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Review Article

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A Review on Analytical Method Development and Validation by Using Rp-HPLC

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Abstract Chromatography is primarily a separation technique, but it is primarily used in chemical analysis. High-performance liquid chromatography (HPLC) is a highly versatile technique that separates analytes by passing them through a column packed with micron-sized particles. Today, reversed-phase chromatography is the most commonly used separation technique in HPLC. Reversed-phase chromatography is used for both analytical and preparative applications in the field of biochemical separation and purification. Moderately hydrophobic molecules such as proteins, peptides and nucleic acids can be separated by reversed-phase chromatography with excellent recovery and resolution. As a result, it has become an integral part of the requirements of the regulatory organization. Analytical method development finally results in official test methods. These methods are used in quality control laboratories to ensure the identity, purity, safety, efficacy and performance of drug products. Regulatory authorities are placing greater emphasis on analytical methods in manufacturing. Drug approval by regulatory authorities requires the applicant to prove control of the entire process of drug development by using validated analytical methods.

Keywords: Chromatography, HPLC, Reversed Phase, Biochemical Separation

Introduction

High-Performance Liquid Chromatography has become one of the most powerful analytical chemistry tools. It is capable of separating, identifying, and quantifying the compounds present in any sample that can be dissolved in a liquid. High-performance liquid chromatography (HPLC) is one of the most accurate analytical methods for both quantitative and qualitative drug product analysis. The principle is that a sample solution is injected into a porous material column (stationary phase), and a liquid (mobile phase) is pumped at high pressure through the column. The sample is separated based on differences in migration rates through the column caused by different partitioning of the sample between the stationary and mobile phases. Elution occurs at different times depending on the partition behavior of different components.

A sample compound with a higher affinity for the stationary layer will travel slower and for a shorter distance than a compound with a lower affinity, which will travel faster and for a longer distance. The purpose of the HPLC method is to separate and quantify the main drug, any reaction impurities, all available synthetic intermediates, and any degradants. High-Performance Liquid Chromatography has become one of the most powerful analytical chemistry tools. It is capable of separating, identifying, and quantifying the compounds present in any sample that can be

dissolved in a liquid. It is one of the most accurate analytical methods for quantitative and qualitative drug product analysis, as well as determining drug product stability.

HPLC principle

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

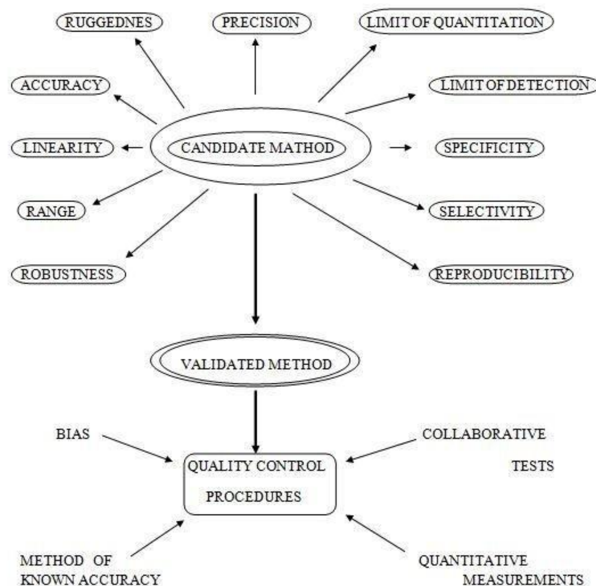


Figure 1: Flow chart of method development and validation

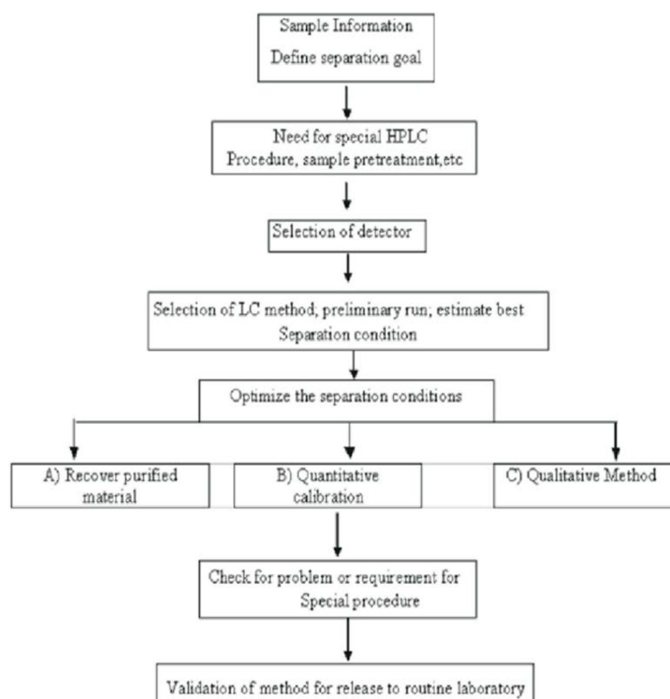


Figure 2: Method of validation by HPLC

Chromatography

Chromatography is a technique to separate mixtures of substances into their components on the basis of their molecular structure and molecular composition. This involves a stationary phase (a solid, or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase flows through the stationary phase and carries the components of the mixture with it. Sample components that display stronger interactions with the stationary phase will move more slowly through the column than components with weaker interactions.

