention are the back-pressure regulator and the fraction collector. The back-pressure regulator is placed immediately posterior to the HPLC detector. It is designed to apply constant pressure to the detector outlet which prevents the formation of air bubbles within the system. This, in turn, improves chromatographic baseline stability. It is usually devised to operate regardless of flow rate, mobile phase, or viscosity. The fraction collector is an automated device that collects uniform increments of the HPLC output.

DETECTOR

The HPLC detector, situated toward the end of the column distinguishes the analytes as they elute from the chromatographic column. Regularly utilized detectors are UV spectroscopy, fluorescence, mass spectrometric, and electrochemical identifiers.

1. Molecular spectroscopic techniques.

UV detectors

Works by measuring the change in UV absorption by the sample. The sample under analysis should have absorbance in the region of ultraviolet radiation. In UV transparent solvent, these

detectors are concentration sensitive. Direct detection has a flaw as not all inorganic ions have appropriate chromophores but this can be compensated by using the method of derivatization. This is done by mixing the effluent with a chromogenic reagent in a post-column reactor. It is shown in figure 5.

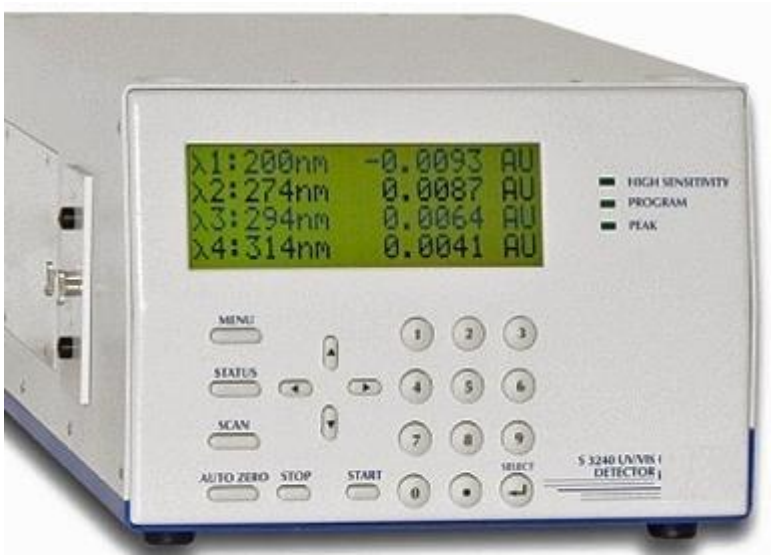

FIG. NO. 5: UV VIS DETECTOR

Refractive index detectors

The refractive index of a medium is the ratio of the speed of light in a vacuum to the speed of light in the medium. These detectors measure the change in refractive indices in the eluent as the solute passes through the sample cell. This method of detection is less sensitive than UV detection, although nonchromatographic compounds can be measured directly without derivatization.

Fluorometric detection

The solute is excited by UV radiation at a particular wavelength and the emission wavelength is detected. Used with naturally fluorescent compounds but compounds can be reacted to produce fluorescent derivatives.

2. Atomic spectroscopic techniques

Atomic spectroscopy includes atomic absorption spectroscopy as well as atomic emission spectroscopy. The spectroscopic determination of atomic species can only be performed in the gaseous medium in which the individual atoms are well separated from one another.

Thus, the first step in the atomic spectroscopic technique is atomization, a process in which the sample is volatilized in such a manner so as to produce an atomic gas.

DATA COLLECTION DEVICES

Data collection devices are also called integrators. Signals from the detector might be gathered on graph recorders or electronic integrators that fluctuate in many-sided quality and in their capacity to process, store and reprocess chromatographic information. The graph so produced is used to interpret the result of the experiment. The PC coordinates the reaction of the indicator to every part and places it into a chromatograph that is anything but difficult to interpret.

