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AN OVERVIEW OF THE ANALYTICAL STRATEGY FOR HPLC METHOD DEVELOPMENT

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Abstract

Chromatography is a potent analytical technique widely utilized by modern chemists for its ability to quantitatively analyze multiple components in a single procedure. HPLC relies on the differential partitioning of sample components between stationary and mobile phases, resulting in their separation over time. This versatile technique offers several advantages, including simultaneous analysis of multiple compounds, high resolution, sensitivity, repeatability, small sample sizes, and moderate operating conditions. HPLC can be classified based on various factors, such as scale of operation, separation principles, elution techniques, and modes of operation. Method development for HPLC involves a systematic approach. Initially, physicochemical properties of the drug molecule are assessed, including solubility, polarity, pKa, and pH. The choice of stationary phase, mobile phase, detectors, organic modifiers, ion-pair reagents, flow rate, and column temperature is then crucially determined to optimize separation. Diluents and extraction methods are selected to ensure effective sample preparation. System suitability parameters like retention time, theoretical plates, tailing factor, and resolution are evaluated to gauge method performance. Linearity is assessed to establish the concentration range where the drug exhibits a linear response. The method's practicality is confirmed through laboratory mixture analysis and the examination of marketed formulations. Sample preparation is a critical step, aiming to create a homogeneous, interference-free solution compatible with the HPLC method. Finally, method optimization is carried out, identifying weaknesses and enhancing performance through experimental design, considering varying conditions, instrument setups, and sample types. In conclusion, HPLC is a powerful tool in pharmaceutical analysis, enabling precise quantification and identification of compounds in diverse samples. Method development is a systematic process that ensures the reliability and effectiveness of HPLC analysis for pharmaceutical applications.

Key Words: Chromatography, HPLC, Method development, Separation, Pharmaceutical analysis

1- Introduction [1-10]

Chromatography is probably the most powerful analytical technique available to the modern chemist. Its power arises from its capacity to determine quantitatively many individual components present in mixture by single analytical procedure. It has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid. High performance liquid chromatography (HPLC) is the most accurate analytical methods widely used for the quantitative as well as qualitative analysis of drug product. The separation of sample is based on the differences in the rates of migration through the column arising from different partition of the sample between the stationary and mobile phase. Depending upon the partition behaviour of different components, elution at different time takes place. The High Performance Liquid Chromatography is more versatile than gas chromatography since (a) it is not limited to volatile and thermally stable samples, and (b) the choice of mobile and stationary phases is wider.

1.1 Advantages HPLC

• Simultaneous Analysis

• High Resolution

• High Sensitivity

• Good repeatability

• Small sample size

• Moderate analysis condition.

• Easy to fractionate the sample and purify

1.2 Classification of HPLC

- Preparative HPLC and analytical HPLC (based on scale of operation)
- Affinity chromatography, adsorption chromatography, size exclusion chromatography, ion exchange chromatography, chiral phase chromatography (based on principle of separation)
- Gradient separation and isocratic separation, (based on elution technique)
- Normal phase chromatography and reverse phase chromatography (based on modes of operation).

A. Normal phase chromatography: In normal phase chromatography, mobile phase is non-polar and stationary phase is polar. Hence, the station phase retains the polar analyte. An increase in polarity of solute molecules increases the adsorption capacity leading to an increased elution time. Chemically modified silica (cyanopropyl, aminopropyl and diol) is used as a stationary phase in this chromatography. For example. A typical column has an internal diameter of around 4.6 mm, and a length in the range of 150 to 250 mm. Polar compounds in the mixture that are passed through the column will stick longer to

the polar silica than the non-polar compounds. Therefore, the non-polar ones will pass more quickly through the column

B. RP-HPLC (Reversed phase HPLC): RP-HPLC has a non-polar stationary phase and polar or moderately polar mobile phase. RP-HPLC is based on the principle of hydrophobic interaction .In a mixture of components those analytes which are relatively less polar will be retained by the non-polar stationary phase longer than those which are relatively more polar. Therefore the most polar component will elute first.