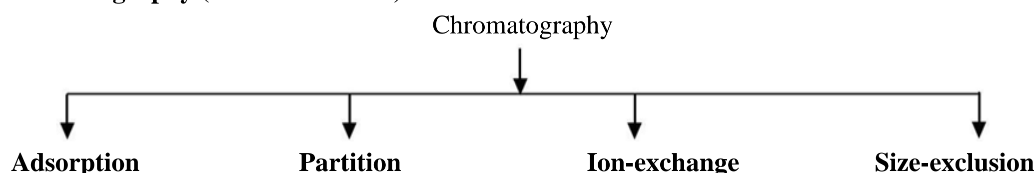
This difference in rates cause the separation of various components Chromatographic separations can be carried out using a variety of stationary phases, including immobilized silica on glass plates (thin-layer chromatography), volatile gases (gas chromatography) and liquids (liquid chromatography)

Classification of chromatographic methods

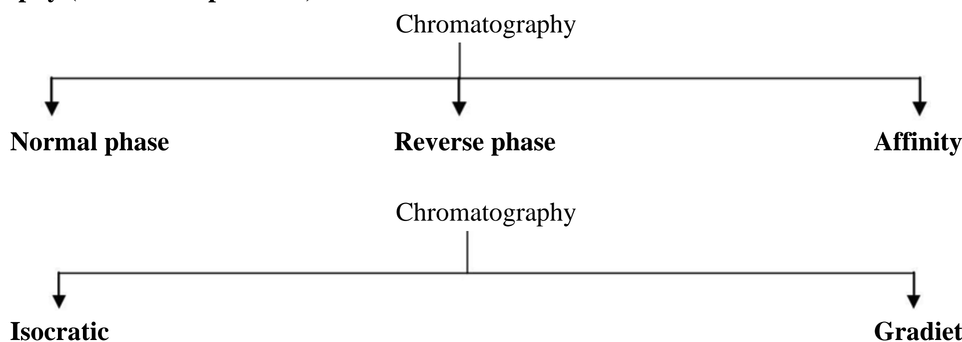
- Solid – Liquid (TLC, HPLC, Column chromatography)
- Solid – Gas (Gas solid chromatography)
- Liquid – Gas (Gas liquid chromatography)
- Liquid – Liquid chromatography (Paper and column partition chromatography)

Out of all liquid chromatographic methods HPLC and UPLC are mainly used for qualitative and quantitative analysis.

Types of chromatography (Based on nature)



Chromatography (Based on separation)



High Performance Chromatography

High performance liquid chromatography (HPLC) is basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster all chromatographic separations, including HPLC operate under the same basic principle; separation of a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation.

Types of HPLC:

There are following variants of HPLC, depending upon the phase system (stationary) in the process:

1. Normal Phase HPLC

This method separates analytes on the basis of polarity. NP-HPLC uses polar stationary phase and non-polar mobile phase. Therefore, the stationary phase is usually silica and typical mobile phases are hexane, methylene chloride,

chloroform, diethyl ether, and mixtures of these Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

2. Reverse Phase HPLC

The stationary phase is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. It works on the principle of hydrophobic interactions hence the more nonpolar the material is, the longer it will be retained.

3. Size-exclusion HPLC:

The column is filled with material having precisely controlled pore sizes, and the particles are separated according to its their molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later.

4. Ion-Exchange HPLC

The stationary phase has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

Fundamentals of RP-HPLC

A number of chromatographic parameters are also used to gauge the effectiveness of RP-HPLC separations. The retention time is the length of time it takes for a molecule to elute from the column after injection, whereas the selectivity factor is the ratio of the distance between two peaks. Resolution measures the space between two successive peaks, while capacity factors measure the compound's relative affinity for the stationary phase.

The first stage in developing a successful separation using RP-HPLC technology is selecting the stationery and mobile phases. It is crucial to pick a stationary phase that works well with the mobile phase and can successfully trap the target analyte. It's critical to choose a mobile phase that elutes the target analyte from the column and is compatible with the stationary phase.

To get the best separation conditions, it is possible to adjust a number of parameter including column temperature, mobile phase pH, flow rate, and gradient elution. The optimization process aims to achieve maximum resolution, minimal analytical time, and optimal solvent consumption. To guarantee that RP-HPLC methods are reliable reproducible, and precise, validation is crucial. Some of the factors that are investigated during validation include accuracy, precision linearity, salience, and limitations of detection and quantification.

