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Review Article

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A review on analytical method development and validation of novel HPLC technique in pharmaceutical dosage form

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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. Reasons include the reversed-phase method's simplicity, versatility, and applicability. This is because the reversed-phase method can handle compounds of different polarities and molecular weights. 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. This review presents the importance of RP-HPLC in analytical method development, its strategies, and a brief knowledge of the critical chromatographic parameters that need to be optimized for efficient method development.

Keywords: Chromatography, HPLC, Reversed Phase, biochemical separation

Introduction

Market is flooded with combination of drugs in various dosages forms. The multi component formulations have gained a lot of important nowadays, due to greater patient acceptability, increased potency, multiple action and quicker relief. There is plethora of analysis of such drug combinations. For the analysis of these formulations various techniques are used. Analysis plays a major role today, and it can be considered as an interdisciplinary subject. Pharmaceutical analysis derives its principles from numerous branches of science like chemistry, Physics, microbiology, nuclear science, electronics etc [1,2].

Drugs or pharmaceuticals are chemicals which are of organic, inorganic or other origin. Whatever may be the origin, some property of the medicinal agent is used to measure them qualitatively and quantitatively. E.g. gravimetric analysis, acidimetry and alkalimetry methods.

Analytical chemistry is the study of the separation, identification, and quantification of the chemical components of naturals and artificial materials. Analytical chemistry deals with the methods for determining the chemical composition of samples of matters. It deals with quantitative analysis of composition of substances and complex materials in various matrices, by measuring a physical or chemical property of a distinctive constituent of the component or component of interest. The identification of a substance, the elucidation of its structure and

quantitative analysis of its composition are the aspects covered by analytical chemistry. It is an interdisciplinary branch of science where in a large number of research workers have contributed to developmemt [3,4].

Analytical chemistry has been important since the early days of chemistry, providing methods for determining which elements and chemicals are presents in the object in question. During this period significant analytical contributions to chemistry include the development of systematic elemental analysis and systematized organic analysis based on the specific reactions of functional groups. Most of the major developments in analytical chemistry take place after 1900. During this period instrumental analysis becomes progressively dominant in the field. In particular many of the basic spectroscopic and spectrometric techniques were discovered in the early 20th century and refined in the late 20th century. Analytical chemistry is dominated by instrumental analysis. Analytical chemistry play an important role in the pharmaceutical industry where, asude from QA, it is used in discovery of new drug candidates and in clinical applications where understanding the interactions between the drug and the patient are critical [3].

Analytical chemistry can be divided in two general areas of analysis

Quantitative: Determines the amount of one or more of these components

Qualitative: It gives an indication of the identity of the chemical species in the sample However, devising an accurate analytical method for each ingredient of complex dosage formulation is very challenging task because of following reasons.

- Combined dosage forms contain several therapeutically and chemically compatible species with very similar chemical nature
- They are present in the formulations in widely divergent concentration depending upon their relative potency and therapeutic need of patient.
- They are present in the additives, excipients and decomposition products which further complicate the development of analytical procedure

The following steps are involved in the analysis of a drug samples

- Procurement of samples in pure form
- Preparation of sample
- Identification of suitable analytical procedures
- Any chemical or physical methods used for qualitative identification and quantitative measurement of sample
- Conditions determined by analytical problems
- Interpretation of analytical results

General criteria for selection of analytical method [5,6]:

A vital first step in any quantitative analysis is the selection of method. This will require careful consideration of following criteria.

- The type of analysis required
- Problems arising from the nature of material to be investigated.
- Possible interference from components of the material other than those of interest.
- The concentration range, which needs to be investigated.
- The accuracy required.
- The facilities available.
- The time required to complete the analysis.
- The time required to complete the analysis.
- The number of analysis of similar types, which have to be performed.

Finally, the choice of method is always govered by complexity of sample as well as number of components in the samples.

Analytical method are generally classified into two categorie

- **Instrumental Method**
- **Non-Instrumental Method**

1) **Instrumental Method:** A physical property is made to determine the contents of composition of a substance. E.g. Electrochemical method change in the electrical properties of the system. Examples are Electrogravimetry, Potentiometry, Conductometry.

