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A REVIEW FOR METHOD DEVELOPMENT AND VALIDATION PARAMETERS BY USING HPLC TO DIFFERENT PHARMACEUTICAL DOSAGE FORMS

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1. INTRODUCTION

The techniques through which the chemical components present in complex mixtures are Separated, identified, and determined is termed chromatography. This technique is widely used like spectroscopy and is a very powerful tool not only for analytical methods but also for preparative methods. Compounds of high-grade purity can be obtained by this method. Chromatography can be simply as follows: “It is the technique in which the components of a mixture are separated based upon the rates at which they are carried or moved through a stationary phase (column) by a gaseous or liquid mobile phase”.

High-Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) was developed in the late 1960s and early 1970s. Today it is widely applied for separations and purificationsin a variety of areas including pharmaceuticals, biotechnology, environmental, polymer and food industries. HPLC has over the past decade become the method of choice for the analysis of a wide variety of compounds. Its main advantage over GC is that the analytes do not have to be volatile, so macromolecules are suitable for HPLC analysis. HPLC is accomplished by injection of a small amount of liquid sample into a moving stream of liquid (called the mobile phase) that passes through a column packed with particles of the stationary phase. The separation of a mixture into its components depends on different degrees of retention of each component in the column.

The extent to which a component is retained in the column is determined by its partitioning between the liquid mobile phase and the stationary phase. 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 your separation. Since the compounds have different mobilities, they exit the column at different times; i.e., they have different retention times, R High-Performance Liquid Chromatography (HPLC) High-Performance Liquid Chromatography (HPLC) was developed in the late 1960s and early 1970s.

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HPLC is accomplished by injection of a small amount of liquid sample into a moving stream of The retention time is the time between injection and detection. 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.[1] HPLC has gained its quality primarily because of its reliability (use of pressure-driven liquid support) and suppleness (possibility of adjusting the composition of every mobile and stationary phase).

The activity mode or separation mechanism depends on the inal interactive relationships between the stationary half, the mobile half, and additionally the analyte.

Particle-packed columns with either entirely porous- or the newly developed core-shell particles and monolithic columns are utilized in normal or miniaturized HPLC. Quantitative analysis is often accomplished with HPLC. An automatic injector providing reproducible injection volumes is extremely beneficial is standard on modern commercial systems.

HPLCs are rather simple. Good separation of a given pair of compounds by HPLC depends on the choice of column and the efficiency of the overall system. The relative position of the various components in the sample on the chromatogram is affected by a solute-solvent type of interaction with the column substrate competing with a solute-solvent interaction with the mobile phase. Column efficiency is concerned with the broadening of an initially compact band of solutes as it passes through the column. The broadening is a result of column design and column operating conditions.

For samples with a broad range of retention times, it is often desirable to employ solvent programming, whereby the mobile phase composition is varied continuously or in steps as the separation proceeds. The analysis of mixtures of widely varying compositions frequently leads to a very widespread retention time. HPLC is just one type of liquid chromatography, meaning the mobile phase is a liquid. Reversed-phase HPLC is the most common type of HPLC. What reversed-phase means is that the mobile phase is relatively polar, and the stationary phase is relatively non-polar.

Thus non-polar compounds will be more retained (i.e. have longer retention times) than a polar compounds. In normal phase HPLC, the mobile phase is relatively non-polar and the stationary phase is relatively polar.

Other more general types of HPLC include partition, adsorption, ion-exchange, size-exclusion, and thin-layer chromatography. The goal of the HPLC method is to try, separate, and quantify the main drug, any reaction impurities, all available synthetic intermediates, and any degradants. High-Performance Liquid Chromatography is now one of the most powerful tools in analytical chemistry.

It has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid. HPLC is the most accurate analytical method widely used for the quantitative as well as qualitative analysis of drug products and used for determining drug product stability.[2]

Principle of HPLC

HPLC is a separation technique that involves:

The injection of a small volume of liquid sample into a tube packed with tiny particles (3 to 5 micron (μm) in diameter called the stationary phase) where individual components of the sample are moved down the packed tube (column) with a liquid (mobile phase) forced through the column by high pressure delivered by a pump.

These components are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles.

These separated components are detected at the exit of this tube (column) by a low-through device (detector) that measures their amount. Output from this detector is called an “HPLC” In principle, LC and HPLC work the same way except for the speed, efficiency sensitivity, and ease of operation of HPLC are vastly superior.

