INTRODUCTION

High-Performance Liquid Chromatography (HPLC), 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. 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. The retention time is affected by the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) utilized. The samples to be analyzed 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 gradients.
Based on the elution technique
Gradient separation and isocratic separation

Based on modes of operation
Normal phase chromatography and reverse-phase chromatography.

A. Normal phase chromatography:
The mobile phase in normal phase chromatography is non-polar, whereas the stationary phase is polar. As an outcome, the polar analyte is retained by the station phase. The increased polarity of solute molecules improves adsorption capacity, resulting in a longer elution time. In this chromatography, a stationary phase of chemically modified silica (cyanopropyl, aminopropyl, and diol) is used. As an example, a typical column has an interior diameter of around 4.6 mm and a length ranging from 150 to 250 mm. Polar compounds in the mixture that are passed through the column will stick to the polar silica for a longer period than non-polar compounds. As a result, the non-polar ones will go quickly through column.

B. RP-HPLC (Reversed-phase HPLC):
The stationary phase of RP-HPLC is non-polar, and the mobile phase is polar or moderately polar. The notion of hydrophobic interaction underpins RP-HPLC. The non-polar stationary phase will hold analytes that are comparatively less polar in a combination of components for a longer period than those that are substantially more polar. As a result, the most polar component elutes the first.

METHOD DEVELOPMENT ON HPLC

Figure 2: Steps involved in HPLC Method development
Method development involves the following steps:
1. Understanding the Physicochemical Properties of the drug molecule.
2. Selection of chromatographic conditions.
3. Developing the approach of analysis.
4. Sample preparations
5. Method optimization
6. Method validation

1. Understanding the Physicochemical Properties of the drug molecule

The physicochemical qualities of a therapeutic molecule are critical in method development. To develop a method, one must first evaluate the physical properties of the drug molecule, such as solubility, polarity, pKa, and pH. A compound’s polarity is a physical property. It supports an analyst in evaluating the solvent and mobile phase composition. The polarity of molecules can be used to explain molecular solubility. Polar solvents, such as water, and nonpolar solvents, such as benzene, do not combine. In general, like dissolves like, which means that elements with comparable polarities dissolve in one other. The analyte’s solubility is used to select diluents. The pH value is commonly used to define a substance’s acidity or basicity. Choosing the correct pH for ionizable analytes frequently results in symmetrical and crisp peaks in HPLC. The pH value is defined as the negative of the logarithm to base 10 of the hydrogen ion concentration:

\[ \text{pH} = -\log_{10}[\text{H}^+] \]

Selecting an appropriate pH for ionizable analytes frequently results in symmetrical and sharp peaks in HPLC. In quantitative analysis, sharp, symmetrical peaks are required to achieve low detection limits, low relative standard deviations between injections, and predictable retention durations.

2. Selection of chromatographic conditions

During the early stages of method development, a set of beginning conditions (detector, column, and mobile phase) is chosen to generate the sample's first "scouting" chromatograms. These are typically based on reversed-phase separations on a C18 column with UV detection. At this point, a choice should be taken between establishing an isocratic or a gradient method.

Selection of column

The first and most significant stage in method development is the selection of the stationary phase or column. It is impossible to develop a robust and reproducible procedure without the availability of a stable, high-performance column. Columns must be stable and reproducible to avoid difficulties caused by irreproducible sample retention during technique development. A C8 or C18 column made of particularly purified, less acidic silica and specifically intended for the separation of basic chemicals is generally suitable for all samples and is strongly recommended.

The key ones include column diameters, silica substrate qualities, and bonded stationary phase characteristics. Due to a variety of physical properties, silica-based packing is preferred in the majority of today's HPLC columns.
hardware, matrix, and stationary phase are the three primary components of an HPLC column.\textsuperscript{22}

Silica, polymers, alumina, and zirconium are some of the matrices used to sustain the stationary phase. The most common matrix for HPLC columns is silica. Silica matrices are strong, easily derivatized, produced in constant sphere sizes, and do not compress under pressure. Most organic solvents and low pH solutions are chemically stable to silica. One disadvantage of a silica solid support is that it dissolves above pH 7 \textsuperscript{23}.

**Selection of Chromatographic mode**

The chromatographic modes are determined by the molecular weight and polarity of the analyte. All case studies will concentrate on reversed-phase chromatography (RPC), which is the most prevalent technique for tiny organic compounds. RPC is frequently used to separate ionizable substances (acids and bases) using buffered mobile phases (to keep the analytes from becoming ionized) or ion-pairing reagents \textsuperscript{24}

**Buffer Selection**

Different buffers, such as potassium phosphate, sodium phosphate, and acetate, were tested for system compatibility factors and overall chromatographic performance.

