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A REVIEW ON EXPERIMENTAL DESIGNS IN HPLC METHOD DEVELOPMENT AND VALIDATION

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Abstract

The development and validation of High-Performance Liquid Chromatography (HPLC) methods are critical steps in ensuring the accurate, reliable, and reproducible quantification of analytes in various matrices. This study outlines a systematic approach to HPLC method development and validation, including the selection of appropriate stationary and mobile phases, optimization of chromatographic conditions, and evaluation of method performance. The developed method demonstrated excellent performance with linear calibration curves (R^2>. 0.999), high precision (%RSD < 2%), and recoveries reliability under slight variations in analytical conditions. These findings confirm the suitability of the method for routine analysis in pharmaceutical, clinical, or industrial applications. Every day many chromatographers face the need to develop a high-performance liquid chromatography (HPLC) method. This review describes systematic procedures for developing an HPLC method, based on the best information available. HPLC method development involves several essential steps: sample pretreatment, detection of sample bands, choosing separation conditions, quantitation, and method validation. HPLC is an analytical tool which is able to detect, separate and quantify the drug, its various impurities and drug related degrades that can form on synthesis or storage. This article describes strategies & an issue comes in development of a HPLC method development & validation.

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Introduction

High-Performance Liquid Chromatography, also known as High-Pressure Liquid Chromatography, is a type of column chromatography that is commonly used in biochemistry and analysis to separate, identify, and quantify active chemicals. It is a popular analytical technique for separating, identifying, and quantifying each element of a mixture. HPLC is a sophisticated column liquid chromatography technology [1]. The solvent normally flows through the column due to gravity, but in the HPLC process, the solvent is pushed under high pressures of up to 400 atmospheres so that the sample can be separated into different constituents based on differences in relative affinities. HPLC generally comprises a column that contains packing material (stationary phase), a pump that drives the mobile phase(s) through the column, and a detector that detects the molecule retention times [2]. The retention time is affected by the interactions between the stationary phase, the molecules being analysed, and the solvent(s) utilized. The samples to be analysed are added in small quantities to the mobile phase stream and are slowed by specific chemical or physical interactions with the stationary phase. The amount of retardation is determined by the nature of the analyte as well as the composition of both the stationary and mobile phases. The retention time is the time it takes for a certain analyte to elute. Any miscible combination of water or organic liquids is a common solvent. Gradient elution has been used to change the mobile phase composition during the analysis. The gradient separates analyte mixtures based on the analyte's affinity for the current mobile phase. The nature of the stationary phase and the analyte influence the choice of solvents, additives, and gradient.

Objective

- To learn the separation techniques of HPLC chromatography.
- To use these techniques to separate and identify o-nitroaniline and p-nitroaniline in sample mixture.

- To identify the compounds-based TLC Rf and HPLC Rt.
- To develop rapid. Sensitive and selective method
- Economic and accurate method
- Method validation according to ICH guidelines

Classification of HPLC

- HPLC is classified into analytical and preparatory categories based on the scale of operation.
- Various chromatographic techniques include size exclusion, affinity, and adsorption chromatography.
- Chiral phase and ion exchange chromatography are categorized based on the principle of separation.
- Isocratic and gradient separation methods distinguish chromatography based on elution technique.
- Chromatography operates in normal and reverse phases, determined by modes of operation [4].

HPLC Principle

High-performance liquid chromatography (HPLC) relies on the distribution of the analyte between a stationary phase and a mobile phase (eluent), typically within the column's packing material. The chemical structure of the analyte dictates its movement rate through the stationary phase, forming the basis for separation. This principle enables precise separation and analysis of diverse compounds, making HPLC a fundamental technique in analytical chemistry, particularly in pharmaceutical and chemical industries

The principle is that a solution of the sample is injected into a column of a porous material (stationary phase) and a liquid (mobile phase) is pumped at high pressure through the column. 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 behaviours of different components, elution at different time takes place. In a separation column between a stationary and a mobile phase, the purification happens. A separation column contains a granular substance with incredibly small porous particles as the stationary phase. On the other hand, the mobile phase is a solvent or solvent mixture that is pushed through the separation column under high pressure. The sample is injected into the mobile phase flow from the pump to the separation column via a valve with a connected sample loop, which is a tiny tube or a stainless steel capillary [5].

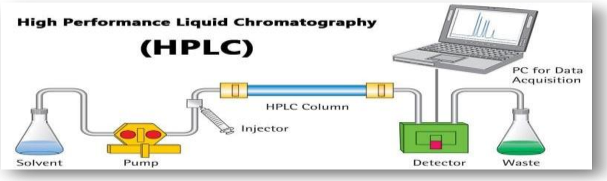

Fig. 1: FLOW DIAGRAM OF HPLC[4]

HPLC of Method Development

Stationary Phase

HPLC is the most advanced form of modern TLC. It uses HPLC plate featuring small particles with a narrow size distribution which results in homogenous layers with a smooth surface to be obtained. HPLC uses smaller plates (10 × 10 or 10 × 20 cm). HPLC plates provide improved resolution, higher detection sensitivity, and improved in situ quantification and are used for industrial pharmaceutical densitometric quantitative analysis. Normal phase adsorption TLC on silica gel with a less polar mobile phase, such as chloroform– methanol, has been used for more than 90% of reported analysis of pharmaceuticals and drugs [10].

