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A Comprehensive Review of HPLC: Techniques, Applications, and Advancements

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ABSTRACT
High-performance Liquid Chromatography, HPLC for short, is an essential tool that is used in all levels of drug discovery, development and production. Together with other technologies, HPLC remains one of the most distinguished tools of analytical chemistry today. The goal of this article is to describe the progress made with the development of HPLC methods for the routine analysis of formoterol fumarate and mometasone furoate in metered dose inhalers. O-Linked oligosaccharides microsomes including neutral sialylated and sulfated oligonucleotides were estimated. This method achieved the quantitative characterization of isomeric products for N-acetylglucosamine-6Osulphotransferases-catalyzed in vitro reactions as well as the O-glycosylation profiling of serum IgA as a model glycoprotein. Validated HPLC-UV methods for lipoic acid measurement in human plasma can now use this method as an approach for HPLC. The first simple and sensitive procedure for the determination of clobetasol in rat plasma and its application to skin penetration was developed recently. The cleavage of the outlined isomers is performed primarily by means of chromatographic and electrophoretic processes. This process provides the means for obtaining biologically and pharmaceutically significant nonproteinogenic amino acid enantiomers and compounds related to them.

I. INTRODUCTION

The modern pharmaceutical industry, high-performance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development, and production[1]. High Performance Liquid Chromatography (HPLC) was derived from the classical column chromatography and, is one of the most important tools of analytical chemistry today[2]. HPLC is the method of choice for checking peak purity of new chemical entities, monitoring reaction changes is in synthetic procedures or scale up, evaluating new formulations and carrying out quality control / assurance of the final drug products[3].HPLC is a modern form of LC that uses small-particle columns through which the mobile phase is pumped at high pressure[4].HPLC is an analysis Method that yields high performances and high speed compared with traditional column Chromatography because of the forcibly pumped mobile phase [5].

The name 'high performance liquid chromatography originates from the late 1960s, when technology was developed to make both stationary phase column packings of very small particle size and sophisticated instruments which could generate the high pressures required for the flow of mobile phase through these packings."High performance' was used to distinguish the new technique from previous liquid chromatographic procedures involving glass columns packed with larger particle size material [6].

. 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, tR. 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[7].

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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 [8].

Principal

High-Performance Liquid Chromatography operates through the interaction of analytes between a liquid mobile phase and a solid stationary phase under high pressure [9]. The separation mechanism relies on various molecular interactions, including hydrophobic interactions in reverse-phase chromatography, polar interactions in normal-phase chromatography, and ionic interactions in ion-exchange chromatography [10]. The efficiency of separation is governed by theoretical plates, resolution factors, and capacity factors, which are influenced by operational parameters such as mobile phase composition, flow rate, and column characteristics [11].

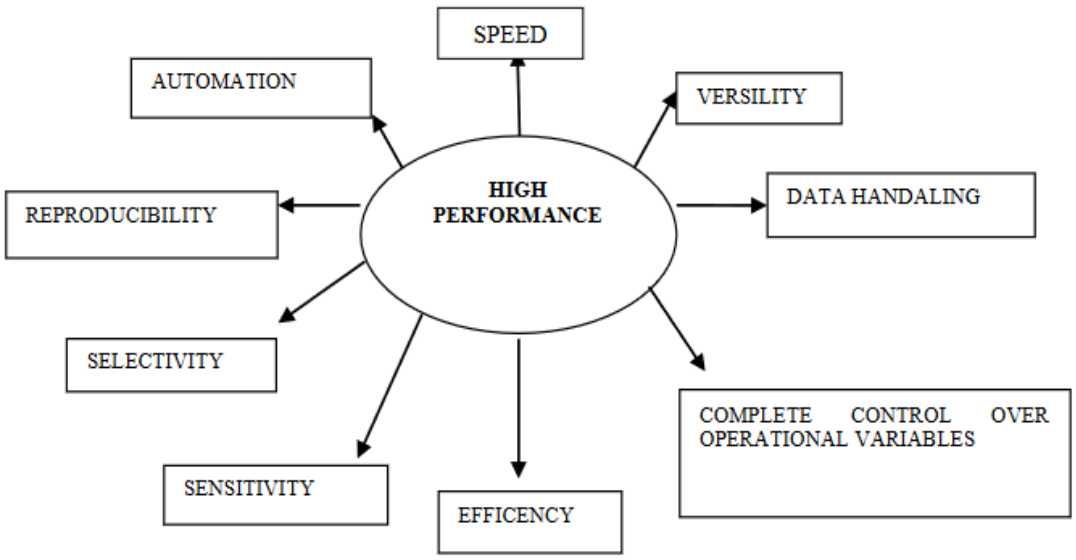

Fig 1: High Performance liquid chromatography (HPLC).