Though HPLC retains major of the credits for the analytical side, the earlier one of simple Liquid Chromatography still applications for the preparative purposes Solvent reservoir Mobile phase contents are contained in a glass reservoir. The mobile phase, or solvent, in HPLC, is usually a mixture of polar and non-polar liquid components whose respective concentrations are varied depending on the composition of the sample.

The type and composition of the mobile phase affect the separation of the components. For HPLC we use high-grade solvent. Different solvents are used for different types of HPLC. For normal-phase HPLC, the solvent is usually non-polar, and, in reverse-phase HPLC, the solvent is normally a mixture of water and a polar organic solvent. The purity of solvents and inorganic salts used to make the mobile phase is paramount.[3]

The most common solvent reservoirs are as simple as glass bottles with tubing connecting them to the pump inlet.

Degassers may consist of

✓ Vacuum pumping system

✓ Distillation system

✓ Devices for heating and stirring

✓ System for sparging, in which the dissolved gases are swept out of solution by the bubbles of an inert gas that is not soluble in the mobile phase.

✓ Often the systems also contain a means of filtering dust and particulate matter from the solvents to prevent these particles from damaging the pumping or injection Systems or clogging the column.

Pump

A pump can be compared to the human heart which continuously pumps blood throughout the body but through the human heart can withstand changes in blood pressure within a limit due to stress and strain the HPLC pump is required to deliver a low of mobile phase at constant pressure and low rate.

Changes in both these parameters can lead to errors in the results. In simple language, the HPLC pump has to have ruggedness and at the same time should be able to provide reproducible low characteristics run after run.

The operational pressure limits have a vast range depending upon analysis requirements. In normal analytical operation the pressure can vary between 2000 – 5000 psi but in applications covered under UHPLC mode operating pressure can be as high as 15000 – 18000 psi.

All HPLC systems include at least one pump to force the mobile phase through whose packing is fairly compact. The result of this is a pressure increase at the injector which can attain 20 000 kPa (200 bars) depending upon the low rate imposed upon the mobile phase, its viscosity, and the size of the particles of the stationary phase. Pumps are designed in order to maintain a stable low rate, avoiding pulsations even when the composition of the mobile phase varies. These low rate metered pumps contain, in general, two pistons in series, working in opposition, to avoid interruptions to the low rate.[4]

Constant pressure pumps

This provides a consistent continuous low rate through the column with the use of pressure from a gas cylinder. Valving arrangement allows rapid re illof the solvent chamber. A low-pressure gas source is needed to generate high liquid pressures.

Syringe type pumps

It is suitable for small bore columns. The constant low rate is delivered to the column by a motorized screw arrangement. The solvent delivery rate is set by changing the voltage on the motor. These pumps deliver pulse less low independent of column back pressure and changes in viscosity but major disadvantages are limited solvent capacity and limitation on gradient operation.

Reciprocating piston pumps

These deliver solvent(s) through the reciprocating motion of a piston in a hydraulic chamber. On the backstroke, the solvent is sucked in and gets delivered to the column in the forward stroke. Flow rates can be set by adjusting piston displacement in each stroke. Dual and triple head pistons consist of identical piston chamber units which operate at 1800 or 1200 phase differences. The solvent delivery of reciprocating pump systems is smooth because while one pump is in the billing cycle the other is in the delivery cycle. High-pressure output is possible at a constant low rate and gradient operation is possible. However, pulse dampening is required for further elimination of pressure pulses.

Sample injector

The injector can be a single injection or an automated injection system. An injector for an HPLC system should provide an injection of the liquid sample within the range of 0.1-100 mL of volume with high reproducibility and under high pressure (up to 4000 psi). The injector must also be able to withstand the high pressures of the liquid system. An autosampler is an automatic version for when the user has many samples to analyze or when the manual injection is not practical.

Injectors are used to provide constant volume injection of samples into the mobile phase stream. Inertness and reproducibility of injection are necessary to maintain a high level of accuracy.[5] In HPLC, the injection of a precise volume of sample onto the head of the column must be made as fast as possible in order to cause the minimum disturbance to the dynamic regime of the mobile phase whose low must be stable from column to detector. This is done by a special high-pressure valve, either manual or motorized, possessing several low paths, which is situated just prior to the column. This must be a component of precision able to resist pressures greater than 30 000 kPa. The valve functions in two positions

Manual injection

(Rheodyne/Valco injectors): The injection is done through a specially designed 6-port rotary injection valve. The sample is introduced at atmospheric pressure by a syringe into a constant volume loop. In the LOAD position, the loop is not in the path of the mobile phase. By rotating to the INJECT position the sample in the loop is moved by the mobile phase stream into the column. It is important to allow some samples to low into waste from the loop so as to ensure there are no air bubbles in the loop and the previously used sample is completely washed out to prevent memory effects.

