

Recent Analytical Method Developed by RP-HPLC

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Abstract: Reversed-phase chromatography is the mainly used in chromatographic mode, it is used to separate neutral molecules in solution based on their hydrophobicity. As the name suggested that, reversed-phase chromatography (often referred to as RP chromatography) is the reverse of normal-phase chromatography in the intelligence that it involves the employ of a polar mobile phase and a non-polar stationary phase. It ensures that a decrease in the polarity of the mobile phase results in a decreases in solute retention. Where a functional group is bonded to the silica in reversed-phase chromatography (RPC) generally refers to the used of chemically bonded stationary phases. In the partitioning model of retention, the stationary phase plays an important role in the retention process. The solute is consideration to be fully embedded in the stationary phase chains, somewhat adsorbed on the surface and consequently is considered to be partitioned between the mobile phase and a "liquid-like" stationary phase. The mobile phases used in reversed-phase chromatography are based on a polar solvent, typically water, to which a less polar solvent such as acetonitrile or methanol is added. The solvent selectivity are controlled by the nature of the additional solvents for example methylene chloride and 1,2-dichloroethane, cooperate preferentially with solutes that have large dipole moments, such as nitro- compounds, nitriles, amines and sulfoxides.

Key words: Reverses Phase -High Performance Liquid Chromatography • Elution • Parameters • Mobile Phase

INTRODUCTION

The word Chromatography is derived from Greek word chroma meaning "color" and graphein meaning "to write." Chromatography is a series of techniques that are used to separate components individually from a sample mixture and the separation takes place between two phases: stationary phase and mobile phase [1]. In chromatography, the extracting phase that moves in a definite direction is known as mobile phase and, the extracting phase that remains in a fixed position is known as stationary phase. Mobile phase can be a liquid, a gas, or a supercritical fluid. The mobile phase includes the sample which have to be separated or analyzed and the solvent that moves the sample through the column. The stationary phase can be only a liquid or a solid and the mobile phase moves over its surface [2]. The components which have more affinity towards stationary phase will eluted last while the components which have

less affinity to stationary phase eluted first. Chromatography was invented by the Russian botanist Mikhail Tswett in the late 1890's. He analyzed solutions containing plant pigments, such as chlorophylls and xanthophylls, through glass columns packed with finely divided calcium carbonate. The separated species appeared as colored bands on the column [3].

High Performance Liquid Chromatography: High Performance Liquid Chromatography (HPLC) is a separation method of great importance to the pharmaceutical industry. The principle involves the injection of a small quantity of sample into the flowing liquid (mobile phase) at or close to the head of a stainless steel tube (column) packed with very small particles (3-5µm in diameter called the stationary phase) where individual components of the sample are moved down the column with mobile phase, forced through the column by high pressure at a constant rate delivered by a pump [4].

HPLC Are Superior to LC Because of its Speed, Sensitivity, Efficiency and Ease of Operation: The components are separated from one another by the disparity in the comparative distribution ratio of the components between the two phases. These separated components are then detected after elution from column by a detector that measures their quantity [5].

The technique was originally developed by Russian botanist M.S.Tswett in 1903 and since then there has been an enormous development of the technique. The real breakthrough to liquid chromatography of low molecular weighted compounds was developed in the late 1960s by the introduction of small diameter particles (3 to 10 μm) e.g., octadecyl groups bound to silica (C18 column). The new technique became speedily a dominant separation technique and today is termed as high performance liquid chromatography (HPLC) [6]. For pharmaceutical formulations where the analytes often are non-volatile and/or occur in an aqueous matrix, the reversed-phase mode, using a hydrophobic stationary phase and an aqueous mobile phase is extremely useful. Further, the popularity and usefulness of HPLC was increased as it became automatic and computerize the systems providing information of success and failure of operations and high sample capacities. Many scientists got Nobel Prize on their work in which chromatography played an important role. Most recently, the 2002 Nobel Prize in chemistry was went to the method development for identification and structure analysis of biological macromolecules in which HPLC is used [7].