Advantages:

- Small samples can be used.
- High sensitivity can be obtained.
- Determination is very fast.
- Even complex samples can be handled easily.

2) **Non instrumental Method:** In these methods chemical reactions gravimetric is involved. These are volumetric and method. These are also known as classical method of analysis.

Advantages:

- Methods are based on absolute measurement.
- Specialized training is usually not required.
- Equipment needed is cheap.

The pharmaceutical analysis can be done with various methods, which are classified accordingly as in figure 1.

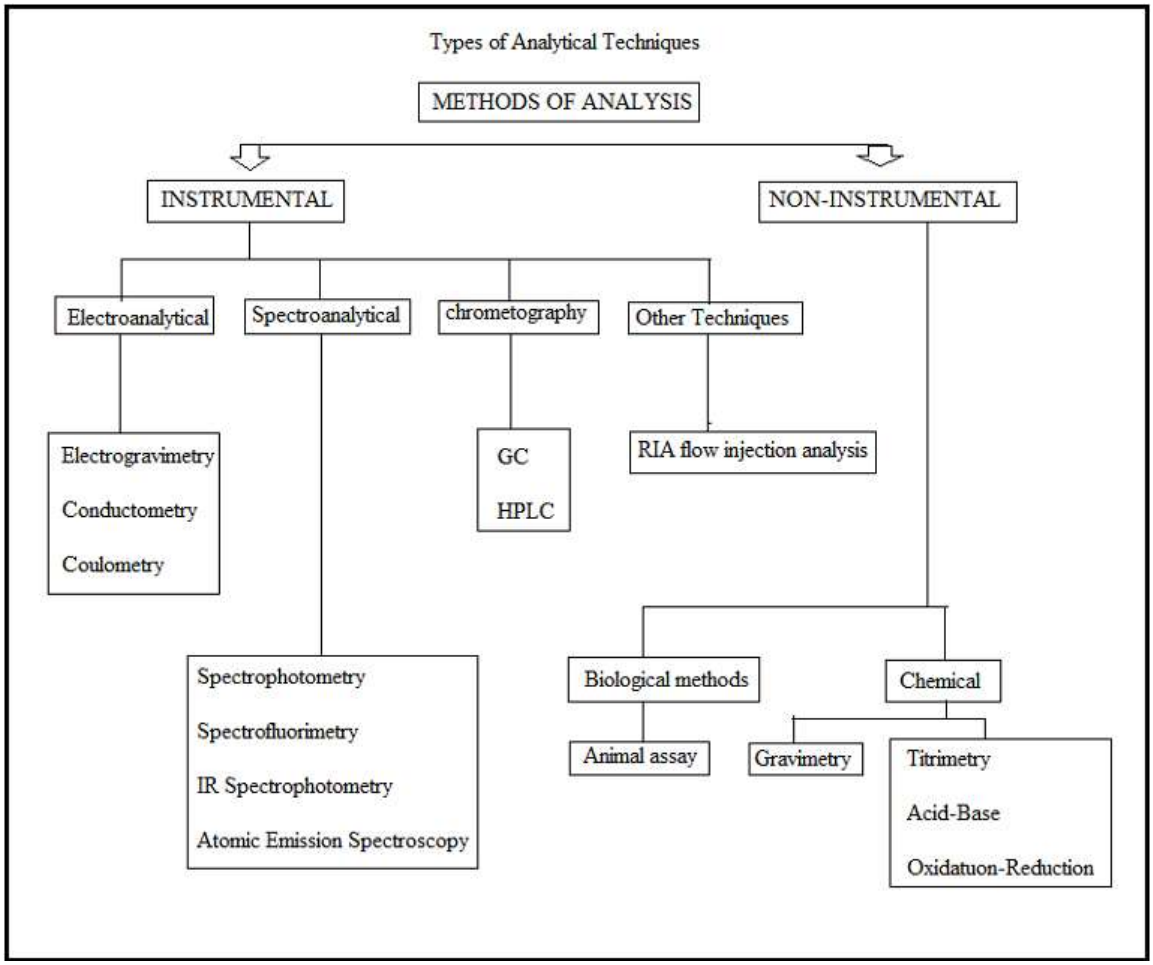

Figure 1: Pharmaceutical Method of Analysis [5]

Analytical Method Development

The number of drugs introduced into the market are increasing every year. These drugs may be either new entities or partial structural of modification the existing one Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs.