Operation

The sample to be analyzed is injected in a small volume into the stream of the mobile phase.

The motion of analyte through the column is slowed by specific chemical or physical interactions with the stationary phases as it traverses the length of the column. The amount he analyte is slowed depends on the nature of the analyte and on the compositions of the stationary and mobile phases. Time taken by a specific analyte to elute is called retention time; the retention time under particular conditions is considered a reasonably unique identifying characteristic of a given analyte. Smaller particle size column packing (which creates a higher back-pressure) increases the linear velocity giving the components less time to diffuse ithin the column, which leads to improved resolution in the resulting chromatogram. Commonly used solvents include any miscible combination of water or various organic liquids (most common being methanol and acetonitrile). Water may contain buffers or salts to help in separation of the analyte components or compounds such as trifluoroacetic acid which acts as an ion pairing agent. A further refinement to HPLC has been to change the mobile phase composition during the analysis. This is known as gradient elution. A general gradient for reversed phase chromatography might start at 5% methanol and progresses gradually to 50% methanol over 25 minutes; the gradient chosen depends on the hydrophobicity of the analyte. The analyte mixtures

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are separated as a function of the affinity of the analyte for the current mobile phase composition relative to the stationary phase. This process of portioning is similar to that which occurs during a liquid-liquid extraction but this is continuous and not step-wise. For example, when using a low water/ high methanol gradient, the more hydrophobic components will elute from the column due to a relatively hydrophobic mobile phase. The hydrophilic compounds will elute under conditions of relatively low methanol/high water. The choice of solvents, additives and gradient depend on the nature of the analyte and the stationary phase. Generally, a series of tests are performed on the analyte and a number of trial runs may be processed in order tofind the optimum HPLC method giving the best separation of peaks [12].