In HPLC, a sample solution is injected into a loop which contains a specified volume (20 μL). The sample moves into the stream of mobile phase (the eluent) onto a column which is packed with a appropriate stationary phase, where the separation occurs. Thus mobile phase is delivered as of a pump at a constant rate, (regulate of 1 mL/min) with pressure. Pressures of 1000-2000 psi are generally used with an upper limit of 4000 psi [8]. The high pressures are required to force the mobile phase through a tightly packed column filled with tiny particles. The combination of flow and pressure loops provides accurate and pulse less flow over a broad range of different solvent combinations and pressures, is directly responsible for the "high performance". A suitable column chosen for the separation of significance, all the components of sample must exceed throughout the column and "elute" at different times (disparity immigration). It is widely used for assay and impurity analysis for pharmaceutical quality control. The requirements for methods usually depend on the stage of development of the drug [9].

The essential components of HPLC system consists of:

- Reservoir for the mobile phase.
- A pump to travel the eluent and sample.
- An injector to permit sample foreword,
- A column(s) where taking apart of components take place.
- A detector to identify the separated analytes.
- A data collection device for elucidation and storage of results.

Types of HPLC:

- Reversed-phase chromatography
- Normal-phase and adsorption chromatography
- Ion exchange chromatography
- Size exclusion chromatography
- Bio-affinity chromatography

Reversed-Phase Chromatography (RPC): Reversed phase HPLC (RP-HPLC) has non-polar stationary phase for examples phenyl, C18, C8, C3, etc. and an aqueous, moderately polar mobile phase water (buffer) + water-miscible organic solvent (e.g. methanol, acetonitrile). RPC is the most popular mode of chromatography used by the researchers. This technique can be used to separate neutral molecules in solution [10]. RPC works on the principle of hydrophobicity, which results from repulsive forces between a polar mobile phase, the relatively non-polar components and the non-polar stationary phase. The polar components gets eluted first as they have less affinity to stationary phase and non-polar components are retained for longer time as they have more affinity to stationary phase [11].

Normal Phase or Adsorption Chromatography: Normal phase HPLC (NP-HPLC) has polar stationary phase (e.g. silica gel, cyanopropyl-bonded, amino-bonded, etc.) and non-polar mobile phase (e.g. hexane, iso-octane, methylene chloride, ethyl acetate). This technique is capable of water-responsive compounds, geometric isomers, cis-trans isomers and chiral compounds [12]. The polar analyte interacted by means of and retained by the polar stationary phase. The strength of adsorption increases with increased polarity of analyte and the interaction between the non-polar analyte and the polar stationary phase decreases the elution time as they have less affinity to each other. Separation takes place on the basis of their polarity; the more non-polar solute, the

lesser is its retention on the column and vice-versa. By increasing the polarity of the mobile phase results in decreased solute retention, since the mobile phase is less polar than the stationary phase [13].

Ion Exchange Chromatography: In Ion-exchange chromatography, the column packing contains ionic groups (e.g. sulfuric, tetra alkyl ammonium) and the mobile phase is an aqueous buffer (e.g. phosphate, formate, etc.). The retention is based on the hold between solute ions and charged sites leap to the stationary phase of the column [14]. This technique is widely used in purifying water, separation of Ionic dyes, separation of amino acids, separation of proteins, separation of carbohydrates and oligosaccharides, etc [15].

Size Exclusion Chromatography (SEC): Size exclusion chromatography (SEC) mainly separates particles on the basis of size relative to the pore size of column. Larger molecules elute earliest and smaller molecules elute afterward. The SEC technique is used mainly for polymer characterization, for the molecular weight determination of polysaccharides and for determination of structure of proteins [16].

There Are Two Modes: Gel Permeation Chromatography (GPC) and Gel Filtration Chromatography (GFC).

Bio-Affinity Chromatography: In this technique Separation occurs on the basis of specific reversible interaction of proteins with ligand. Ligands are covalently close to solid support on a bio-affinity matrix, retains proteins by means of interaction to the column-bound ligands [17].