2- METHOD DEVELOPMENT ON HPLC

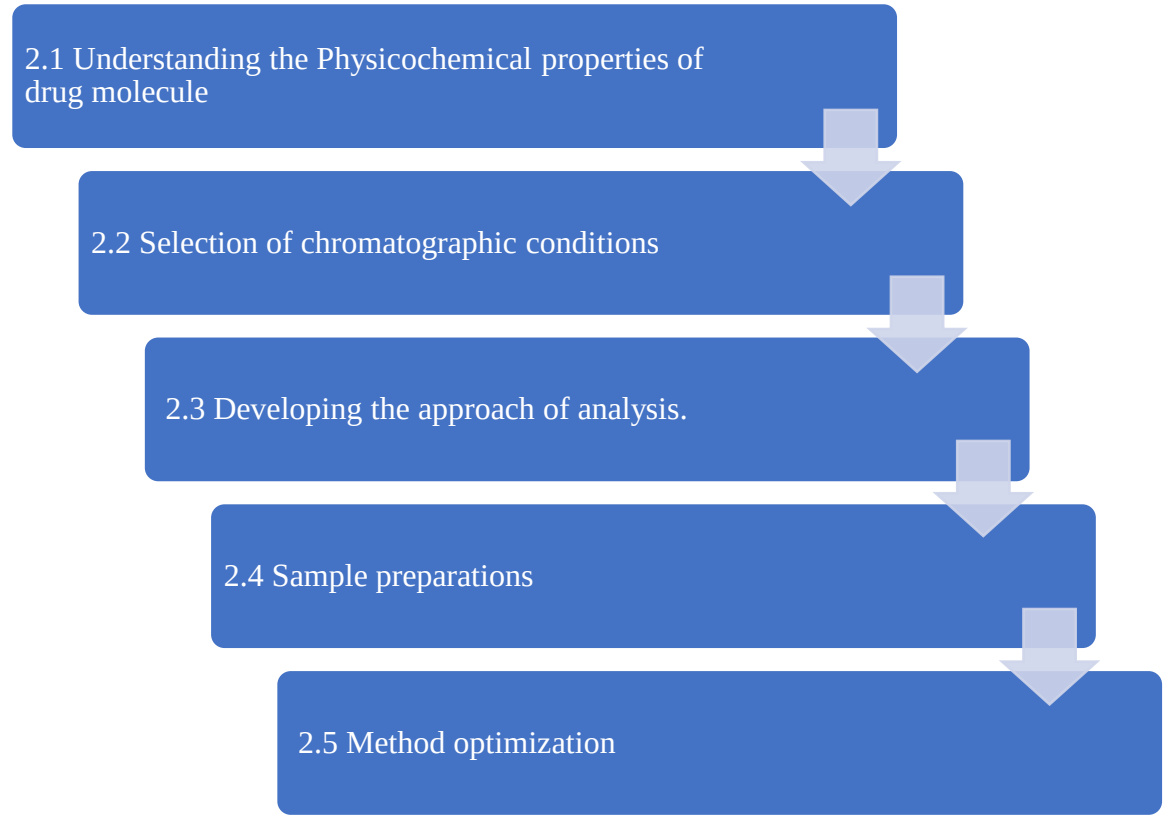

Figure 1- Steps of Method development

2.1 Understanding the Physicochemical properties of drug molecule[11-14]

Physicochemical properties of a drug molecule play an important role in method development. For Method development one has to study the physical properties like solubility, polarity, pKa and pH of the drug molecule

Molecular weight - Identification of the mode of HPLC testing i.e. a regular RP HPLC or Normal HPLC OR Molecular Weight separation technique.

Molecular structure- Identification of the nature of the molecule i.e. acidic, basic, neutral, stereo-isomers or Chiral molecule; which dictates the method input variables eg. stationary phase and operating pH, Identification of functional group - which dictates the choice of column chemistry and detector - whether UV/Vis, fluorescent, refractive index detector or ECD, Knowledge of functional group will also help predicting the possible degradation route

Polarity - physical property of a compound. It helps an analyst, to decide the solvent and composition of the mobile phase

Solubility – It can be explained on the basis of the polarity of molecules, Selection of suitable diluent and mobile phase.

pH and pKa - It plays an important role in HPLC method development. The pH value is defined as the negative of the logarithm to base 10 of the concentration of the hydrogen ion.

pH = - log10[H3O+].