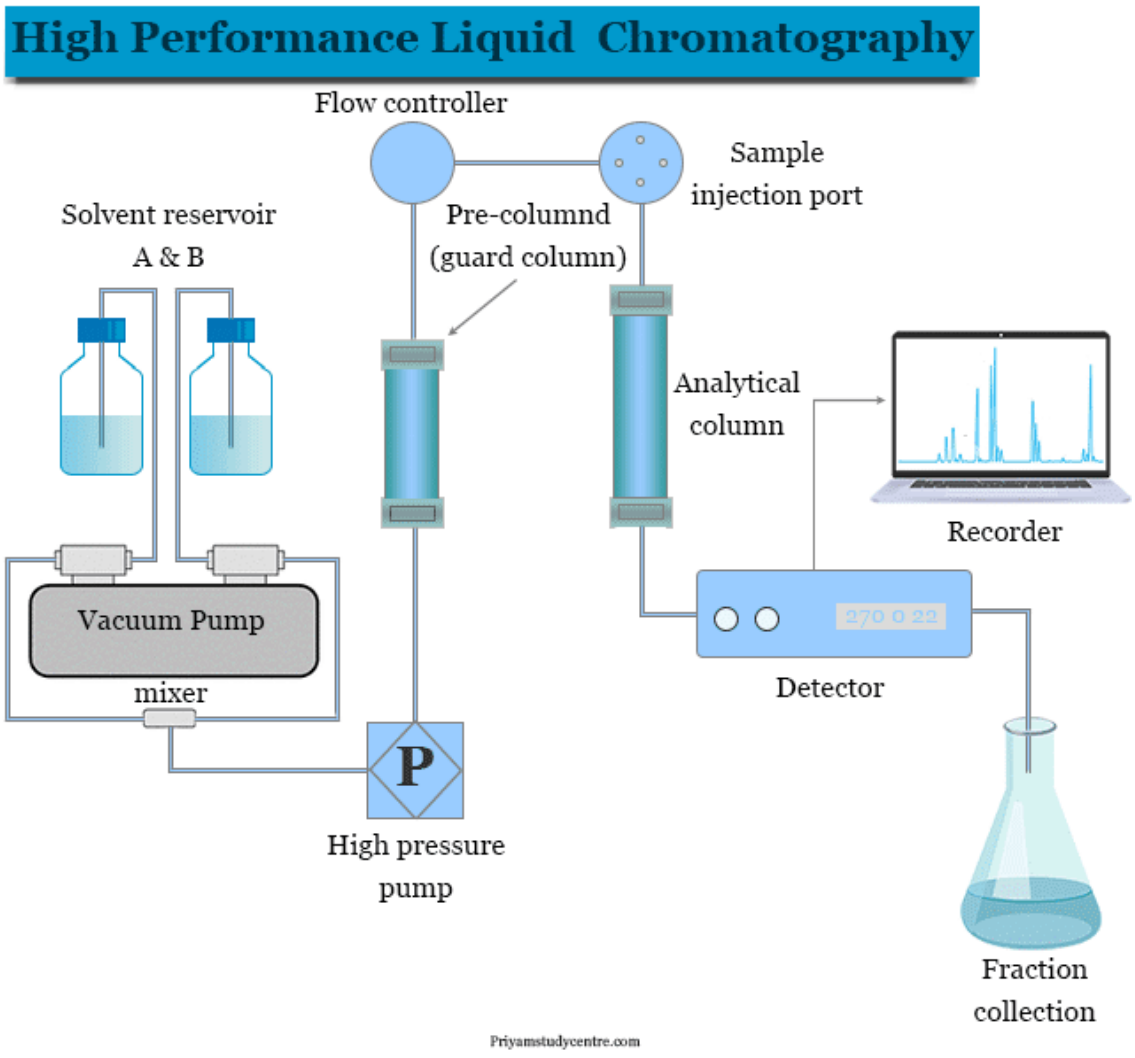

Fig 2: Overview Of HPLC

Mobile Phase:

Themobile phase in HPLC refers to the solvent being continuously applied to the column, or stationary phase. The mobile phase acts as a carrier for the sample solution. A sample solution is injected into the mobile phase of an assay through the injector port. As a sample solution flows through a column with the mobile phase, the components of that solution migrate according to the non-covalent interactions of the compound with the column [13].

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Mobile phase/solvent reservoir

The reservoir that holds the mobile phase is often no more than a glass bottle. Often, the reagent bottle that holds our HPLC solvent can be used as a reservoir. Solvent is delivered from the reservoir to the pump by means of Teflon tubing -- called the "inlet line" to the pump. Some HPLC systems like the Agilent 1100 shown at the right have special compartments to hold one or more mobile phase reservoirs. The reservoirs in these systems may have additional features that allow the mobile phase to be degassed and isolated from contact with air [14]. In HPLC the mobile phase or 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 [15].

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 specified limit due to stress and strain the HPLC pump is required to deliver a flow of mobile phase at constant pressure and flow 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 flow 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 flow 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 flow rate, avoiding pulsations even when the composition of the mobile phase varies. These flow rate metered pumps contain, in general, two pistons in series, working in opposition, to avoid interruptions to the flow rate[16].

A pump aspirates the mobile phase from the solvent reservoir and forces it through the system's column and detector. Depending on a number of factors including column dimensions, the particle size of the stationary phase, the flow rate and composition of the mobile phase, operating pressures of up to 42000 kPa (about 6000 psi) can be generated. The role of the pump is to force a liquid (called the mobile phase) through the liquid chromatography at a specific flow rate, expressed in milliliters per min (mL/min). Normal flow rates in HPLC are in the 1-to 2-mL/min range. Typical pumps can reach pressures in the range of 6000-9000 psi (400-to 600- bar). During the chromatographic experiment, a pump can deliver a constant mobile phase composition (isocratic) or an increasing mobile phase composition (gradient) variations in flow rates of the mobile phase affect the elution time of sample components and result in errors. Pumps provide a constant flow of mobile phase to the column under constant pressure[17].

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. 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 flow must be stable from column to detector. [18].