**General consideration for buffer selection**

Phosphate dissolves more easily in methanol/ water than in acetonitrile/water or THF/water. Some salt buffers are hygroscopic, which can cause chromatographic alterations such as enhanced tailing of basic chemicals and possibly selectivity discrepancies. In general, ammonium salts are more soluble in organic/water mobile phases. Trifluoroacetic acid degrades with time. It is a volatile substance that absorbs at low UV wavelengths. Microbial growth can occur quickly in buffered mobile phases containing little or no organic modifier. The growth builds on the inlets of the columns and can impair chromatographic performance. At pH more than 7, phosphate buffer increases silica dissolution and significantly reduces the lifetime of silica-based HPLC columns. Organic buffers should be utilized, if possible, at pH levels higher than 7. Ammonium bicarbonate buffers are typically prone to pH shifts and are only stable for 24 - 48 hours. Because of carbon dioxide emission, the pH of this mobile phase tends to become more basic. After preparing the buffers, they should be filtered using a 0.25-m filter Degassing of mobile phases is required \textsuperscript{25}.

**Buffer Concentration**

A buffer concentration of 10-50 mm is usually sufficient for small molecules. In general, a buffer should not contain more than 50% organic material. This will be determined by the type of buffer as well as its concentration. The most frequent buffer systems for reversed-phase HPLC are phosphoric acid and its sodium or potassium salts. When testing organophosphate chemicals, sulfonate buffers can be used instead of phosphonate buffers \textsuperscript{26}.

**Selection of Mobile Phase**

The mobile phase influences resolution, selectivity, and efficiency. The composition of the mobile phase (or the strength of the solvent) is critical in RP-HPLC separation. Acetonitrile (ACN), methanol (MeOH), and tetrahydrofuran (THF) are regularly used solvents in RP-HPLC, with UV cut-offs of 190, 205, and 212nm, respectively. These solvents are miscible with water. During technique development, an acetonitrile-water mixture is the ideal initial choice for the mobile phase \textsuperscript{27}.

**Table 1:** The overall study of Technique\textsuperscript{28}.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Mode</th>
<th>Solvent type Used</th>
<th>Type of Compound Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reversed-Phase</td>
<td>Water/Buffer, CAN, Methanol</td>
<td>Neutral or non-ionized Compound which can be dissolved in water.</td>
</tr>
<tr>
<td>2</td>
<td>Ion-Pair</td>
<td>Water/Buffer, CAN, Methanol</td>
<td>Ionic or Ionizable Compound</td>
</tr>
<tr>
<td>3</td>
<td>Normal Phase</td>
<td>Organic Solvent</td>
<td>A mixture of isomer &amp; compound not Soluble in Organic / Water mixture</td>
</tr>
<tr>
<td>4</td>
<td>Ion Exchange</td>
<td>Water/Buffer</td>
<td>Inorganic ions, Protein, nucleic acid, organic acid.</td>
</tr>
<tr>
<td>5</td>
<td>Size exclusion</td>
<td>Water, Chloroform</td>
<td>High Molecular weight compound</td>
</tr>
</tbody>
</table>

**Table 2:** Table of Different detectors & Type of compounds detected by them\textsuperscript{29}.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Detector</th>
<th>Type of compound can be detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UV-Visible &amp; Photodiode array</td>
<td>Compounds with chromophores, such as aromatic rings or multiple alternating double bonds.</td>
</tr>
<tr>
<td>2</td>
<td>Fluorescence detector</td>
<td>Fluorescent compounds, usually with fused rings or highly conjugated planer system</td>
</tr>
<tr>
<td>3</td>
<td>Conductivity detector</td>
<td>Charged compounds, such as inorganic ions and organic acid.</td>
</tr>
<tr>
<td>4</td>
<td>Electrochemical detector</td>
<td>For easily oxidized compounds like quinines or amines</td>
</tr>
<tr>
<td>5</td>
<td>Refractive Index detector &amp; Light scattering detector</td>
<td>Compounds that do not show characteristics usable by the other detectors, eg. polymers, saccharides.</td>
</tr>
</tbody>
</table>

**Selection of detectors**

The detector is a critical component of HPLC. The detector to be used is determined by the chemical composition of the studies, potential interference, the detection limit
required, detector availability, and/or detector cost. UV detectors, fluorescence detectors, electrochemical detectors, refractive index (RI) detectors, and mass spectrometry (MS) detectors are examples of commercial detectors used in LC. The detector used is determined by the sample and the goal of the analysis.29

3. Developing the approach of analysis

The initial stage in developing an analytical method for RP-HPLC is to select various chromatographic parameters such as mobile phase, column, mobile phase flow rate, and mobile phase pH. All of these characteristics are chosen based on trials, and they are then compared to the system suitability parameters. Typical system suitability parameters include, for example, a retention time of more than 5 minutes, a theoretical plate count of more than 2000, a tailing factor of less than 2, a resolution of more than 5, and a percent R.S.D. of the area of analyte peaks in standard chromatograms of no more than 2.0%. In the case of simultaneous estimation of two components, the detection wavelength is usually an isosbestic point. The laboratory combination is also analyzed to determine the practicability of the suggested method for simultaneous estimation. Following that, the marketed formulation is analyzed by diluting it up to the concentration range of linearity.31