Selecting a proper pH for ionizable analytes often leads to symmetrical and sharp peaks in HPLC. Sharp, symmetrical peaks are necessary in quantitative analysis in order to achieve low detection limits, low relative standard deviations between injections, and reproducible retention times. Log P should be considered for selecting appropriate mode of chromatography.

Sensitivity to heat- Sonication generates heat which could cause degradation of heat sensitive analytes in such cases alternate techniques for dissolving analyte needs to be evaluated

Sensitivity to light- Appropriate measures for sample preparation for light sensitive material eg. use of amber coloured glassware, subdued light is advised

2.2 Selection of chromatographic conditions[15-19]

2.2.1 Selection of the stationary phase/column

Selection of the stationary phase/column is the first and the most important step in method development. The development of a rugged and reproducible method is impossible without the availability of a stable, high performance column.

A C8 or C18 column made from specially purified, less acidic silica and designed specifically for the separation of basic compounds is generally suitable for all samples and is strongly recommended.

The column is selected depending on the nature of the solute and the information about the analyte. Reversed phase mode of chromatography facilitates a wide range of columns like dimethyl silane (C2), butylsilane (C4), octylsilane (C8), octadecylslane (C18), base deactivated silane (C18) BDS phenyl, cyanopropyl (CN), nitro, amino, etc. Generally longer columns provide better separation due to higher theoretical plate numbers.

As the particle size decreases the surface area available for coating increases. Columns with 5-µm particle size give the best compromise of efficiency, reproducibility and reliability.

Factor(s)	Effect on column efficiency
Column length	Choose shorter column for shorter analysis time, lower back pressure and fast equilibration and less solvent consumption Choose longer columns for enhanced resolution
Column internal diameter	Choose wider diameter column for greater sample loading Choose narrow column for more sensitive and reduced mobile phase consumption

Particle size	Choose larger particle (5-10 µm) for sample with structurally different compounds Choose very large particle (15-20 µm) for preparative separation
Particle shape	Choose irregular particles when high surface area and high capacity is required Choose spherical particles for lower back pressure, column stability and greater stability
Pore size	Choose a pore size of 150 or less for sample with molecular weight less than 2000 Choose a pore size of 300 or less for sample with molecular weight greater than 2000
Carbon load	Choose high carbon loads for greater column capacities and resolution Choose low carbon loads for fast analysis
Surface area	Choose end capped packing to eliminate unpredictable secondary interaction with the base materials Choose non-end capped phase for selectivity differences for polar compounds by controlling secondary interaction

Table 1- Various Factors for column Selection

2.2.2 Selection of Mobile Phase[20]

- The mobile phase effects resolution, selectivity and efficiency.
- Mobile phase composition (or solvent strength) plays an important role in RP-HPLC separation.
- Acetonitrile (ACN), methanol (MeOH) and tetrahydrofuran (THF) are commonly used solvents in RP-HPLC having low UV cut-off of 190, 205 and 212nm respectively. These solvents are miscible with water. Mixture of acetonitrile and water is the best initial choice for the mobile phase during method development.
- In many cases, the colloquial term used for the mobile phases in reversed phase chromatography is "buffer"

2.2.3 Buffer Selection[21]

Choice of buffer is governed by the pH that is desired. The typical pH range for reversed phase on silica based packing is pH 2 to 8. It is important that the buffer has a pKa close to the desired pH since buffer controls pH best at their pKa.

General consideration for buffer selection:

- Phosphate is more soluble in methanol/water than in acetonitrile/water or THF/water.
- Ammonium salts are generally more soluble in organic/water mobile phases
- Microbial growth can quickly occur in buffered mobile phases that contain little or no organic modifier at all. The growth accumulates on column inlets and can damage chromatographic performance
- Ammonium bicarbonate buffers usually are prone to pH changes and are usually stable for only 24 - 48 hrs. The pH of this mobile phase tends to become more basic due to the release of carbon dioxide.