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 flow rates. The pump must push hard to

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move the mobile phase through the column and this resistance causes a high pressure within the chromatograph[19].
It is a vital component and should be maintained properly as per supplier instructions for getting reproducibility separation efficiency run after run. The types of columns are[20]:

- **Reverse-Phase Columns**: The most commonly used, these columns contain a non-polar stationary phase and separate compounds based on hydrophobic interactions.
- **Normal-Phase Columns**: These columns use a polar stationary phase and are used to separate polar compounds.
- **Ion-Exchange Columns**: These are used to separate charged molecules (ions) based on their charge.
- **Size-Exclusion Columns**: These columns separate molecules based on size, commonly used for proteins or polymers.

Detectors

A detector in HPLC is placed at the end of the system. Its work is to analyse the solution which is eluting from the column. The concentration of individual component of the analyte is proportional to the electronic signal coming out of the component of the mixture[21,22].

❖ **Electrical Conductivity HPLC Detectors[23]:**

Electrical Conductivity HPLC detectors are designed to detect all ions present, whether they come from the solute or the mobile phase. These detectors measure the conductivity of both the mobile phase and the solute, requiring appropriate electronic adjustments to account for interference from the mobile phase. The electronic resistance measured is directly proportional to the concentration of ions in the solution.

❖ **Refractive Index HPLC detectors[24,25]:**

Refractive Index Detectors (RID) are also considered bulk property detectors and function by detecting changes in the refractive index of the eluent from the column, in comparison to the pure mobile phase. Various types of refractive index detectors include the Christiansen effect detector, interferometer detector, thermal lens detector, and dielectric constant detector. These detectors are primarily used for detecting non-ionic compounds that do not fluoresce or absorb in the UV region. However, they have some limitations, including lower sensitivity, the need for temperature control, and reduced compatibility with gradient elution.

❖ **Electrochemical HPLC Detectors[26,27]:**

Electrochemical HPLC detectors are named for their ability to measure the current generated during the oxidation or reduction of solutes. These detectors function as either amperemeters or coulometers in HPLC. They are divided into equilibrium and dynamic detectors, with their suitability depending on the volumetric properties of the solute molecules in either aqueous or organic mobile phases. These detectors are sensitive to variations in the flow rate or composition of the eluent and require a working electrode, reference electrode, and auxiliary electrode for proper operation.

❖ **Light Scattering HPLC Detectors[28]:**

Light scattering HPLC detectors are useful for large molecular weight molecules like surfactants, lipids and sugar. It measures the scattered light coming out of the eluent. Low angle laser light scattering detector and the multiple angle laser light scattering detectors are the two types of Light scattering detectors available. They are also called as Evaporative light scattering detector because in this the beam of light by particles of compound remaining after evaporation of the mobile phase. The importance of such type of detector is growing with time because it acts as universal detector and does not require a compound to have a chromophore for detection. They can be used with gradient elution.

❖ **Ultraviolet/Visible Detectors[29]:**

Ultraviolet/Visible (UV/Vis) detectors are among the most commonly used HPLC detectors due to the fact that most compounds absorb in the UV or visible regions. They provide a specific response to either classes of compounds or individual compounds based on the functional groups of the eluting molecules. The principle behind optical detectors is the change in intensity when a beam of electromagnetic radiation passes through the detector flow cell. There are three main types of UV/Vis detectors: fixed wavelength detectors, variable wavelength detectors, and diode array detectors.

❖ **Diode Array Detectors[30]:**

In a diode array detector, the sample is exposed to light across all wavelengths generated by the lamp simultaneously. The emitted light is

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collimated by an achromatic lens system, passes through the detector cell, and then onto a holographic grating. From there, the light falls onto a diode array consisting of hundreds of diodes. The chromatogram is generated using the UV wavelength corresponding to the particular diode at the end of the analysis. DAD allows for the observation of the analyte's response at various wavelengths in a single run, thus saving both time and energy.

❖ **Fluorescence HPLC Detectors[31]:**

Fluorescent detectors are the most selective, sensitive and specific than all other HPLC detectors. Specific wavelength is used to excite and then emit light signal in analyte atoms. They intensity of light is monitored continuously to quantify the analyte concentration. Fluorescence is 10-1000 times sensitive than UV detector for strong UV absorbing compounds. Even a single analyte in the cell can be detected by the fluorescence detector. For some compounds which do not have fluorescence absorbance or low absorbance, they can be treated with fluorescence derivatives such as dansylchloride.