Automatic injection

Automatic injection improves laboratory productivity and also eliminates personal errors. Present-day advanced HPLC systems are equipped with an auto-injector along with an auto-sampler. The software programs of the loop and delivery the sample to the column. The computer also controls the sequence of samples for injection from vials kept in numbered positions of the autosampler. It is important to adopt precautions to ensure consistency of results and also prolong the service life of the automated system.

Prime injector with solvents to be used but it should be ensured that solvent is compatible with the solvent used earlier. Needle wash between samples will prevent carry-over between injections. Before the start and at end of analysis ensure tubing is completely washed of buffers or previously used solvents. Do not forget to feed the vial number correctly on the autosampler rack and list out the Sequence correctly on the computer.[6]

Columns

Columns are usually made of polished stainless steel, are between 50 and 300 mm long, and have an internal diameter of between 2 and 5 mm. They are commonly filled with a stationary phase with a particle size of 3–10 µm. Columns with internal diameters of less than 2 mm are often referred to as microbore columns. Ideally, the temperature of the mobile phase and the column should

be kept constant during an analysis. Considered the "heart of the chromatograph" the column's stationary phase separates the sample components of interest using various physical and chemical parameters.

The small particles inside the column are what causes the high back pressure at normal low rates. The pump must push hard to move the mobile phase through the column and this resistance causes a high pressure within the chromatograph.[7]

It is a vital component and should be maintained properly as per supplier instructions for getting reproducibility separation eficiency run after run.

Guard columns

A guard column is introduced before the analytical column to increase the life of the analytical column by removing not only particulate matter and contaminants from the solvents but also sample components that bind irreversibly to the stationary phase. The guard column serves to saturate the mobile phase with the stationary phase so that losses of this solvent from the analytical column are minimized. The composition of the guard-column packing is similar to that of the analytical column; the particle size is usually larger. When the guard column has become contaminated, it is repacked or discarded and replaced with a new one.

Analytical columns

It is the heart of High-performance liquid chromatography. Liquid-chromatographic columns range in length from 10 to 30 cm. normally, the columns are straight, with added length, where needed, being gained by coupling two or more columns together. The inside diameter of liquid columns is often 4 to 10 mm; the most common particle size of packing is 5 or 10 m. The most common column currently in use is one that is 25 cm in length, 4.6 mm inside diameter, and packed with 5 mm particles. Columns of this type contain 40,000 to 60,000 plates/meter HPLC columns are mostly made up of smooth bore stainless steel. HPLC columns are sometimes made from heavy walled glass tubing and polymer tubing, such as polyether ether ketone.

Column temperature control

For some applications, close control of column temperature is not necessary and columns are operated at room temperature. Often, however, better, more reproducible chromatograms are obtained by maintaining constant column temperature.[8]

Detector

The HPLC detector, located at the end of the column detects the analytes as they elute from the chromatographic column. Commonly used detectors are evaporative light scattering, UV-spectroscopy, luorescence, mass-spectrometric and electrochemical detectors. The detector can see (detect) the individual molecules that come out (elute) from the column. A detector serves to measure the amount of those molecules so that the chemist can quantitatively analyze the sample components.

The detector provides an output to a recorder or computer that results in the liquid chromatogram (i.e., the graph of the detector response). A detector gives a speci ic response for the components separated by the column and also provides the required sensitivity. It has to be independent of any changes in mobile phase composition. Monitoring the mobile phase as it emerges from the column.[9]

Type of detector

Evaporative light scattering detectors (ELSDs)

ELSD is ideal for detecting analytes with no UV chromophore as they do not rely on the optical properties of a compound.

➢ High sensitivity provides superb responses for all compounds, down to low nanogram levels.

➢ Real-time control during an injection using pro grammable Dimension Software maintains maximum sensitivity throughout the run.[10]

➢ Real-time gas programming using Dimension Software eliminates solvent enhancement effects during gradient elution, for excellent quantification

➢ Low dispersion and high-speed data output rates are the perfect match for fast LC applications.