1. Simple and precise HPLC methods were developed for the simultaneous estimation of two anti-inflammatory drugs (curcumin andgalangin). The method was tailored to analyze both drugs in their commercial dosage form(capsules)with no interference forming residents. Chromatographic separation was performed over precoated hplc plate [F254,20 HPLC plate (F254,20cm×10cm,250µm thickness, Merck, Darmstadt, Germany) viaalineara sending technique using n-hexane, ethyl acetate, acetic acid, and methanol as the mobile phase.
2. The report of HPLC densitometric method, which has been developed and validated for quantification of stigmasterol petroleum ether extract of leaves and stems of Bryophyllumpinnatum. The separation was performed on HPLC aluminum plates precoated with silica gel 60 F254. Good separation was achieved in mobile phase using Chloroform: Ethanol (9.8:0.2 v/v). Determination and quantitation were performed by densitometric scanning at 490 nm in reflection/absorbance mode.

Mobile Phase

The selection of mobile phase is based on adsorbent material used as stationary phase and physical and chemical properties of analyte. The mobile-phase systems are used based on their diverse selectivity properties are diethyl ether, methylene chloride, and chloroform combined individually or together with hexane as the strength adjusting solvent for normal-phase HPLC and methanol, acetonitrile, and tetrahydrofuran mixed with water for strength adjustment in reversed-phase HPLC. Separations byion pairing on C-18 layers are done with a mobile phase such as methanol–0.1 M acetate buffer (pH 3.5) containing 25 mM sodium pentane sulfonate.

Method optimization

Identify the method's "weaknesses" and optimize the method using experimental design. Understand how the approach performs under varied settings, with different instrument setups, and with different samples. The majority of HPLC technique development optimization has been focused on the optimization of HPLC conditions.34The compositions of the mobile phase and stationary phase must be considered. Optimization of mobile phase parameters is always prioritized above optimization of stationary phase parameters since it is considerably easier and more comfortable. Only the parameters that are likely to have a substantial effect on selectivity in the optimization must be studied to reduce the number of trial chromatograms involved. The different components of the mobile phase defining acidity, solvent, gradient, flow rate, temperature, sample quantities, injection volume, and diluents solvent type are primary control variables in the optimization of liquid chromatography (LC) procedures.

Methodology for Robustness Testing:

Experimental Design

A designed experiment (e.g., Box-Behnken Design or Plackett-Burman Design) can be used to systematically alter the parameters and evaluate the resulting effects on method performance.

Data Collection

Record chromatographic data for each variation, focusing on parameters like retention time, peak symmetry, resolution, and baseline noise.

Statistical Analysis

Use statistical tools (e.g., ANOVA, response surface methodology) to evaluate the significance of each factor and determine how sensitive the method is to each parameter variation [12].

Method of Validtion

The act of demonstrating that a method's presentation characteristics meet the requirements for the anticipated scientific application through research facility inspections is the approval of an insightful procedure. Each new or modified approach must first receive approval in order to verify that it will provide results that are predictable and trustworthy when applied by various administrators using the same hardware in the same or a different research institution. The method being discussed and its anticipated goals will determine exactly what kind of approval programme is needed. Method approval research can be used to assess the calibre, dependability, and consistency of scientific findings; it is an essential component of any competent perceptive practise.

Accuracy

Accuracy is the nearness of a measured value to the true or accepted value. Accuracy indicates the deviation between the mean value found and the true value. Let is determined by applying the method to samples to which known amounts of analyte have been added. These should be analysed against standard and blank solutions to ensure that no interference exists.

Specificity

The precision of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample. Precision is a measure of the reproducibility of the whole analytical method [23]. It consists of two components: repeatability and intermediate precision.

Precision

An analytical procedure's precision expresses the degree of agreement (degree of scattering) between a series of measurements acquired from multiple samplings of the same homogenous sample under the required conditions.43 An analytical procedure's precision expresses the degree of agreement (degree of scattering) between a series of measurements acquired from multiple samplings of the same homogenous sample under the required conditions. Precision is classified into three categories: repeatability, intermediate precision, and reproducibility [44]. The standard deviation or relative standard deviation of a sequence of data is commonly used to express the precision of an analytical technique.