Parameters Involved During Analytical Method Development on RP-HPLC

Nature of the Sample: Before starting the method development, sample properties must be known. The chemical composition of the test sample can help for the best choice of initial state for an HPLC separation.

Some Important information concerning sample composition and properties are follows:

Number of compounds present, chemical structures (functionality) of compounds, molecular weights of compounds, pK_a values of compounds, UV spectra of compounds and maximum wavelength of absorbance, sample solubility [18].

Optimization of Mobile Phase: The selection of the mobile phases is based on the nature of the sample and the retention behaviour. For normal-phase HPLC, lipophilic solvents (non-polar) should be employed. The occurrence of water in the mobile phase will decrease the efficiency of the stationary phase so it must be avoided. In reverse-phase HPLC, aqueous (polar) mobile phases are used, with and without organic modifiers [19].

For mobile phase optimization, these steps should be follows:

- Define the most excellent modifier type: tetrahydrofuran > acetonitrile > methanol.
- Define most favorable solvent strength so that retention for all components are in the range of 1-20.
- Perform firstly isocratic reading in 20% steps of development, preparatory at 100% organic.
- Perform gradient determination of % organic mobile phase. [20]

Selection of Buffers: Obtaining acceptable peak shape for analytes requires the proper preference of a mobile phase and buffer. Buffers get better peak shape of basic compounds and able to adjust the band spacing (or selectivity) and retention of acidic or basic compounds. The strongly suggest 10 - 20 mM of an ammonium salt of for the selection as a preliminary buffer i.e. acetate, carbonate, formate and phosphate salts designed for electrolyte solutes. Further considerations for the proper selection of a buffer system and the operation of buffers as a method development implement are given in the section method development for ionizable compounds.

pH Selection: By changing the pH of mobile phase, better separation of peaks were observed. The pH of mobile phase was adjusted to 2.0, 3.0, 7.0 and 10.0. At low pH range 2-3.0, satisfactory separation of the drug with proper resolution and short run time was achieved.

They are ideal for the following:

- Ionizable compounds.
- Improving peak shape of acidic or basic compounds.
- Changing selectivity or retention of acidic or basic compounds.

Detector Wavelength: The wavelength selected should be a wavelength of active ingredient in the UV spectrum. At all times use a wavelength more than 10 nm from the mobile phase UV cut-off.

Flow Rate Programming: For the purpose of rapid analysis of the drug, mobile phase flow rate programming is necessary for good resolution. Best results were obtained with flow rate in the range of 0.5 - 2.0 ml/min of selected mobile phase. Select suitable flow rate according to the column diameter e.g. 1.0 ml/min for 4.6mm id, 0.5 ml/min for 3.2mm id.

Columns: The selection of column is the most important parameter for separating a mixture into components. The capacity factor, selectivity and the efficiency of the column are all affected by the environment of the packing material or the materials of construction [21].

Stationary Phases: Silica gel is the most common stationary phase for adsorption chromatography, although the use of other metal oxides, such as alumina and zirconium, as well as carbon and hydroxyapatite has been reported. Unchanged silica, alumina, porous graphite, a selection of chemically personalized supports prepared from polymers, silica, resins with acid or basic groups are usually used as supports for bonded phases. Silica packings in column are popular because they can hold up at the high pressures. Silica is of low cost, abundant and available in a variety of sizes, shapes and degrees of porosity [22].

Pore Size: The stationary phases are generally porous to afford greater surface area therefore; small pore size provides greater surface area and vice-versa. For the majority of the chromatographic modes, a narrow distribution of pore sizes is advantageous.

Internal Diameter of Column: The internal diameter (ID) of HPLC column is an important parameter which will affect the sample load, the peak dilution and the flow rate. That larger the inner diameter, the mass of analyte that able to loaded onto the column is superior and the higher is the flow rate [23]. A larger inner diameter of column over 10 mm is used to purify utilizable amounts of material since of their large loading capacity and higher is the flow rate. Most of the analytical columns ranges from 2 to 5 mm in diameter accept narrow-bore columns (1–2 mm) which are used when more sensitivity is desired [24].