GENERAL METHOD OF DEVELOPMENT OF HPLC

Steps involved in the method development of HPLC are as follows:

1 Understanding the Physicochemical Properties of the drug molecule

2 Selection of chromatographic conditions

3 Developing the approach of analysis

4 Sample preparations

5 Method optimization

6 Method validation

UNDERSTANDING THE PHYSICOCHEMICAL PROPERTIES OF DRUG MOLECULE

The physicochemical properties of a drug molecule play an important role in method development. For Method development, one has to study the physical properties like solubility, polarity, pKa, and pH of the drug molecule. Polarity is a physical property of a compound. It helps an analyst, to decide the solvent and composition of the mobile phase. The solubility of molecules can be explained on the basis of the polarity of molecules. The analyte must be soluble in diluents and must not react with any of its components. pH and pKa play an important role in HPLC method development. [6]

SELECTION OF CHROMATOGRAPHIC CONDITION

Selection of column

Selection of the stationary phase/column is the first and the most important step in method development. To avoid problems from irreproducible sample retention during method development, it is important that columns be stable and reproducible. A C8 or C18 column made from specially purified, less acidic silica and designed specifically for the separation of basic compounds is generally suitable for all samples and is strongly recommended. The use of silica-based packing is favored in most of the present HPLC columns due to several physical characteristics.[8]

Buffer selection

Choice of the buffer is governed by the desired pH. The typical pH range for reversed-phase on silica-based packing is pH 2.0 to 8.0. It is important that the buffer has a pKa close to the desired pH since the buffer controls pH best at their pKa. A rule is to choose a buffer with a pKa value <2 units of the desired mobile phase pH.

Buffer Concentration

Generally, a buffer concentration of 10-50 mM is adequate for small molecules no more than 50% organic should be used with a buffer. This will depend on the specific buffer as well as its concentration. Phosphoric acid and its sodium or potassium salts are the most common buffer systems for reversed-phase HPLC. Sulfonate buffers can replace phosphonate buffers when analyzing organophosphate compounds.[9]

Isocratic and Gradient Separations

Isocratic mode of separation includes constant eluent composition; which means equilibrium conditions in the column and the actual velocity of compounds moving through the column are constant. As mentioned earlier isocratic elution is normally successful in the partition of sample components that are not altogether different in their proclivity for the stationary stage and in gradient elution the organization of the mobile phase is fluctuated ordinarily from low to high eluting quality.

Internal Diameter

The range of internal diameter of columns in standard columns for reversed-phase and normal phase is from 3.9 to 4.6 and length should be 15cm, 25cm and25cm. It is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution[10]. It also determines the quantity of analyte that can be loaded into a column.

Particle size and pore size

The smaller particles usually provide more surface area and better separations but the pressure required for the optimum linear velocity increases by the inverse of the particle diameter squared. Larger particles are used in preparative HPLC. The pore size of the column defines the ability of the analyte molecules to penetrate inside the particle and interact with its inner surface.

Selection of Mobile Phase

The mobile phase affects resolution, selectivity, and efficiency. Mobile phase composition (or solvent strength) plays an important role in RP-HPLC separation. A mixture of acetonitrile and water is the best initial choice for the mobile phase during method development.

TABLE NO. 1: SELECTION OF MOBILE PHASE

MODE	SOLVENT TYPE	TYPE OF COMPOUND
Reversed-phase	Water/buffer, ACN, methanol	The neutral or non-ionized compound can be dissolved in water or organic solvent
Ion pair	Water/buffer, ACN, methanol	Ionic or ionizable compound
Normal phase	Organic solvent	A mixture of isomers and compounds not suitable inorganic or water mixtures
Ion exchange	Water/buffer	Inorganic ions, proteins, nucleic acid, organic acids.
Size exclusion	Water, tetrahydrofuran, chloroform	High molecular weight compound.

Selection of detectors

The detector is a very important part of HPLC. Selection of detector depends on the chemical nature of analyses, potential interference, the limit of detection required, availability, and/or cost of detector. UV visible detector is a versatile, dual-wavelength absorbance detector for HPLC. Photodiode Array (PDA). The detector offers advanced optical detection for waters analytical HPLC, preparative HPLC, or LC/MS system solutions. Its integrated software and optics innovations deliver high chromatographic and spectral sensitivity. Refractive index chromatographic and spectral sensitivity, stability, and reproducibility make this detector the ideal solution for the analysis of components with limited or no UV absorption. Multi-wavelength Fluorescence Detector offers high sensitivity and selectivity fluorescence detection for quantitating low concentrations of target compounds.