❖ **Data collection devices /Recorder[32]:**

Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and re-process chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret. Frequently called the data system, the computer not only controls all the modules of the HPLC instrument but it takes the signal from the detector and uses it to determine the time of elution (retention time) of the sample components (qualitative analysis) and the amount of sample (quantitative analysis)

Applications of High Performance Liquid Chromatography

○ Pharmaceuticals[33]

High-performance liquid chromatography (abbreviated as HPLC or simply LC) is a method of choice for assessment of a huge variety of samples in the pharmaceutical industries. It is used throughout the process of designing a new drug from assessing new formulations, scrutinizing purity of new chemical subsistence, auditing changes in synthetic procedures or extent up to bearing out quality control of the eventual drug product.

Common applications in pharmaceutical analysis are:

¬ To regulate drug stability.

¬ Tablet dissolution application of pharmaceutical dosages form.

¬ To control quality of Pharmaceuticals: HPLC is used to see whether the prepared and manufactured drugs or pharmaceutics are according to the standards laid down by the regulating bodies of drugs like pharmacopoeia and others. When particularly specified HPLC solvents and mobile phases are utilized it will give an idea of how the peak of the drug will look in the formulation to be determined.

¬ To determine the shelf life of pharmaceutical products.

¬ To recognize the specific constituent or molecules in the mixture and for bio availability investigations etc. After the preparation of the specific formulation, the discharge of the drug during a particular period of time is analyzed for bio availability investigations.

¬ To evaluate the concentration of plasma, metabolic profile etc of chemical moieties or formulation during development or preclinical trials. The analysis is done to examine their blood concentration after certain periods of administration.

○ Medical[34]

Clinical utilization of HPLC can incorporate medication investigation, yet falls all the more intently under the classification of supplement examination. While pee is the most widely recognized mode for dissecting drug fixations, blood serum is the example gathered for most clinicalinvestigations with HPLC. Other techniques for identification of particles that are valuable for clinical examinations have been tried against HPLC, to be specific immunoassays. In one illustration of this, cutthroat protein restricting examines (CPBA) and HPLC were looked at for responsiveness in discovery of vitamin D. Valuable for diagnosing lacks of vitamin D in youngsters, it was observed that responsiveness and particularity of this CPBA came to just 40% and 60%, separately, of the limit of HPLC. While a costly device, the precision of HPLC is almost unrivaled.

○ Legal[35]

This strategy is additionally utilized for recognition of illegal medications in pee. The most widely recognized strategy for drug identification is an immunoassay. This technique is substantially more helpful. Nonetheless, comfort comes at the

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expense of explicitness and inclusion of a wide scope of medications. As HPLC is a technique for deciding (and potentially expanding) immaculateness, utilizing HPLC alone in assessing centralizations of medications is to some degree inadequate. With this, HPLC in this setting is frequently acted related to mass spectrometry. Utilizing fluid chromatography rather than gas chromatography related to MS avoids the need for derivatizing with acetylating or alkylation

Advantages of HPLC[36]:

The predominance of HPLC as a head scientific strategy is no mishap. The most noticeable benefit is its relevance to different analytes types, from little natural atoms and particles to huge biomolecules and polymers. The fruitful coupling of HPLC to MS gave it an invulnerable edge as "the ideal insightful instrument" — joining amazing division ability with the phenomenal affectability and particularity of MS. HPLC–MS is quickly turning into the standard stage innovation for bioanalytical testing (drugs in natural liquids), follow examination for deposits in food, scientific and ecological examples and life science research. At last, the phenomenal accuracy and strength of HPLC with UV location makes it a crucial device for Quality Control (QC). This last point is represented by a contextual analysis on steadiness assessment of a drug item. Utilizing a HPLC lab can grows better items, gain a superior comprehension of contenders items and can be utilized to help address/forestall item reviews.