Recent advancements in RP-HPLC include its downsizing, the introduction of hyphenated methodologies like LC-MS, and the creation of new stationary and mobile phases. More precise separation and analysis of complex compounds may be possible with newly constructed stationery and mobile phases with increased selectivity and efficiency.

Miniaturized RP-HPLC can analyze smaller sample volumes, reduce run durations and solvent consumption. By combining the advantages of RP-HPLC with mass spectrometry, hybrid techniques provide more accurate and sensitive analyte detection.

In conclusion, RP-HPLC is a vital analytical chemistry technique, particularly for the investigation of medicines. The creation of accurate and dependable RP-HPLC techniques is necessary for determining the quality, safety, and efficacy of drug products. The stationery and mobile phases must be carefully considered, the separation parameters must be fine-tuned, and RP-HPLC methodologies must be validated in order to achieve the perfect separation. One way that RP-HPLC is changing to become more adaptable and potent in the analytical environment is through the addition of new stationery and mobile phases, downsizing, and hyphenated techniques.

Steps involved in method development:

Documentation starts at the very beginning of the development process. A system for full documentation of development studies must be established. All data relating to these studies must be recorded in laboratory notebook or an electronic database.

1. Analyte standard characterization

a) All known information about the analyte and its structure is collected i.e., physical and chemical properties.
b) The standard analyte (100 % purity) is obtained. Necessary arrangement is made for the proper storage (refrigerator, desiccators and freezer).
c) c)When multiple components are to be analyzed in the sample matrix, the number of components is noted, data is assembled and the availability of standards for each one is determined.
d) Only those methods (spectroscopic, MS, GC, HPLC etc.,) that are compatible with sample stability are considered.

2. Method requirements: The goals or requirements of the analytical method that need to be developed are considered and the analytical figures of merit are defined. The required detection limits, selectivity, linearity, range, accuracy and precision are defined.

3. Literature search and prior methodology: The literature for all types of information related to the analyte is surveyed. For synthesis, physical and chemical properties, solubility and relevant analytical methods, books, periodicals, chemical manufacturers and regulatory agency compendia such as USP / NF, are reviewed. Chemical abstracts service (CAS) automated computerized literature searches are convenient.

4. Choosing a method: Using the information in the literatures and prints, methodology is adapted. The methods are modified wherever necessary. Sometimes it is necessary to acquire additional instrumentation to reproduce, modify, improve or validate existing methods for in-house analytes and samples. a) If there are no prior methods for the analyte in the literature, from analogy, the compounds that are similar in structure and chemical properties are investigated and are worked out. There is usually one compound for each analytical method that already exist that is similar to the analyte of interest.

5. Instrumental setup and initial studies: The required instrumentation is to be setup. Installation, operational and performance qualification of instrumentation using laboratory standard operating procedures (SOP's) are verified. Always new consumables (e.g. solvents, filters and gases) are used. For example, method development is never started on a HPLC column that has been used earlier. The analyte standard in a suitable injection / introduction solution and in known concentrations and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix. If the sample is extremely close to the standard (e.g., bulk drug), then it is possible to start work with the actual sample.

Choice of the Column:

Column is the heart of HPLC system. Good silica and bonding process will provide the reproducible and symmetrical peak necessary for accurate qualification. Commonly used RP columns include C18 (USP L1), C8 (USPL8), Phenyl (USP L11) and Cyno (USP L18). There is no good or bad column. They are chemically different boned phases and demonstrate significant changes in selectivity using same mobile phase. Column vary from manufacturer to manufacturer relative to their pore volumes, pore size, surface area, particle size, carbon load and whether they are end capped or not. Column length also plays a vital role in the separation resolution. Various types of columns and their applications are shown in Table 2.

Table 1: Various types of columns and their applications

Column	Phase	Solvents	Application
C18	Octadecyl	ACN, MeOH, H2O	General, nonpolar
C8	Octyl	ACN, MeOH, H2O	General, nonpolar
Phenyl	Styrl	ACN, MeOH, H2O	Fatty acid, Double bond
Cyano	Cyanopropyl	ACN, MeOH, H2O, THF	Ketones, aldehydes
Amino	Aminopropyl	ACN, MeOH, H2O, THF, CHCl3, CH2Cl2	Sugars, anions
Diol	Dihydroxy hexyl	ACN, MeOH, H2O, THF	Proteins
SAX	Aromatic quaternary amine	SALT Buffers, ACN, MeOH, H2O	Anions
SCX	Aromatic sulfonic acid	SALT Buffers, ACN, MeOH	Cations

DEAE	Alkyl ether, ethyl 2°amine	SALT Buffers, ACN, MeOH, H2O	Protein cations

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