➢ Superb reproducibility below 2% gives reliable and accurate results

➢ Multivendor software control and data acquisition using Agilent Chem Station chromatography data system, and other vendors' interfaces, eliminates the need for an analog to digital converter.

➢ Rapid heating and cooling of the evaporator tube minimize equilibration time.

➢ Full DMSO transparency ensures that responses from early eluting compounds are not hidden.

➢ Fully integrated with all Agilent analytical and preparative LC systems for the complete chromatographic solution.

➢ Complementary to LC/MS.

Refractive index detectors

✓ The nearly universal but poor detection limit

✓ Passes visible light through 2 compartments, sample &reference.

✓ When the solvent composition is the same the light passed through the compartments the light beam that passes through is recorded as zero.

✓ When a solute is in the sample compartment, refractive index changes will shift the light beam from the detector.

✓ Limit of detection (LOD) 10 ng of solute.

U.V detectors

- Based on electronic transitions within molecules.
- A most common type of detector for LC

- The ixed wavelength, Hg lamp 254 nm (π = > π*
- The tunable wavelength is selectable for specific wavelengths, monochromators, or filters.[11]

Clasification of HPLC

A. Based on modes of chromatography

1. Normal–Phase chromatography

Normal–phase chromatography was one of the first kinds of HPLC that chemists developed. Also known as normal-phase HPLC (NP HPLC) this method separates analytes based on their affinity for a polar stationary surface such as silica; hence it is based on the analyte's ability to engage in polar interactions (such as hydrogen-bonding or dipole-dipole type of interactions) with the sorbent surface. NP-HPLC uses a non-polar, non aqueous mobile phase (e.g. Chloroform, Octane), and works effectively for separating analytes readily soluble in non-polar solvents. The analyte associates with and is retained by the polar stationary phase.[12]

2. Hydrophilic interaction chromatography

Hydro philic interaction chromatography (HILIC) can be described as a reversed reversed-phase chromatography performed using a polar stationary phase (for example, unmodiliedsilica, amino, or diol bonded phases). The mobile phase employed is highly organic in nature (> 70% solvent, typically acetonitrile) containing also a small percentage of aqueous solvent/buffer or another polar solvent. The water/polar solvent forms an aqueous-rich sub-layer adsorbed to the polar surface of the stationary phase into which analytes partition. The retention mechanisms in HILIC are complex but are believed to be a combination of hydrophilic partitioning interaction and secondary electrostatic and hydrogen-bonding interactions. These mechanisms result in an elution order that is roughly the opposite of that in the reversed-phase. Although the organic modifier/aqueousratio is the predominant factor in providing the necessary separation selectivity in HILIC, the choice of stationary phase is also important in matching the column chemistry to the analyte functional groups.

3. Reversed-Phase Chromatography (RPC)

Reversed phase HPLC (RP-HPLC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been surface-modi ied with RMe2 SiCl, where R is a straight-chain alkyl group such as C18 H37 or C8 H17. With such stationary phases, retention time is longer for molecules that are less polar, while polar molecules elute more readily (early in the analysis).

An investigator can increase retention times by adding more water to the mobile phase; thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase. Similarly, an investigator can decrease retention time by adding a more organic solvent to the eluent.[13]

B. Based on the principle of separation Ion exchange chromatography

1.In ion-exchange chromatography

(IC), retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Solute ions of the same charge as the charged sites on the column are excluded from binding, while solute ions of the opposite charge of the charged sites of the column are retained on the column. Solute ions that are retained on the column can be eluted from the column by changing the solvent conditions (e.g. increasing the ion effect of the solvent system by increasing the salt concentration of the solution, increasing the column temperature, changing the pH of the solvent, etc...).

In general, ion exchangers favor the binding of ions of higher charge and smaller radii. An increase in counter ion (concerning the functional groups in resins) concentration reduces the retention time. A decrease in pH reduces the retention time in cation exchange while an increase in pH reduces the retention time in anion exchange. By lowering the pH of the solvent in a cation exchange column, for instance, more hydrogen ions are available to compete for positions on the anionic stationary phase, thereby eluting weakly bound cations.[14]

This form of chromatography is widely used in the following applications: water puri ication, preconcentration of trace components, ligand-exchange chromatography, ion-exchange chromatography of proteins, high-pH anion-exchange chromatography of carbohydrates, and oligosaccharides, and others.