Disadvantages of HPLC[37]:

HPLC can be a costly strategy, it required countless costly organics, needs a force supply and ordinary support is required. It can be muddled to investigate issues or grow new methods. The absence of a general identifier for HPLC, nonetheless, the UV-Vis locator just identifies chromophoric compounds. The division in High-execution fluid chromatography has less effectiveness than GC. It is harder for the beginner. HPLC siphon process unwavering quality depends on of neatness of the example, portable stage and legitimate activity of the framework. The expense of HPLC is undeniably more costly than its archetypes. Consequently, in case you're working at an exploration office or lab that has low financing, you might discover HPLC hardware hard to buy. As a rule, HPLC is flexible and amazingly exact with regards to distinguishing and

specialists, which can be an oppressive additional progression. This strategy has been utilized to recognize an assortment of specialists like doping specialists, drug metabolites, glucuronide forms, amphetamines, narcotics, cocaine, BZDs, ketamine, LSD, weed, and pesticides. Performing HPLC related to Mass spectrometry diminishes the outright requirement for normalizing HPLC exploratory runs.

measuring synthetic parts. With many advances included, the accuracy of HPLC is to a great extent down to the cycle being mechanized and thusly exceptionally reproducible. HPLC has low affectability for specific mixtures and some can't be recognized as they are irreversibly adsorbed. Unstable substances are better isolated by gas chromatography.

Use of HPLC in Pharmacy

1. **Validated method development for estimation of formoterol fumarate and mometasone furoate in metered dose inhalation form by HPLC:** A simple stability-indicating method was developed and validated for analysis of formoterol fumarate and mometasone furoate in metered dose inhalation formulations. This is a simple, accurate, precise, and stabilityindicating HPLC method for the routine analysis of formoterol fumarate and mometasone furoate in metered dose inhalation formulations. The results of stress testing undertaken according to the ICH guidelines reveal that the method is selective and stability-indicating[38].
2. **Development and application of multidimensional HPLC mapping method for O-linked oligosaccharides:** An HPLC mapping method was developed for detailed identification of O-glycans including neutral, sialylated, and sulfated oligosaccharides. Furthermore, using this method, it was able to quantitatively identify isomeric products from an in vitro reaction catalyzed by N-acetylglucosamine-6Osulfotransferases and obtain O-glycosylation profiles of serum IgA as a model glycoprotein. The HPLC map provides a glycomics tool for unambiguous identification and quantitative profiling of O-glycans expressed on a variety of proteins of physiological and pathological interest[39].
3. **A simple and specific method for estimation of lipoic acid in human plasma by HPLC:** A rapid HPLC method for determination of lipoic

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acid (LA) in human plasma was developed and validated.This HPLC method for the estimation of LA in human plasma is simple, sensitive, reproducible and precise. The assay is less laborious, economical and less time consuming than other reported methods previously. The validated HPLC-UV method may be applied to the measurement of LA levels in clinical practice. Further the LA concentrations can be confirmed by LC-MS quantification; thereby this method may be applied in future studies with shorter run time thereby large number of samples can be quantitated[40]

4. **HPLC method for determination of clobetasol in rat plasma and its application to skin penetration:** A simple and sensitive HPLC method was developed for quantification of clobetasol (CLB) in rat plasma. This method was successfully applied to the study of penetration of clobetasol from different zinc salts in rat for the first time. The method is also proficient in determination of large number of biological samples[41].
5. **Enantiomeric separation of nonproteinogenic amino acids by HPLC:** Amino acids are essential for life, and have many functions in metabolism. One particularly important function is to serve as the building blocks of peptides and proteins. Peptides are frequently cyclic and contain proteinogenic as well as nonproteinogenic amino acids in many instances. Since most of the amino acids contain a chiral carbon atom, the stereoisomers of all these amino acids and the peptides in which they are to be found may possess differences in biological activity in living systems. The development of methods for the separation of enantiomers is of great interest, since the potential biological or pharmacological applications are mostly restricted to one of the enantiomers. The separation of these isomers is achieved mainly by chromatographic and electrophoretic methods. Indirect and direct HPLC methods are useful for separation of biologically and pharmaceutically important enantiomers of nonproteinogenic amino acids and related compounds.

II. CONCLUSION

High-Performance Liquid Chromatography (HPLC) remains the bedrock of analytical chemistry today, providing unparalleled accuracy and sensitivity for the examination of intricate biological and pharmaceutical samples. The establishment of reliable HPLC methods for analytes like formoterol fumarate, mometasone furoate, and clobetasol illustrates its importance in drug development and pharmacokinetic research. In addition, its use for glycosylation analysis and isomer identification shows its increasing relevance in biochemical investigation. These developments reaffirm the significance of HPLC in guaranteeing the quality, effectiveness, and safety of therapeutic products.

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