Column Length: Column length affects both the efficiency and the speed of the separation. Shorter columns result in shorter analysis times however; the column efficiency tends to decreases with length. In general, short columns are used for simple separations and large columns are used for complex samples. An analytical column comes in ranges from 30-300 mm in length [25, 26].

Selection of Column: The selection of the column in HPLC is based on the mode of separation. In HPLC partition and adsorption modes are used for separation, the separation mechanism is based on inductive forces, dipole-dipole interaction and hydrogen bond formation [27].

- In case of normal phase chromatography, it is the pore size and particle size that determine the separation efficiency. The smaller pore size, the better is the separation efficiency, but one also has to keep the molecular weight in mind while selecting an appropriate pore size of column. General rule is to select a column having a pore size 60 to 80 Å, for compound having molecular weight up to 350 and to pore size 100-200 Å for compound having molecular weight up to 1500.
- In case of reverse phase packing's, the separation as based on the differences in hydrophobic character of a molecule and degree of hydrophobicity dependent on the chemistry used for deactivation of silanol, deactivation of silanol is done by using hydrocarbons. The length of carbon chain used for deactivation known as carbon loading. The carbon loading 8% to 12% is ideal for the components that show moderate to moderately high hydrophobicity.

Isocratic and Gradient Pumping Systems (J.M. Cintron)

Isocratic Pumping Systems: Isocratic elution is commonly used for the elution of analytes from the column. In isocratic elution, the mobile phase does not change with time throughout the analysis. The mobile phase may be a single solvent or a solution of two or more miscible solvents. The most important of isocratic pumps are accuracy and smoothness of flow because the pump delivers only one solvent system, simple, inexpensive pulse dampeners and elementary flow or pressure opinion control circuits can be used [26, 28].

Gradient Pumping Systems: In gradient elution, the mobile phase composition changes with time during the analysis. Two mobile phases are used- eluent-A & eluent -B. The combination of two solvents to form a gradient is referred to as a binary gradient, three solvents make a ternary gradient and a quaternary gradient that formed by the use of four different solvents [26, 28].

System Suitability: The system suitability test is used to ensure that proper concert of the selected chromatographic system. The all parameters i.e. efficiency, capacity factor, resolution factor and asymmetry factor are generally capable in assessing the column performance; these parameters are defined below [26, 29].

Efficiency (N): Efficiency of a chromatographic column is describing in termed as the number of theoretical plates (N) and then calculated by formula [22, 23]:

$$N = 5.54 \frac{t_R^2}{W_h^2}$$

where

t_R = Retention time or the baseline distance between the point of injection and the perpendicular dropped from the maximum of the peak of interest.

W_h = The width of the peak of interest determined at half peak height, measured in the same units as t_R .

The number of theoretical plates may be expressed per metre (N'):

$$N' = \frac{N}{l}$$

where

l = length of column in metres.

Capacity Factor (Mass Distribution Ratio, D_m): The capacity factor or mass distribution ratio can be expressed as follows:

$$D_m = \frac{\text{Amount of solute in stationary phase}}{\text{Amount of solute in mobile phase}}$$

The factor is determine the retention of a solute and intended from the chromatogram by the following formula:

$$D_m = \frac{t_R - t_M}{t_M}$$

where

t_R = Retention time of the solute

t_M = Retention time of an unretained component

A low D_m value expressed the peak elutes near to the solvent front, which can be compromise selectivity. A minimum D_m value is 1 which used for the peak of interest.

Retention time of the test product can be checked, if necessary, by changing the relative proportion of solvents in the mobile phase. Usually, an increase in the compositions of more polar solvents will lead to the shorter retention time on a normal-phase column and a longer retention time on a reversed-phase column [26].