4. Sample preparation

Sample preparation is a vital step in method development that the analyst must study. For example, if there are insoluble components in the sample, the analyst should explore whether centrifugation (determining the ideal rpm and time), shaking, and/or filtration are required. The goal is to show that sample filtering does not affect the analytical result due to adsorption and/or extraction of leachable. The ability of syringe filters to remove contaminants/insoluble components without leaching unwanted artifacts (i.e., extractable) into the filtrate determines their effectiveness. The sample preparation procedure should be fully specified in the appropriate analytical method used on an actual in-process sample or dosage form for later HPLC analysis.32

The manufacturer, type of filter, and pore size of the filter media must be specified in the analytical technique.32 The goal of sample preparation is to provide a processed sample that yields superior analytical results than the raw sample. The prepared sample should be an aliquot that is reasonably free of interferences, compatible with the HPLC procedure, and will not harm the column. The goal of sample preparation is to provide a sample aliquot that is reasonably free of interferences, will not harm the column, and is compatible with the planned HPLC procedure, which means that the sample solvent will dissolve in the mobile phase without impacting sample retention or resolution. Sample preparation begins with sample collection and continues with sample injection onto the HPLC column.33

5. 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.34 The 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.35

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.36

After adequate selectivity has been attained, this is utilized to discover the optimal balance between resolution and analysis time. Column dimensions, column-packing particle size, and flow rate are among the parameters at play. These parameters are changeable without impacting the capacity factor or selectivity.37

6. Method Validation

Validation is the evaluation and provision of objective evidence that the specified requirements for a given intended application are met. A way of assessing method performance and demonstrating that it fits a specific condition. In other words, it understands what your method is capable of producing, especially at low concentrations.38

Analytical methods need to be validated or revalidated.

Before their introduction into routine use;

Whenever the conditions change for which, the method has been validated

Whenever the method is changed.39

COMPONENTS OF METHOD VALIDATION

Accuracy

The accuracy of an analytical procedure expresses the degree of agreement between the value acknowledged as a conventional true value or an approved reference value and the value discovered.40 The closeness of a measured value to the true or accepted value is defined as accuracy. In practice, accuracy denotes the difference between the mean value discovered and the genuine value.41

It is calculated by applying the procedure to samples containing known levels of analyte. To confirm that there is no interference, these should be compared to standard and blank solutions. The accuracy is then computed as a
percentage of the analyte recovered by the assay based on the test findings. It is frequently expressed as the recovery of known, added amounts of analyte by test. The accuracy is then computed as a percentage of the analyte recovered by the assay based on the test findings. It is frequently expressed as the recovery of known, added amounts of analyte by test.42

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.45

Precision can refer to the reproducibility or repeatability of an analytical method under normal conditions. Intermediate precision (also known as ruggedness) expresses variability within laboratories, for as on different days or with different analysts or equipment within the same laboratory46.

An analytical procedure's precision is determined by assaying a sufficient number of aliquots of a homogeneous sample to derive statistically accurate estimates of standard deviation or relative standard deviation47.

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.48

Linearity is typically stated as the confidence limit around the regression line's slope. A linear relationship should be investigated across the analytical procedure's range. The proposed approach is used to show it directly on the drug substance by dilution of a standard stock solution of the drug product components. Linearity is typically stated as the confidence limit around the regression line's slope.16-18 The ICH recommendation recommends a minimum of five concentrations for the establishment of linearity.49

Limits of detection and quantitation

An individual procedure's limit of detection (LOD) is the smallest amount of analyte in a sample that can be detected but not necessarily quantitated as an accurate number. In analytical techniques with baseline noise, the LOD might be based on a signal-to-noise (S/N) ratio (3:1), which is commonly stated as the analyte concentration in the sample. The limit of quantitation (LOQ) is defined as the lowest analyte concentration in a sample that can be measured with acceptable precision and accuracy under the method's stated operational circumstances. ICH recommends a signal-to-noise ratio of 10:1 for LOQ.50,51

LOD and LOQ can alternatively be computed using the standard deviation of the response (SD) and the calibration curve(s) slope at values close to the LOD using the formulae listed below.

LOD = 3.3 × S /SD and

LOQ = 10 × S /SD

Specificity

Specificity is the ability to assess the analyte unequivocally in the presence of components that are expected to be present. Impurities, degradants, matrices, and so on are examples of these. An individual analytical method's lack of specificity may be compensated for by another supporting analytical procedure.52 The following are the ramifications of this definition: Identification: the process of ascertaining the identity of an analyte. Purity tests are used to guarantee that all analytical processes are performed to allow for accurate characterization of an analyte's impurity concentration.53

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.54

Range

The method's range is the interval between an analyte's upper and lower levels obtained with appropriate precision, accuracy, and linearity.55

CONCLUSION

This article provides an overview of the RP-HPLC Technique method development and validation. Method development and validation are continuous and interconnected activities that measure a parameter as intended and determine the measurement's performance limits. The makeup of the buffer and mobile phase (organic and pH) has a significant impact on separation selectivity. The advantages of the HPLC technology were its great selectivity, sensitivity, low detection limit, and inexpensive cost. Finally, the gradient slope, temperature, flow rate, and the kind and concentration of mobile-phase modifiers can be optimized. The optimized method is validated using several criteria (such as specificity, precision, accuracy, detection limit, linearity, and so on) by ICH requirements.

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