The technique is well suited for

✓ The separation of inorganic and organic anions and cations is an aqueous solution.

✓ Ionic dyes, amino acids, and proteins can be separated by ion exchange because such compounds are salt in brine water,

2. Size Exclusion Chromatography (SEC)

It is also useful for determining the tertiary structure and quaternary structure of puri ied proteins. SEC is used primarily for the analysis of large molecules such as proteins or polymers. SEC works by trapping these smaller molecules in the pores of a particle. The larger molecules simply pass by the pores as they are too large to enter the pores. Larger molecules, therefore, low through the column quicker than smaller molecules, that is, the smaller the molecule, the longer the retention time.

This technique is widely used for the molecular weight determination of polysaccharides. In SEC, there is no interaction between the sample compounds and the column packing material. Instead, molecules diffuse into the pores of a porous medium. Depending on their size relative to the pore size, molecules are separated. Molecules larger than the pore opening do not diffuse

into the particles, while molecules smaller than the pore opening enter the particle and are separated. Large molecules elute Smaller molecules elute later.[15]

It is classified into two categories based on the nature of the columns and their packing.

Gel Filtration Chromatography
Which uses hydrophilic packing to separate polar species and uses mostly aqueous mobile phases. This technique is mostly used to identify the molecular weights of large sized proteins & bio-molecules.

Gel Permeation Chromatography
Which uses hydrophobic packing to separate non-polar species and uses non-polar organic solvents. This technique is used to identify the molecular weights of polymers.

3. Affinity chromatography
Afiinity chromatography involves covalently bonding a reagent called an af inity ligand, to a solid support. Typical afinity ligands are antibodies, enzyme inhibitors, cofactor/ coenzyme, or other molecules that reversibly and selectively bind to analyte molecules in the sample. The principle is that the stationary phase consists of a support medium (e.g. cellulose beads) on which the substrate (or sometimes a coenzyme) has been bound covalently, in such a way that the reactive groups that are essential for enzyme binding are exposed. As the mixture of proteins is passed through the chromatography column, those proteins that have a binding site for the immobilized substrate will bind to the stationary phase, while all otter proteins will be eluted in the void volume of the column.

The factors which influence the HPLC performance
Internal diameter
The internal diameter
(ID) of an HPLC column is a critical aspect that determines the quantity of analyte that can be loaded onto the column and also influences sensitivity. Larger columns are usually seen in industrial applications such as the purification of a drug product for later use. Low ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity.

Particle size
Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). Smaller particles generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter squared.[16]

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

Pump pressure
Pumps vary in pressure capacity, but their performance is measured by their ability to yield a consistent and reproducible low rate. 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.

Temperature
For proper function of the HPLC the temperature has its own in luence. Mostly HPLC columns can work at room temperature or around (25-35 ◦c) are good. But there is also an unexceptional case that requires a higher temperature.

Parameter used in HPLC
For the accurate analysis of a compound, there are some parameters that are used as a standard for a particular compound. If there is a change occurs in the parameters the result may be affected greatly. The most commonly used parameters are internal diameter, particle size, pore size, and pump pressure. For different compounds, the parameters can be changed according to their nature and chemical properties.
1. Retention time: Retention time is the difference in time between the point of injection and the appearance of peak maxima. It is also defined as the time required for 50% of a component to be eluted from a column. It is measured in minutes and seconds.
2. Retention volume: Retention volume is the volume of carrier gas required to elute 50% of the component from the column. It is the product of retention time and low rate.

Advantage and disadvantages of HPLC
Advantages
✓ Separations are fast and efficient (high-resolution power)
✓ It can be applied to the separation and analysis of very complex mixtures
✓ Accurate quantitative measurements.
✓ Repetitive and reproducible analysis using the same column.
✓ Adsorption, partition, ion exchange, and exclusion column separations are excellently made.
✓ HPLC is more versatile than GLC in some respects because it has the advantage of not being restricted to volatile and thermally stable solute and the choice of mobile and stationary phases is much wider in HPLC.
✓ Aqueous and non-aqueous samples can be analyzed with little or no sample pre-treatment. ✓ A variety of solvents and column packing are available, providing a high degree of selectivity for specific analyses.

✓ It provides a means for the determination of multiple components in a single analysis and etc.

Disadvantages

✓ Column performance is very sensitive, which depends on the method of Packing.

✓ Further, no universal and sensitive detection system is available.