Resolution Factor (R_s): Resolution between nearer peaks of related height in a chromatogram can be calculated by the following formula:

$$R_s = \frac{1.18 (t_{R2} - t_{R1})}{(W_{b1} + W_{b2})}$$

where

t_{R1} and t_{R2} = Retention times or baseline distances between the point of injection and the perpendicular dropped from the maximum of each of the two peaks.

W_{b1} and W_{b2} = The respective peak widths determined at half peak height, measured in the same units as t_{R1} and t_{R2} . [22, 26]

The value of R_s for a baseline separation between peaks of similar height should be at least 1.5.

Relative Retention [3, 14]: The relative retention (r) is calculated as an estimate using the following formula:

$$r = \frac{t_{R2} - t_M}{t_{R1} - t_M}$$

where

t_{R2} = Retention time of the peak of interest

t_{R1} = Retention time of the reference peak

t_M = Retention time of an unretained component

The unadjusted relative retention (rG) is calculated from the expression:

$$rG = t_{R2}/t_{R1}$$

Unless indicated, values for relative retention affirmed in the monographs correspond to unadjusted relative retention.

METHOD DEVELOPMENT BY RP-HPLC

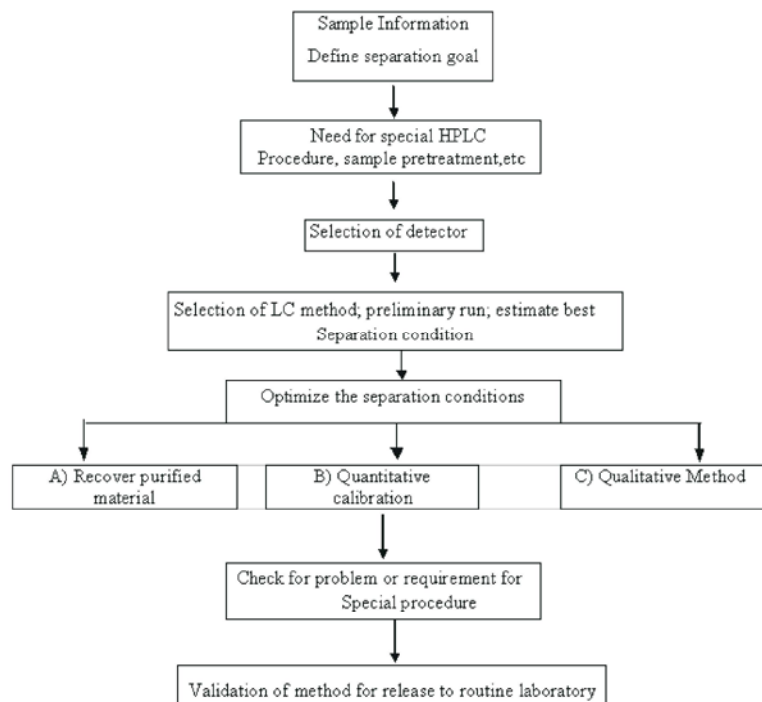


Fig. 1: Flow Chart for Method Development by RP-HPLC [4]

Asymmetry Factor (As): The symmetry factor for a peak can be calculated using the following formula:

$$As = W_x/2d$$

where

W_x = Peak width at 5% of peak height, measured from the baseline.

d = Baseline distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 5% of the peak height, measured in the same units as W_x .

Values for which are more than 2 may lead to erroneous integration, resulting in incorrect quantitation. The main factors that manipulate peak symmetry depend upon retention, solvent effects, incompatibility of the solute with the mobile phase, or expansion of an excessive void at the inlet of the column.

Repeatability: Unless stated in the "Assay" of the individual monograph, the relative standard deviation of peak areas for a series of injections of reference solutions bracketing groups of test solutions must not exceed 2.0%. In a "Related substances" test the relative standard deviation of peak areas for three replicate injections of the

reference solution must not exceed 5.0%, unless otherwise stated in the individual monograph. The sequence of injections the relative standard deviation of the retention time of the principal peak should not exceed 1.0% [30].

Completing the RP-HPLC Method: The absolute procedure should meet all the goals that were define at the establishment of method development. Developed method should be properly validated.