DEVELOPING THE APPROACH FOR ANALYSIS

While developing the analytical method on RP-HPLC the first step which is followed is the selections of various chromatographic parameters like the selection of mobile phase, selection of column, selection of flow rate of mobile phase, selection of pH of the mobile phase. Detection wavelength is usually the isosbestic point in the case of simultaneous estimation of two components. After this, the linearity of the drug is studied to know the range of concentrations up to which the drug follows the linear pattern.[11]

SAMPLE PREPARATION

Sample preparation is an essential part of HPLC analysis, intended to provide a reproducible and homogenous solution that is suitable for injection onto the column.[12] The aim of sample preparation is a sample aliquot that, is relatively free of interferences, will not damage the column, and is compatible with the intended HPLC method that is, the sample solvent will dissolve in the mobile phase without affecting sample retention or resolution. Sample preparation begins at the point of collection, extends to sample injection onto the HPLC column.

METHOD OPTIMIZATION

Here the identification of the “weaknesses” of the method is done and the same is optimized through experimental design.[13] Here one has to understand the method performance with

different conditions, different instrument setups, and different samples. This enables better reproducibility of the obtained results.

METHOD VALIDATION

Validation is the confirmation by examination and the provision of objective evidence that the particular requirements for specific intended use are fulfilled.[5] A process of evaluating method performance and demonstrating that it meets a particular requirement. In essence, it knows what the method is capable of delivering, particularly at low concentrations.

PARAMETERS

Typical parameters recommended by FDA, USP, and ICH are as follow:

- ➢ Specificity
- ➢ Linearity & Range
- ➢ Precision
- ➢ Method precision (Repeatability)
- ➢ Intermediate precision (Reproducibility)
- ➢ Accuracy (Recovery)
- ➢ Solution stability
- ➢ Limit of Detection (LOD)
- ➢ Limit of Quantification (LOQ)
- ➢ Robustness
- ➢ System suitability

SPECIFICITY

The specificity of an analytical method is its ability to measure accurately an analyte in the presence of an interference sample matrix.[14] Specificity is the function of selectivity towards

selective samples. It is assured by the complete separation of the peak of analytes from other peaks originating from the sample matrix.

LINEARITY AND RANGE

It's the ability to obtain test results, which are directly proportional to the concentration of analyte in the sample. A linear relationship should be evaluated across the range of the analytical procedure. Linearity is usually expressed as the confidence limit around the slope of the regression line. For the establishment of linearity, a minimum of five concentrations are recommended by the ICH guideline.

PRECISION

Expresses the closeness of agreement (degree of scattering) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

ACCURACY

Expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

SOLUTION STABILITY

During validation, the stability of standards and samples is established under normal conditions, normal storage conditions, and sometimes in the instrument to determine if special storage conditions are necessary.[15]

LOD

LOD stands for a limit of detection. It's the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value.

LOQ

LOQ stands for a limit of quantification. Quantitation limit - the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy.

ROBUSTNESS

The measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters provides an indication of its reliability during normal usage.

SYSTEM SUITABILITY

Used to verify that the detection sensitivity, resolution, and reproducibility of the chromatographic system are adequate for the analysis.[7] Here a liquid sample is passed over an absorbent material to test its efficacy. It is required to be done before every sample analysis.

APPLICATIONS OF HPLC

PHARMACEUTICAL APPLICATION

The pharmaceutical applications include controlling drug stability, dissolution studies, and quality control. Although expected at first to be used as a complementary method to gas chromatography, the pharmaceutical industry now almost exclusively uses HPLC as a chromatographic technique. Other forms of HPLC that are used in the pharmaceutical industry include reversed-phase, denaturing, and immobilized enzyme reactor HPLC. However, one of the disadvantages of HPLC is that it must be preceded by calibration tests which can increase costs.[16]