✓ Very costive, have low sensitivity for certain compounds, and some cannot be detected as they are irreversibly adsorbed.

Applications of High-Performance Liquid Chromato graphy (HPLC)

The information that can be obtained using HPLC includes

Identification, quantification, and resolution of a compound. Preparative HPLC refers to the process of isolation and puri ication of compounds. This differs from analytical HPLC, where the focus is to obtain information about the sample compound. The major applications are the following.

Pharmaceuticals

High-Performance Liquid Chromatography provides reliable quantitative precision and accuracy along with a high linear dynamic range to allow the determination of API and related substances in a single run. A convenient method for sample preparation for solid dosage forms is dispersion in water or aqueous media modi ied with acetonitrile or methanol. HPLC offers several possibilities for the separation of chiral molecules into their respective enantiomers. These include precolumn derivatization to form diastereomers.

Manufacturing

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

According to the European pharmacopeia, HPLC is used in only 15.5% of syntheses. However, it plays a role in 44% of syntheses in the United States pharmacopeia. This could be due to differences in monetary and time constraints, as HPLC on a large scale can be an expensive technique. An increase in specificity, precision, and accuracy that occurs with HPLC, unfortunately, corresponds to an increase in cost.

Research

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 the results of synthesis reactions, as purity is essential in this type of research. 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. In one example of this, competitive protein binding assays (CPBA) and HPLC were compared for sensitivity in the detection of vitamin D. 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. While an expensive tool, the accuracy of HPLC is nearly unparalleled.

High-Performance Liquid Chromatography (HPLC) analysis

HPLC is optimum for the separation of chemical and biological compounds that are non-volatile. Typical non volatile compounds are:

✓ Pharmaceuticals like acetaminophen (Tylenol) aspirin, ibuprofen,

✓ Salts like sodium chloride and potassium phosphate

✓ Proteins like egg white or blood protein

✓ Organic chemicals like polymers (e.g. polystyrene, polyethylene)

✓ Heavy hydrocarbons like asphalt or motor oil

✓ Many natural products such as ginseng, herbal medicines, plant extracts

✓ Thermally unstable compounds such as trinitrotoluene (TNT), enzymes.

2. METHOD DEVELOPMENT AND VALIDATION PARAMETERS OF HPLC

METHOD DEVELOPMENT AND VALIDATION PARAMETERS BY USING HPLC FOR TABLET

A simple, specific, accurate and precise reverse phase high performance liquid chromatographic method is developed and validated for the estimation of telmisartan in tablet dosage form. The expected separation and peak shapes were obtained on chromosil C18 (250 mm x 4.6 mm, 5 µm) column. To have an ideal separation of the drug under isocratic conditions, mixtures of solvents like methanol, water and acetonitrile with or without different buffers in different combinations were tested as mobile phases on a chromosil C18 column. A mixture of methanol : 0.1% orthophosphoric acid : acetonitrile in the ratio of 80:05:15 v/v/v was proved to be the most suitable of all the combinations since the chromatographic peak obtained was better defined and resolved and was almost free from tailing. The flow rate was 1.5 ml/min and effluents were monitored at 256 nm. The retention time for telmisartan was 2.7 min. The method was validated and found to be accurate, and

precise. Recovery of telmisartan from tablet formulation was found to be 99.41%. The proposed method was successfully applied for the quantitative determination of telmisartan in tablet formulation.

METHOD DEVELOPMENT AND VALIDATION PARAMETERS BY USING HPLC FOR CAPSULE

It describes a precise, accurate and reproducible Reverse phase High Performance Liquid Chromatographic (RP-HPLC) method for simultaneous estimation of Amlodipine besylate (AMLB) and Valsartan (VAT) on RP C-18 Column (Kromasil, 250 x 4.6 mm) using acetonitrile: phosphate buffer (0.02M, pH 3.0), (56:44 v/v) as mobile phase at a flow rate of 1.0 ml/min and the detection wavelength was 234 nm. The retention time for AMLB and VAT was found to be 3.07 and 6.20 min, respectively. The method was also applied for the determination of AMLB and VAT in the presence of their degradation products formed under variety of stress conditions. Proposed method was validated for precision, accuracy, linearity range, robustness and ruggedness.