Linearity

The capacity of an analytical process to produce test results that are directly proportional to the concentration of analyte in the sample (within a certain range) is referred to as linearity. If the method is linear, the test findings are proportional to the concentration of analyte in samples within a given range, either directly or through a well-defined mathematical transformation [13].

Repeatability

Repeatability is the variation experienced by a single analyst on a single instrument. It does not distinguish between variation from the instrument or system alone and from the sample preparation process. During validation, repeatability is performed by analysing multiple replicates of an assay composite sample by using the analytical method. The recovery value is calculated.

Intermediate

Intermediate precision is the variation within a laboratory such as different days, with different instruments, and by different analysts. The precision is then expressed as the relative standard deviation. Accuracy and precision are not the same, as the diagram below indicates. A method can have good precision and yet not be accurate.

Range

Range of an analytical method is the interval between the upper and lower concentration of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The range is normally derived from the linearity studies and depends on the intended application of the procedure. The following minimum specified ranges should be considered.

Robustness

The capacity of an analytical method to remain unaffected by minor but deliberate adjustments in method parameters (e.g., pH, mobile phase composition, temperature, and instrumental settings) is characterized as robustness, and it indicates its reliability during typical operation [13].

Application of HPLC

The information that can be obtained using HPLC includes identification, quantification, and resolution of a compound. Preparative HPLC refers to the process of isolation and purification of compounds. This differs from analytical HPLC, where the focus is to obtain information about the sample compound.

Chemical Separations: It is based on the fact that certain compounds have different migration rates given a particular column and mobile phase, the extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase.

Purification

Purification is defined as the process of separating or extracting the target compound from a mixture of compounds or contaminants. Each compound showed a characteristic peak under certain chromatographic conditions. The migration of the compounds and contaminants through the column need to differ enough so that the pure desired compound can be collected or extracted without incurring any other undesired compound.

Identification

Generally, assay of compounds is carried using HPLC. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels which the assay will be performed.

Pharmaceutical Applications: The pharmaceutical applications the include controlling of drug stability, dissolution studies and quality control.

Environmental Applications: Structure elucidation and Monitoring of unknown pollutants and detecting components of drinking water. steroids in biological samples.

Pharmaceutical applications

- Quality control
- Content Uniformity Test (CUT
- Identity- and purity checks
- Stability tests, etc.

Clinical applications

- Lipids
- Metabolism studies
- Drug screening
- Doping control, etc.

Cosmetics

- Identity of raw material
- Preservatives, coloring materials, etc.
- Screening for illegal substances, etc.

Herbal medicines and botanical dietary supplements

- Identification
- Stability tests
- Detection of adulteration
- Assay of marker compounds, etc.

Food and feed stuff

- Quality control
- Additives (e.g. vitamins)
- Pesticides
- Stability tests (expiration), etc [16].

Advantage of HPLC

Separations are fast and efficient (high-resolution Continuous monitoring of the column effluent.

- It can be applied to the separation and analysis of very complex mixtures
- Accurate quantitative measurements.
- Repetitive and reproducible analysis using the same column.
- Adsorption, partition, ion exchange, and exclusion column separations are excellently made.
- HPLC is more versatile than GLC in some respects because it has the advantage of not being restricted to volatile and thermally stable solute and the choice of mobile and stationary phases is much wider in HPLC.
- Aqueous and non-aqueous samples can be analyzed with little or no sample pre- treatment.
- A variety of solvents and column packing are available, providing a high degree of selectivity for specific analyses.
- It provides a means for the determination of multiple components in a single analysis and etc [17].

Disadvantages of HPLC

- Column performance is very sensitive, which depends on the method of Packing.
- Further, no universal and sensitive detection system is available.
- Very costive, have low sensitivity for certain compounds, and some cannot be detected as they are irreversibly adsorbed.
- Low sensitivity for some compounds [18].

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No

Informed Consent

Not Applicable.

Ethical Statement

Not Applicable.

Author Contribution

All authors are contributed equally.

Conclusion

The successful development and validation of the HPLC method provide a robust, reliable, and accurate analytical tool for the quantification of target analytes in pharmaceutical and other industries. The optimization of chromatographic parameters, including the choice of mobile phase, column type, and detection wavelength, has led to a method that is both efficient and reproducible. Validation results, in compliance with ICH guidelines, demonstrated that the method is specific, precise, accurate, and linear over a wide concentration range. Furthermore, the low LOD and LOQ, coupled with high recovery rates, affirm its suitability for routine analysis. Robustness tests showed minimal sensitivity to variations in method parameters, further proving the method's reliability. This method is now deemed suitable for the quality control of raw materials, intermediate products, and final formulations. It offers a valuable tool for ensuring product consistency and safety, facilitating regulatory compliance, and advancing analytical capabilities in various sectors. The study highlights the importance of method validation in confirming analytical method quality and reinforces the significance of continuous method refinement in scientific research and industrial applications.

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