ENVIRONMENTAL APPLICATION

Monitoring of pollutants and detecting components of drinking water. Coupling of HPLC to NMR was applied for the first time to the analysis of environmental samples, i.e., water samples from an ammunition hazardous waste site.[17] Using the continuous flow mode at very low flow rates (≤0.017 mL/min) and large volume injection (400 μL), the confirmation of many nitroaromatic compounds could be achieved down to the microgram-per-liter level after solid-phase extraction of a groundwater sample from a former ammunition production site[18]. The results obtained by HPLC−NMR are compared to those obtained by HPLC−PDA (photodiode array) of the same sample[19], demonstrating that many more compounds can be identified by the former compared to the latter method as a result of coelution of major and minor components in the HPLC chromatogram.[20]

FORENSIC APPLICATION

Analysis of textile dyes, quantification of drugs and steroids in biological samples, and several varieties of blue ballpoint pen inks were analyzed by high-performance liquid chromatography in different studies. Applications of HPLC to the analysis of cannabis, opium alkaloids, amphetamine-related materials, LSD, and polynuclear hydrocarbons were also estimated in other studies.[25]

FOOD AND FLAVOUR APPLICATION

Sugar analysis in fruit juices, detecting polycyclic compounds in vegetables, analysis of preservatives. Even though the food fortification campaign was working, a more precise recommendation is pursued to stop having such a huge variation as discovered in many studies.[21]

CLINICAL APPLICATION

Detecting endogenous neuropeptides, analysis of biological samples like blood and urine. A sensitive HPLC–APCI–MS method for the determination of vitamin K_1 (VK-1) in human plasma was established in a study.

PREPARATIVE HPLC

This differs from analytical HPLC, where the focus is to obtain information about the sample compound. The information that can be obtained includes identification, quantification, and resolution of a compound.

CHEMICAL SEPARATION

It can be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase. The method of high-performance liquid chromatography (HPLC) has been successfully used in the analysis of amino acids and vitamins added to the PN admixtures[22]. The need for tandem mass spectrometry was due to the UV detector's low selectivity and the lack of a suitable chromophore for some vitamins, such as pantothenic acid. In a study, they examined vitamins B1 and B6 by HPLC using a photodiode array detector.

PURIFICATION

It refers to the process of separating or extracting the target compound from other (possibly structurally related) compounds or contaminants. Depending on what needs to be separated and how closely related the samples are, the chromatographer may choose the conditions, such as the proper mobile phase. The migration of the compounds and contaminants through the column needs to differ enough so that the pure desired compound can be collected or extracted without incurring any other undesired compound[23].

IDENTIFICATION

Identification of compounds by HPLC is a crucial part of any HPLC assay. In order to identify any compound by HPLC, a detector must first be selected. Once the detector is selected and is set to optimal detection settings, a separation assay must be developed. It is accomplished by researching the literature and by trial and error. Following the ICH guidelines for analytical method validation[24]. A sample of a known compound must be utilized in order to assure identification of the unknown compound. Identification of compounds can be assured by combining two or more detection methods.

DISCUSSION

HPLC is an advanced technique of column liquid chromatography. It is a very popular technique and is mostly used analytically. Isocratic elution decreases the loading capacity whereas gradient elution does not. HPLC is normally classified based on the mode of chromatography, based on elution technique, based on the scale of operation, and based on the type of analysis. The instrumentation parts include pump, injector, column, detector, integrator, and display system. The different types of columns are guard columns derivatizing columns, capillary columns, fast columns, and preparatory columns. Columns are the main part of HPLC and the efficiency depends upon the type of column. Detectors used are UV detectors, refractive index detectors, etc among which UV detector is more compatible. With the use of HPLC extremely pure compounds can be produced. With the use of HPLC, the accuracy, precision, and specificity of the analytical method can be increased.

CONCLUSION

The HPLC has mostly used an analytical technique. It can be used in both laboratory and clinical science. Reverse phase elution is advantageous over the normal phase. Since isocratic elution decreases the loading capacity, gradient elution is more useful. C8 and C18 columns are generally used. UV detectors are widely used. The typical average pH of reversed-phase on silica-based packing is 5.0. Adequate buffer concentration is 10-50 mM. Different applications are in the field of pharmaceutical analysis, environment, forensic, food, and clinical. Other applications include preparation, chemical separation, purification, and identification. The only disadvantage of HPLC is the high cost.

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