METHOD DEVELOPMENT AND VALIDATION PARAMETERS BY USING HPLC FOR SUSPENSION

The aim of the present study was to develop and validate an analytical method for the estimation of nepafenac as a raw material as well as in dosage form (suspension) by using reverse phase high performance liquid chromatographic (RP-HPLC). The target was to obtain an easy, rapid, reproducible as well as a rugged method. The HPLC system that was used in the proposed study was LC-20AD liquid chromatograph equipped with SPD-20A UV-VIS detector. The separation was performed on C18 column which was attached with loop 20 β l. Elution was done at ambient temperature with a mobile phase consisting of acetonitrile: Water (40: 60v/v) at a flow rate of 1ml/min and at a wavelength of 254 nm. The proposed method was validated as per the ICH guidelines. The retention time for nepafenac was 7.49 minutes (% CV=0.0076). The percentage coefficient variation (CV) of six consecutive peak areas of injections was 0.34% with tailing factor 1.76. The peak area responses were linear within the concentration range of 0.078-20.0 βg/ml (R(2)=0.9993). The sensitivity of the method could be evaluated by limits of detection (LOD) (0.0195 β g/ml) and limits of quantitation (LOQ) (0.039 β g/ml). Nepafenac drug is s in its diluent that could see by intra-day (% CV =0.45-1.96) and inter-day variation (%CV=0.173-1.898%). The accuracy and recovery results of 80%, 100% and 120% were 97.40% to 102.10% with % CV of 0.3201% to 1.3496%. The robustness and ruggedness of the method are significantly broader and is reproducible. It could be used as a more convenient, efficient, easy and time saving method for the analysis of drug in raw material as well as in dosage form (ophthalmic suspension).

METHOD DEVELOPMENT AND VALIDATION PARAMETERS BY USING HPLC FOR SYRUP

A reversed phase HPLC method for rapid and simultaneous identification and quantification of doxylamine succinate, ephedrine sulfate, dextrometorphane hydrobromide, paracetamole and sodium benzoate in cough-cold syrup formulation was described. Separation was carried out on XTerraTM RP 18, Waters (150 mm x 4.6 mm column, 5 µm particle size). For the analysis of investigated substances gradient elution was used employing water, pH adjusted at 2.5 with 85 % ortophosphoric acid as the mobile phase A and acetonitrile as the mobile phase B. Detection was carried out by UV absorbance at 210 nm for doxylamine succinate, ephedrine sulfate, dextromethorphane hydrobromide and sodium benzoate and at 270 nm for paracetamole. The method was validated statistically for selectivity, linearity, precision, accuracy.

METHOD DEVELOPMENT AND VALIDATION PARAMETERS BY USING HPLC FOR CREAM

A rapid, sensitive and specifi c reversed-phase high performance liquid chromatographic method with diode array detection has been developed and validated for the determination of hydroxybenzene (0.494%, w/w) in a commercially available cream pharmaceutical formulation. Isocratic chromatography was performed on a C18 column with methanol-water 60:40 (v/v) containing 0.1% phosphoric acid (v/v) as mobile phase at a fl ow rate of 1.0 ml/min. UV detection was at 254 nm. Linearity of the method was excellent (r2 = 0.9999). The relative standard deviation values for intra- and inter-day precision studies were < 1% and the recovery of hydroxybenzene was >99%. The limit of detection and quantitation for hydroxybenzene was found to be 13.5 η g/ml and 2 µg/ml, respectively. The method was also validated for specifi city and robustness. The method was found to be robust and can be reliably used to determine the hydroxybenzene content of marketed formulations.

METHOD DEVELOPMENT AND VALIDATION PARAMETERS BY USING HPLC FOR INJECTION

Enoxaparin sodium is an anticoagulant medication that is used as a blood thinning agent. It is mostly used for the treatment and prevention of deep vein thrombosis (DVT) and pulmonary embolism (PE). It is also used in certain surgeries and during pregnancy. For the treatment of acute coronary syndrome (ACS) and heart attacks, it may be used. Enoxaparin sodium was validated by the RP-HPLC method. A simple RP-HPLC method was developed in a single HPLC run in a dry powder injection formulation. All injections of HPLC sample were 20 µL volume. The chromatographic separation was completed in the isocratic mode. The used column was USP-L8 (250 mm × 4.6 mm) of BDS type of 10 µm meters in the same mobile phase throughout the analysis by using methanol and ultrapure water with a ratio of 7:93, respectively. The flow rate was 1.0 mL/min. The mobile phase was filtered through 0.45 µm filter paper,