- Some salt buffers are hygroscopic and this may lead to changes in the chromatography like increased tailing of basic compounds and possibly selectivity differences
- Trifluoroacetic acid can degrade with time. It is volatile and absorbs at low UV wavelengths
- At pH greater than 7, phosphate buffer accelerates the dissolution of silica and severely shortens the lifetime of silica-based HPLC columns. If possible, organic buffers should be used at pH greater than 7.
- After buffers are prepared, they should be filtered through a 0.2-µm filter.
- Mobile phases should be degassed

2.2.4 Selection of detectors [22-24]

Detector is a very important part of HPLC. Selection of detector depends on the chemical nature of analyses, potential interference, limit of detection required, availability and/or cost of detector. UV visible detector is versatile, dual wavelength absorbance detector for HPLC. This detector offers the high sensitivity required for routine UV-based applications to low-level impurity identification and quantitative analysis. Photodiode Array (PDA). Detector offers advanced optical detection for Waters analytical HPLC, preparative HPLC, or LC/MS system solutions. Its integrated software and optics innovations deliver high chromatographic and spectral sensitivity. Refractive index chromatographic and spectral sensitivity, stability and reproducibility, which make this detector the ideal solution for analysis of components with limited or no UV absorption. Multi-wavelength Fluorescence Detector offers high sensitivity and selectivity fluorescence detection for quantitating low concentrations of target compounds.

Detector	Type of compound can be detected
UV-Visible &Photodiode array	Compounds with chromophores, such as aromatic rings or multiple alternating double bonds.
Conductivity detector	Charged compounds, such as inorganic ions and organic acid.
Electrochemical detector	For easily oxidized compounds like quinines or amines
Fluorescence detector	Fluorescent compounds, usually with fused rings or highly conjugated planer system.
Refractive Index detector & Evaporative light scattering detector	Compounds that do not show characteristics usable by the other detectors, eg.polymers, saccharides.
Mass Spectroscopic Detector	Mass spectroscopy offers very high sensitivity and selectivity. Detection is based on fragmentation of molecules by electric

	fields and separation on basis of mass to charge ratios of fragmented molecules.
Optical Rotation detector	This detector is specific for the optical isomer measurement. The column can separate R- and L- type optical isomers and the detector can distinguish the same
ICP detector	Inductively coupled plasma–mass spectrometry (ICP-MS)

Table 2- Types of detectors and it's use

2.2.5 Selection of organic modifiers [25-27]

Most widely used solvents in reverse-phase chromatography are methanol and acetonitrile. Tetrahydrofuran (THF) is also used but to a lesser extent. In most of the systems, acetonitrile is used as the default organic modifier because of favourable UV transmittance and low viscosity. It is recommended to mix acetonitrile with 5–10% of the aqueous solution(s) to avoid the pumping problems associated with a higher percent (%) of acetonitrile usage. Methanol is also the second most widely used solvent in liquid chromatography, but it gives the back pressure to LC column. Though THF has some disadvantages like higher UV absorbance, reactivity with oxygen, and slower column equilibration, sometimes it gives very unique selectivity for closely eluting peaks. Intermediate selectivity (if needed for a particular sample) can be obtained by blending appropriate amounts of each of these solvents.

Order of polarity	Methanol > acetonitrile > ethanol > THF > propanol.
Order of solvent strength	Propanol > THF > ethanol > acetonitrile > methanol.

Table 3- Order of Polarity and Solvent Strength

2.2.6 Selection of ion-pair reagents [28-29]

Ion pair reagents are necessary as a mobile-phase additive when structurally or chemically or polarity wise inseparable closely related compounds are to be separated. For example, if a mixture of ionic and non-ionic analyte(s) having the same polarity and same retention time is required to be separated, start by optimizing for one of the analytes by adding an ion pair reagent in a mobile phase which reduces or increases the polarity of component and helps in increasing the elution time difference. Careful choice of an appropriate ion-pair reagent is required in such cases to get the necessary selectivity. A dedicated LC column is used when an ion pair reagent (0.0005 M to 0.02 M) is intended to employ for specific analysis, but an appropriate cleaning procedure has to be established to enhance the lifetime of the column material. Alkyl ammonium salts (tertiary or quaternary) and alkyl sulfonate salts are the most useful in the separation of acidic and basic compounds, respectively. Sodium perchlorate can also be used for acidic components.