Basic criteria for new method development of drug analysis:

- The drug or drug combination may not be official in any pharmacopoeias,
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations,
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipient,
- Analytical methods for the quantitation of the drug in biological fluids may not be available,
- Analytical methods for a drug in combination with other drugs may not be available,
- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable [7].

Introduction to HPLC

HPLC is fastest growing analytical technique for analysis of drugs. Its simplicity, high specificity and wide range of sensitivity makes it ideal for analysis of many drugs in both dosage forms and biological fluids. High performance liquid chromatography (HPLC) is a form of liquid chromatography to separate compounds that are dissolved in solution. HPLC is able to separate macromolecules and ionic species, labile natural product, a wide variety of other high molecular weight poly functional group and polymeric materials.

HPLC is presently used in pharmaceutical research and development in following areas:

1) To purify synthetic or natural products.
2) To characterize metabolism
3) To assay active ingredients, impurities, degradation products and in dissolution studies.
4) In pharmacokinetic and pharmacodynamics studies.

Most of the drugs in multi component dosages forms can be analysed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures. Some of advantages are:

- Speed (analysis can be accomplished in 20 mint or less)
- Greater sensitivity
- Improve resolution
- Reusable columns
- Ideal for the substance of low volatility
- Easy sample recovery, handling and maintenance
- Instrumentation tends itself to automation and quantitation
- Precise and reproducible
- Calculation are done by integrator itself [7, 8, 9]

Basic principle of HPLC

High Performance Liquid Chromatography (HPLC) is a separation technique utilizing differences in distribution of compounds to two phases, called stationary phase and mobile phase. The stationary phase designates a thin layer created on the surface of fine particles mobile phase designates the liquid flowing over particles. Under a certain dynamic condition each component in a sample has difference distribution equilibrium depending on solubility in

the phase and or molecular size. As a result the components move a different speed over the stationary phase and there by separated from each other.

The column is a stainless steel tube, which is packed with spherical particles. Mobile phase is constantly fed into the colun inlet at a constant rate by a liquid pump. A sample is injected from a sample injector, located near the column inlet. The injected samples enter the column with the mobile phase and the components in the sample migrate through it, passing between the stationary phase and mobile phase. Compound move in the column only when is in the mobile phase. Compounds that tend to be distributed in the stationary phase migrate slower. In this way, each component is separated on the column and sequentially elutes from the outlet.

Each component eluting from the column is detected by a detector connected to the outlet of the column. When the separation process is monitored by the recorder starting at the time the sample is injected, a graph is obtained. This graph is called a chromatogram. The time required for a compound to elute (called retention time) and the relationship between compound concentration (amount) and peak area depend on the characteristics of the compound. Retention time is therefor used as an index for qualitative determination and peak surface area (or height) as an index for quantitative determination [10-11].