and isocratic elution was performed. The refractive index (RI) detector was used to analyze this sample. The specific peak of enoxaparin sodium was observed at 5:56 min. The calculated detection limit (LOD) was 0.351 ppm, and the calculated quantitation limit was 1.063 ppm. In repeatability of precision, the average calculated assay (%) was 100.85%, and the calculated RSD (%) was 0.01. In the accuracy test, the RSD (%) was 0.50, and the mean recovery (%) was 100.35. The system's suitability was within the limit. This newly developed method is proposed according to ICH guidelines, and rules and can be applied effectively for the exact estimation of enoxaparin sodium in injection formulation. This newly developed methodology is affordable in cost as long as less time is taken and the consumption of samples is in smaller quantities for every investigation. In medicinal chemistry, the USP (United States Pharmacopeia) and BP (British Pharmacopeia) are directly involved in production as well as in quality testing.

METHOD DEVELOPMENT AND VALIDATION PARAMETERS BY USING HPLC FOR LOZENGES

A simple, sensitive, precise and accurate stability-indicating HPLC method has been developed and validated for determination of Clotrimazole (CLOT) in its lozenges dosage form. The mobile phase consists of 0.1% Tri-ethylamine in water (pH 3.00±0.05 adjusted by using Ortho-phosphoric acid) and Methanol in ratio of (25:75 v/v %) with isocratic programming, Grace Mart C18 (250mm×4.6mm, 5µ) column used as stationary phase with a flow rate of 1.0 mL/minute. The detection wavelength was set at 215 nm. The retention time of clotrimazole was found to be 5.5 ± 0.008 min. Clotrimazole was subjected to different stress testing conditions. The degradation products were well resolved from the drug under the tested conditions. The method was linear (r = 0.9998) at a concentration range of 0.5 to 60 µg/mL. Precision study showed that the percentage relative standard deviation was within acceptable limits, and the mean recovery was found to be 100.75% ± 1.51 for assay of Clotrimazole in lozenges dosage form. The results demonstrated that the method would have a great value when applied in quality control and stability studies for Clotrimazole.

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METHOD DEVELOPMENT AND VALIDATION PARAMETERS BY USING HPLC FOR POWDER

A reversed phase high performance liquid chromatography (RP-HPLC) method for the simultaneous quantification of bedaquiline (TMC207), moxifloxacin and pyrazinamide in a pharmaceutical powder formulation for inhalation has been developed and validated. The powder was simply dissolved in methanol and the analytes separated in a run time of 20 min on a Luna C18 (2) (150 × 4.6 mm, 5 µm) column using gradient elution with methanol and triethylamine phosphate buffer (pH 2.5) delivered at 1.2 mL/min. The detection (with retention time) was carried out at 269 nm (2.9 min) for pyrazinamide, 296 nm (7.0 min) for moxifloxacin and 225 nm (16.3 min) for bedaquiline, respectively. The method was linear for all analytes in the concentration range 1-100 µg/mL with correlation coefficients >0.998. Lower limits of quantitation (µg/mL) of bedaquiline, moxifloxacin and pyrazinamide were 0.56, 0.43 and 0.24, respectively. The method was accurate (relative error in the range −0.2 to 2.2) and precise (%RSD ≤6.2) with recovery in the range 100.0–104.7%. The method was successfully applied to determine the drug content and content uniformity of the three analytes in a spray-dried combination powder formulation for inhalation containing L-leucine.

3. CONCLUSION

High – performance liquid chromatography is one of the most widely used as analytical techniques in pharmaceutical analysis due to its high accuracy, precision, sensitivity, and reliability. Its play a vital role in the method development and validation of various pharmaceutical dosage forms such as tablets, capsules, powder, suspension, injection, syrup, lozenges, creams.

The method development in HPLC involves the selection of suitable mobile phase, column type, flow rate, detection, wave length, and sample preparation techniques to obtain accurate and reproducible results. Proper optimization of these parameters ensures effective separation aand quantification of pharmaceutical compounds.

The method validation is essential to confirm that the developed analytical method is suitable for its intended purpose. Validation parameters such as accuracy, precision, linearity, limit of detection, limit of quantification, robustness and suitability are evaluated according to the guidelines.

Over all HPLC- based analytical methods provide reliable and reproducible results for the quality control and stability studies of pharmaceutical dosage forms .proper development and validation of these methods ensure the safety, efficacy, and quality of pharmaceutical products.

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