2.2.7 Selection of flow rate [30-31]

Separation of mixtures is highly influenced by the flow of mobile phase inside the column. The flow rate is highly crucial in having well-separated peaks with no tailing. The flow rate of the mobile phase can be optimized based on the retention time, column back pressure, and separation of closely eluting adjacent peaks or impurities and peak symmetries from the test run. Preferably the flow rate is fixed not more than 2.0 mL/minute. The flow which gives the least retention times, good peak symmetries, least back pressures, and better separation of adjacent peaks/impurities could be the chosen as an optimized flow rate for the analysis.

2.2.8 Selection of column temperature [32-33]

Temperature is another criterion which has to be optimized for any sample, as the flow rate and the rate of adsorption vary with temperature. It is generally believed that with increasing temperature, it can help to improve the resolution between the adjacent/closely eluting peaks and peak merging. So, a careful choice of the temperature is a must which might change the pressure of the column and ultimately the elution and resolution. Choosing ambient temperature for the analysis is always preferred as it will minimize the degradation of the test sample; however, higher temperatures are also advisable under unavoidable conditions after confirming the stability of the compound. The temperature range which is usually allowed in liquid chromatography is 25 and 60°C. Higher temperatures above 60°C are preferred if the peak symmetry is not good and to increase the retention time for closely occurring peaks.

2.2.9 Selection of diluent [34]

Diluent is an aqueous solution or a solvent used to dissolve and extract the drug moiety for analysis. Select a diluent in which impurities, starting material, by-product, intermediates, degradation products, and the analyte are soluble. It is advisable to check first in the mobile phase. All the analytes should be completely soluble and the solution should be clear. Diluent should be compatible with the mobile phase to obtain the good peak shape.

• Selection of diluent based on extraction efficiency and peak shapes: Select the diluent for finished dosage forms, in which the analyte should be extracted at least 95% for assay and 90% for organic impurities. Calculate the % extraction against pure standard compound in the concentration of linear range, (preferably <1 AU) by diluting the test preparation.

2.2.10 Methods of extraction [35-36]

General methods followed for extraction are sonication, rotary shaking, or seldom both. In some cases where the analyte cannot be extracted by the above procedures, heating can be adapted if the substance is stable and should not precipitate upon cooling to room temperature

2.2.11 Selection of test concentration and injection volume [37]

The test concentration and injection volume are generally chosen based upon the response of API peak at the selected detector wavelength. However, the test concentration shall be finalized after it is proven that drug (API) is completely extractable at the selected test concentration. After finalizing the test concentration and diluent, prepare a test solution, and keep the filtered solution in closed condition on a bench top, and check whether the solution has any precipitation or turbidity after 24 hours. Generally, the test solution must be clear and should not show any turbidity or precipitation.

2.3 Developing the approach of analysis [38-40]

While developing the analytical method on RP-HPLC the first step which is followed is the selections of various chromatographic parameters like selection of mobile phase, selection of column, selection of flow rate of mobile phase, selection of pH of mobile phase. All of these parameters are selected on the basis of trials and followed by considering the system suitability parameters. Typical parameters of system suitability are e.g.

- **Retention time** should be more than 5 min,
- **The theoretical plates** should be more than 2000,
- **The tailing factor** should be less than 2,
- **Resolution** between 2 peaks should be more than 1.5,
- **% R.S.D.** of the area of analyte peaks in standard chromatograms should not be more than 2.0 %. like other.
- **Detection wavelength** is usually isobestic point in the case of simultaneous estimation of 2 components.

After this the linearity of the drug is studied in order to know the range of concentrations up to which the drug follows the linear pattern. Analysis of the laboratory mixture is also carried out in order to know practicability of developed method for simultaneous estimation. After that analysis of marketed formulation is carried out by diluting the marketed formulation up to concentration range of linearity.

2.4 Sample preparation:[42]

Sample preparation is an essential part of HPLC analysis, intended to provide a reproducible and homogenous solution that is suitable for injection onto the column. The aim of sample preparation is a sample aliquot that, is relatively free of interferences, Will not damage the column, and Is compatible with the intended HPLC method that is, the sample solvent will dissolve in the mobile phase without affecting sample retention or resolution. Sample preparation begins at the point of collection, extends to sample injection onto the HPLC column.

2.5 Method optimization:[43]

Identify the "weaknesses" of the method and optimize the method through experimental design. Understand the method performance with different conditions, different instrument set ups and different samples.

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