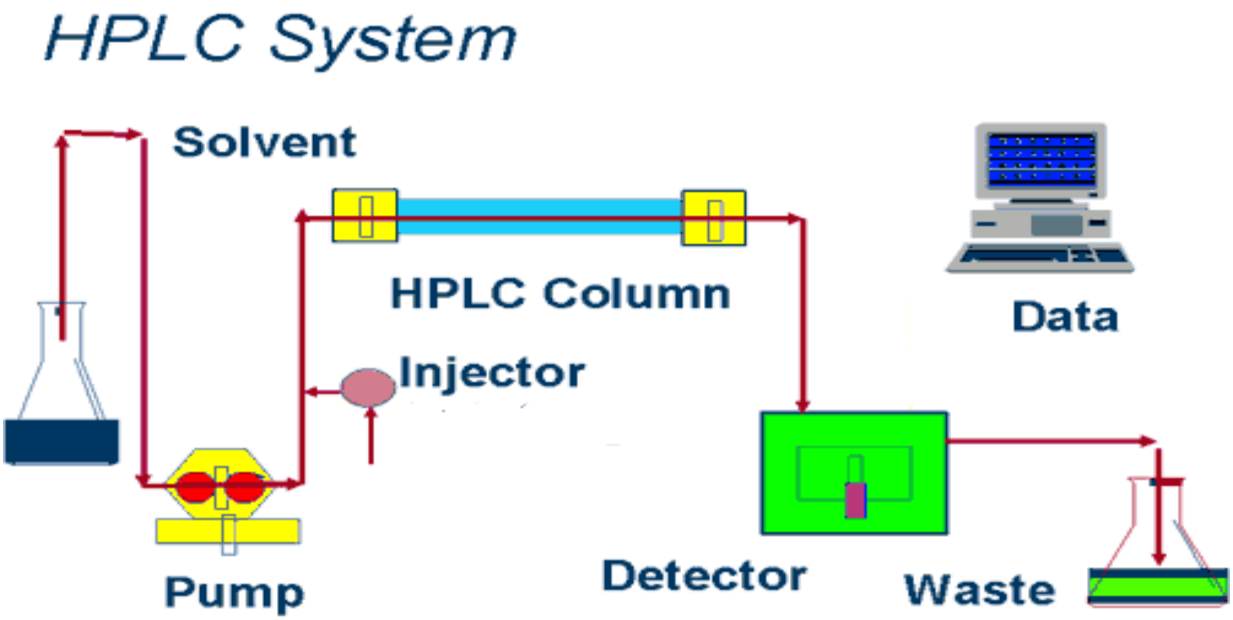

Figure 2: Diagram of HPLC

There are two modes of elution processes, Isocratic elution and Gradient elution

- **Isocratic Elution :** In isocratic elution a sample is injected onto a given column and the mobile phase is unchanged through the time required for the sample components to elute from the column. The isocratic separation of samples widely varying with k' (partition ratio) values long elution times. To adequately handle samples that have both weakly retained and strongly retained substance, the rates of band migrations must be changed during elution.
- **Gradient Elution**: steady changes of the mobile phase composition during the chromatographic run are called gradient elution. The main purpose of gradient elution is to move strongly retained components of the mixture faster, but having the least retained component well resolved. starting with the low content of the organic component in the eluent we allow the least retained components to be separated. Strongly retained component will move very slowly. When we start to increase an amount of organic component in the eluent (acetonitrile) then strongly retained components will move faster and faster, because of the steady increase of the competition for the adsorption sites [12].

Mode of Separation

The schematic of an HPLC instrument typically includes a degasser, sampler, pumps, and a detector. The sampler brings the sample mixture into the mobile phase stream which carries it into the column. The pumps deliver the desired flow and composition of the mobile phase through the column. The detector generates a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components. A digital microprocessor and user software control the HPLC instrument and provide data analysis. Some models of mechanical pumps in an HPLC instrument can mix multiple solvents together in ratios changing in time, generating a composition gradient in the mobile phase. Various detectors are in common use, such as UV/Vis, photodiode array (PDA) or based on mass spectrometry. Most HPLC instruments also have a column oven that allows for adjusting the temperature at which the separation is performed [13].

Normal phase mode, Normal-phase chromatography, alternatively termed adsorption or liquid–solid chromatography, represents the oldest of the chromatographic separation modes. It was first used in the classical open-column form in 1906 by Tweet to separate plant pigments. In normal-phase chromatography, the sample components are retained on the stationary phase through the interaction of permanent dipoles on the component with permanent dipoles on the stationary phase. This results in an adsorption mechanism and gives rise to the general class of adsorption chromatographic methods in which polar stationary phases and non-polar mobile phases are employed [13].