- Preliminary data to show required method performance.
- Written assay method developed for use by other operators.
- Systematic validation of method performance for more than one system and hand.
- Data obtained on anticipated life of column and column-to-column reproducibility.
- Deviant results studied for achievable correction at hidden problems.

All variables (temperature, mobile phase composition, etc.) study for outcome on separation limits distinct for these variables; remedies suggested for possible problems.

Recently Reported Method Developed by RP-HPLC:

Narendra Devanaboyina, *et al.*, (2013) developed and validated a new precise accurate and reliable method for the determination of Capecitabine by using reverse phase high performance liquid chromatography in pharmaceutical dosage forms. Spectrophotometric determination was carried out at an absorption maximum of 230 nm by using methanol. Chromatographic separation was carried out by using a mobile phase of methanol: Acetonitrile: water (80:18:2 V/V) on Zodiac C18 column (250 mm X 4.6 mm, 5 μ m) in an isocratic mode at a flow rate of 1.1 ml/min with UV detection at 230 nm [31].

Raj Kumar Dhawan (2013) developed and validated a simple, precise and accurate stability-indicating reverse-phase high- performance liquid chromatography (RP-HPLC) assay method for the determination of Levodopa and Carbidopa in Levodopa, Carbidopa and Entacapone ER tablets. RP-HPLC separation was achieved on an Inertsil ODS 3V, 250 \times 4.6 mm, 5- μ m column using a mobile phase containing a gradient mixture of mobile phase A and B. The flow rate of mobile phase was 1.5 ml/minute. The detection was carried out at 280 nm using a photodiode array detector [32].

Xing Tang (2012) developed an isocratic reversed phase high performance liquid chromatographic method with UV detection for the analysis of lansoprazole enteric capsules and related impurities. The mobile phase consisted of pH 6.2 phosphate buffer solution and acetonitrile (65:35, v/v). The UV detection was carried out at 285 nm and the flow rate was 1.2 ml/min. The separation was carried out on a Dikma Technologies Diamonsil C18 column, 5 μ m 250 mm \times 4.6 mm, which was maintained at 25°C. LPZ and its related impurities were separated well by this method [33].

Maitreyi Zaveri, *et al.*, (2012) developed a precise and validated RP-HPLC method for the simultaneous determination of Levodopa, Carbidopa and Entacapone in tablets formulation. The quantification was carried out by using Waters XTerra RP18 (250X4.6 mm), 5 μ m column in gradient mode with mobile phase, buffer: Acetonitrile (65:35). The flow rate was 1.5 ml/min. The gradient program throughout HPLC method was adopted to analyze all the three components in a single run [34].

Yusrida Darwis (2012) developed and validated a new, sensitive and specific isocratic reverse phase-high performance liquid chromatography (RP-HPLC) method with fluorescence detection for the determination of sumatriptan in rabbit plasma using sulpiride as an internal standard (IS). Chromatographic separation of the analyte and internal standard was achieved on a Phenomenex C4

(250 \times 4.6 mm, 5 μ m) analytical column maintained at 40°C. The mobile phase was composed of 25 mM ammonium acetate (pH 6.5) and acetonitrile (85:15, v/v), pumped isocratically at a flow rate of 0.9 ml/min. Column eluent was monitored at excitation and emission wavelengths of 225 and 350 nm [35].

CONCLUSION

All of the critical steps in method development have been summarized and prioritized. The steps for method development are discussed in the identical order as they would be investigated in the authentic method development process. These steps will ensure all critical method parameters are optimized before the method validation.

Reversed phase HPLC (RP-HPLC) consists of an aqueous polar mobile phase and a non-polar stationary phase. Reversed phase columns are moderately difficult to damage as compared with normal silica columns, but, many reversed phase columns consist of alkyl derivatized silica particles and they never be used with aqueous bases because it destroys the underlying silica particle. They are mainly used with aqueous acid, but the column would not be exposed to the acid for longer period, as it can rust the metal parts of the HPLC equipment.

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