Reversed phase mode, Reverse phase HPLC is more commonly used compared to NP HPLC. This technique can be used to separate, identify and or quantitate components in mixtures of soluble organic components based on their hydrophobicity. The stationary phase is nonpolar, like C18 bonded silica. The mobile phase is polar, usually being water and polar organic solvent. Compounds with the most hydrophobicity elute later in the chromatogram and those with the least hydrophobicity elute earlier. Isocratic and gradient modes can be used to optimize separation of analytes of interest from matrix components that potentially can interfere with quantitation.

Reversed-phase chromatography is a technique using alkyl chains covalently bonded to the stationary phase particles in order to create a hydrophobic stationary phase, which has a stronger affinity for hydrophobic or less polar compounds. The use of a hydrophobic stationary phase is essentially the reverse of normal phase chromatography, since the polarity of the mobile and stationary phases have been inverted – hence the term reversed-phase chromatography [2,3]. Reversed-phase chromatography employs a polar (aqueous) mobile phase. As a result, hydrophobic molecules in the polar mobile phase tend to adsorb to the hydrophobic stationary phase, and hydro*philic* molecules in the mobile phase will pass through the column and are eluted first [2,4].

Method Development in HPLC

"Best column, best mobile phase, best detection wavelength, efforts in their selection can make a world of difference while developing HPLC method s for routine analysis. Determining the ideal combination of these factors assures faster delivery of desired results a validated method for separation."

Analytical method development is a process of proving that the developed chromatography method is suitable for its intended use in the development and manufacturing of the pharmaceutical drug substance and drug product. The basic separation techniques and principles involved in the analytical method development using the HPLC and UPLC are listed as follows:

- Selection of chromatography mode
- Selection of detector
- Selection of column (stationary phase)
- Selection and optimization of mobile phase
- Buffer and its strength
- pH of buffer
- Mobile-phase composition
- Selection of organic modifiers
- Selection of ion-pair reagents
- Selection of flow rate

- Selection of solvent delivery system (elution mode)
- Selection of diluent
- Methods of extraction
- Samples to be used
- Experimentation to finalize the method
- Selection of test concentration and injection volume
- Forced degradation studies (stress resting)
- Evaluation of stress testing
- Mass balance study
- Finalization of wavelengths
- Stability of solution
- System suitability
- Robustness of the method
- Relative response factor
- Quantification methods

Before starting an analytical method development, literature on some of the column characteristics as mentioned below has to be referred for the target molecules or similar molecules or precursors from open resources like articles, books, pharmacopeia reports, etc. This will give a tentative choice in designing a method for initial or test experiments, which will be further modified or updated to develop a method which fits the separation process for better results in terms of reproducibility, quantification, etc. Solubility profile of drug substance in different solvents at different pH conditions is useful while selecting the diluents for standard solutions and extraction solvents for test solutions [14, 15, 16].

1. *Analytical profile* is useful in understanding the physicochemical properties (e.g., *pKa*, melting point, degradation pathways, etc.) and absorption characteristics of drug in selecting the detector wavelength for analysis.
2. *Stability profile* of the drug substance with respect to storage conditions (sensitivity of the drug towards light, heat, moisture etc.) is useful as it helps in adopting the suitable/adequate precautions while handling drug and its formulated products.
3. *Impurity profile* collects the information of impurities and degradation profile of the drug substance during their formation pathways. This helps a lot in developing the method for separation of all possible impurities and degradation products of targeted analyte. It should be borne in mind that impurity profile may vary depending on the manufacturing process (which uses different methods, precursors, and conditions), which makes it clear that not all manufacturing processes yield the same impurity profile.
4. *Metabolic pathway* is a chemical reaction which occurs within a cell when the drug molecule reacts with an enzyme and forms a metabolite. Metabolic pathway gives the information on oxidation, reduction, and hydrolysis products which gives critical inputs on the possible degradation products.
5. *Checking the polarity* of the drug molecule using the functional groups as elucidated from structural analysis techniques. By comparing the structures of impurities and degradation products with the structure of drug molecule, it will help in understanding the polarity based on the nature of functional groups. This makes the scientists' job easy in choosing the right solvents with either lesser or higher in polarity than the compound of interest.
6. *Estimation of maximum daily dose (MDD).* Calculate the reporting, identification, and qualification thresholds of drug substance and drug product based on the maximum daily dose as per ICH Q3A guideline. MDD info can also be obtained from physical desk reference (PDR), innovator product information leaflet (PIL) [17, 18, 20].

Selection of chromatography mode

Chromatography can be operated by two ways, normal mode and reverse phase modes. The choice of the mode is very important, which is dependent on the type of sample which has to be separated. In general, the usage of reversed-phase chromatography (in which the mobile phase is polar and stationary phase is nonpolar in nature) is the preferred mode for most of the molecules, except in the case of isomer (enantiomers) separation where the normal-phase chromatography (in which the mobile phase is nonpolar and stationary phase is polar in nature) is used. Revered-phase chromatography separates the components with a good resolution based on their hydrophobicity. A compound with a greater polarity elutes earlier, and those with the least polarity elute later [19].

Selection of column stationary phase

The choice of the right column (stationary phase) is the basis of the whole technology. Most chromatographic separations are achieved due to a wide variety of columns available in the market and due to their flexibility in changing and controlling the parameters. A widely used choice of column material is silica either as neat or modified depending on the nature of the solute mixture in normal-phase chromatography, wherein the eluent (mobile phase) is nonpolar an organic solvent. The silanol groups on the surface of the silica give it a polar character.

Though silica remains the most common support for liquid chromatography (LC) columns, other commonly used materials are cross-linked organic polymers, zirconia, etc. The silica support for columns was gradually modified for the betterment through the years by three different manufacturing technologies commonly described as "evolution through three generations."

The initial process started with type A silica where the raw material used is from inorganic sols. A slightly modified type A silica by performing a chemical treatment to remove the metal impurities is termed as a second-generation material which is called as base-deactivated silica. Third generation silica (type B) is an altogether new process which uses organic sols instead of inorganic sols. These materials are similar in properties to the second-generation silica because both have a minimum level of metal impurities. Silica-based liquid chromatography columns with a different percent of cross-linking and functionalization of silanol groups with substituted aliphatic and aromatic moieties were designed for varying polarities of the separating medium.

The following are the parameters of a chromatographic column which need to be considered while choosing a column (stationary phase) for separation of assay, impurities, and degradation products:

Length and diameter of column

Packing material

Shape of particles

Size of particles

Percent (%) of carbon loading

Column dimension: Length and internal diameter of packing bed.

Short (30–50 mm)—can result in short run times and low back pressure

Long (250–300 mm)—can result in higher-resolution and long run times

A column with a diameter of 2.1 mm leads to a high resolution.

Particle size: Decrease in particle size leads to increase in resolution but with a corresponding increase in back pressure. In general smaller particles offer higher efficiency, but there is a chance to get high back pressure limiting the separation efficiency. Less (3 μm) particles are usually used for resolving complex and multicomponent samples, where the lesser surface area induces better resolution and separation characteristics.

Pore size and surface area: Larger pores allow larger solute molecules to be retained for a longer time through maximum surface area exposure. High surface area generally provides greater retention, capacity, and resolution for multicomponent samples. Low surface area materials generally equilibrate quickly and provide lesser separation efficiency but can be highly preferred and important in gradient analyses.

Carbon loading: Higher carbon loads generally offer greater resolution and longer run times. Low carbon loads shorten run times, and many show a different selectivity. A pictorial representation of difference in carbon loading is as shown below.

End capping: End capping reduces peak tailing of polar compounds that interact excessively with the otherwise exposed, mostly acidic silanols. Non-end capped packing provides a different selectivity than do end-capped packing, especially for polar compounds. A pictorial representation of difference in end capping is shown below [20-21].

Selection and optimization of mobile phase

Though adsorption is the principle behind chromatography, real separation happens only when the adsorbed compound is eluted using a mobile phase of the required polarity. The selection of mobile phase is done always in combination with the selection of column (stationary phase). The following are the parameters which shall be taken into consideration while selecting and optimizing the mobile phase.

The right choice of buffer and its eluting efficiency

pH of the buffer or pH of the mobile phase

Mobile-phase composition inclusive of binary and tertiary solvent mixture.

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