Review Article

High Performance Liquid Chromatography: An Overview

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ABSTRACT

The HPLC is the most preferred separation method for identifying, separating, and determining pharmaceuticals. Numerous chromatographic parameters, including sample pretreatment, mobile phase selection, column and detector selection, were examined to optimise the procedure. The HPLC method can be used to analyse the majority of pharmaceuticals in multicomponent dosage forms due to its benefits including rapidity, precision, specificity, accuracy and simplicity of automation. The chemistry of the molecules, the synthetic route, solubility, polarity, pH and pKa values, and the activity of the functional groups are all factors that affect the development of an HPLC method. This article is primarily concerned with the instrumentation as well as method development and validation of HPLC conditions.

Keywords: HPLC, Instrumentation, Method, RP-HPLC, Validation.

INTRODUCTION

The pharmaceutical sector uses high-pressure or high-performance liquid chromatography (both are abbreviated as HPLC) most frequently to assess a wide range of sample types. It is the method of choice for doing quality control and assurance on the finished drug product, keeping track of changes to synthetic processes or scale-up, confirming the purity of new chemical entities, and evaluating new formulations. [1]

Principle of HPLC

While the mobile phase of HPLC entails pumping a liquid through the column at a higher pressure, the stationary phase of HPLC entails injecting the sample's solution into a porous column. The employed separation principle is the adsorption of the solute on the stationary phase based on affinity towards the stationary phase. Depending on the properties of the stationary phase, the separation process may go through multiple steps.

Adsorption chromatography, relies on repeated adsorption and desorption steps for separation; Separation is based on the partition between the mobile and stationary phases in partition chromatography. The Van Demeter equation, which connects the effectiveness of the chromatographic column to the column's particle size, molecular diffusion, and thickness of the stationary phase, serves as the basis for HPLC.[2,3]

HPLC Separation Methods

1. Normal Phase Chromatography (NP)

2. Reversed Phase Chromatography (RP)

1. Normal-Phase HPLC:

In normal phase chromatography, the stationary phase is polar whereas the mobile phase is non-polar. The station phase as a result retains the polar analyte. Longer elution times are produced by the enhanced adsorption capacity brought on by the more polar solute molecules. chemically modified silica (cyanopropyl, aminopropyl, and diol) is utilised as the stationary phase in this chromatography. For instance, A typical column ranges in length from 150 to 250 mm and has an internal diameter of about 4-6 mm. When the mixture is passed through the column, polar silica will retain the adhesion of polar compounds for a longer time than non-polar compounds. The non-polar ones will therefore pass through the column more rapidly.[4,5]

2. Reversed-Phase HPLC:

In RP-HPLC, the mobile phase is polar or moderately polar whereas the stationary phase is non-polar. RP-HPLC is supported by the hydrophobic interaction theory. In a mixture of components, the non-polar stationary phase will hold onto analytes that are comparatively less polar for a longer time than those that are relatively more polar. The most polar component elutes first as a result. Most of these methods employ an aqueous mixture of water and a polar, miscible organic solvent, like acetonitrile or methanol, as the mobile phase. This often ensures that analytes interact properly with the hydrophobic, non-polar surface of the particle. The most common reversed-phase HPLC packing is C18-bonded silica, often known as ODS.[6]

INSTRUMENTATION

A. Mobile Phase Reservoir

The mobile phase reservoir can be any clean, inert container. It usually contains 0.5–2L of solvent, and it should have a cap that allows for a tubing inlet line, which feeds the mobile phase to the solvent delivery system. The cap also serves to keep out dust, reduce solvent evaporation, allow for pressurization of the bottle, and offer ports for additional inlet lines, and sparging (i.e., dispersing He or Ar into the mobile phase to reduce

dissolved air). All mobile phases/solvents should be freshly filtered and preferably degassed. [7]

B. *Degasser*

Fresh filtration and, ideally, degassing should be performed on all mobile phases and solvents. In-line degassers are increasingly common additions to many HPLC systems and eliminate the need to degas mobile phase off-line. They are generally used to remove tiny gas bubbles and minimise dissolved air.[8]

C. *Pump*

Operating pressures for the high-pressure pump range from 500 to 20,000 psi. The purpose of the pump is to supply the column with a precise, accurate, repeatable, continuous, and pulse-free flow of the mobile phase. Constant pressure pumps, syringe-based or displacement pumps, and constant flow pumps are the three main classes of HPLC pumps now in use. Constant flow pumps are by far more prevalent because constant pressure and syringe-based pumps are not easily adaptable to gradient solvent delivery.[9,10]

D. Sample Injector

A single injection or an automated injection system can be the injector. The volume of the liquid sample that can be injected into an HPLC system should be between 0.1 and 100 mL, with good repeatability and high pressure (up to 4000 psi). The injector's type is:

1. Manual injection (Rheodyne/Valco injectors)
2. Automatic injection[11]

E. Column (Stationary Phase)

The HPLC column is where the separation takes place. Because HPLC operates under high pressure, smoothbore stainless steel tubing is typically used to build HPLC columns. Analytical scale columns typically have interior diameters of 4 to 10 mm and lengths between 10 and 30 cm. 3, 5, and 10 m are the most typical packing particle sizes. The above-mentioned columns frequently have the efficiency of 40,000–60,000 plates per meter. The employment of columns with higher performance and higher speeds, which have smaller dimensions than those mentioned above, has become the current trend. These columns have the benefit of speed and low solvent usage, with the efficiency of 100,000 plates per meter. Numerous manufacturers offer hundreds of packed columns in a variety of sizes and packaging materials.[12]

The types of columns are:

1. *Guard columns*

To reduce solvent losses from the analytical column, the guard column works to saturate the mobile phase with the stationary phase. The guard-column packing shares many characteristics with the analytical column, however, the particles are often larger.

2. *Analytical columns*

High-performance liquid chromatography revolves around it. The length of liquid chromatographic columns varies from 10 to 30 cm. The columns are typically straight; but, where necessary, extra length may be achieved by joining two or more columns together.

Column temperature control:

HPLC separations are carried out in ambient temperatures for many applications where perfect control of the column temperature is not required. UHPLC procedures normally need column heating, however, temperature control can improve chromatographic consistency and present opportunities to improve separation efficiency. The column temperature of contemporary instruments can be controlled to within a few tenths of a degree between near ambient and 150°C using column heaters/ovens. The controlled temperature ranges for some column compartments of column chillers range from 4 to 100 degrees Celsius.[8,13,14]

F. Detector

The solutes are observed as they are eluted from the column by passing the column effluent through the detector, which converts some chemical or physical property of the analyte into an electrical signal. The level of a particular property of the mobile phase or solutes is proportional to the electrical signal, which can be amplified and controlled by the appropriate electronics. The two types of HPLC detectors are bulk property detectors, which react to an eluent's bulk properties like conductivity or refractive index (RI), and solute property detectors, which react to analyte properties like UV absorbance. The presence and quantity of the analyte affect the detector's response in both scenarios. The ideal characteristics of an HPLC detector are high sensitivity, stability, linearity, short response time, reliability, non-destructiveness, ease of use, and low dead volume.[15]

- UV/Vis absorbance detectors

  Only UV-absorbing compounds, such as alkenes, aromatics, and compounds that have multiple bonds between C and O, N, or S are detected. Three main types of absorbance detectors are available: fixed-wavelength, variable-wavelength, and photodiode array.

- Electrochemical detectors

  The majority of electroactive substances that are found in nature (such as catechols, quinines, and aryl amines) have an aromatic substituent. Pulsed electrochemical detection (PED) can also be used to directly detect polar aliphatic substances, such as amines, carbohydrates, and other substances.

- Conductivity detectors

  only the charged analyte ions are detected.

- RI detectors

- IR detectors
- Mass spectrometry

  An MS detector detects a compound that is eluting from the HPLC column by first ionising it, followed by mass measurement and/or fragmenting the molecule into smaller, compound-specific fragments.[16,17]

G. Data Collection and Output

The electronic output of the detector is connected to a device for data collecting and output, such as a computer, integrator, or recorder. The data collecting tool outputs a response versus a time plot using the electronic signal generated by the detector. The chromatogram results can then be assessed for both qualitative and quantitative data. Nowadays, recorders are seldom ever used by themselves. The peaks of a chromatogram can be integrated by both integrators and computers, and both have the benefit of being able to store chromatograms for post collection processing. It is also possible to export data from computer-based collection systems to several different programmes and applications. The computer can often interface with and operate the complete HPLC system. Hence, the majority of modern HPLC systems are outfitted with computer control, data collection, and output systems. [8]

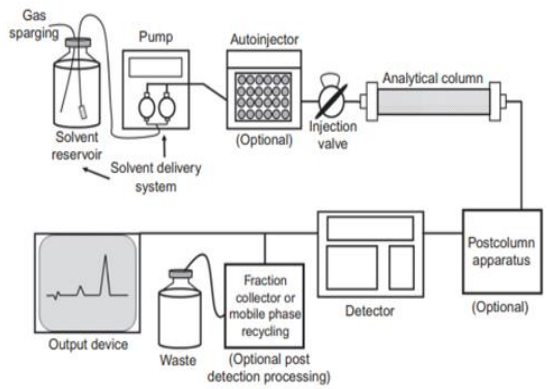

Figure 1: Instrumentation of HPLC

HPLC METHOD DEVELOPMENT

The following is a step in HPLC method development:

1. Information on sample

First, investigate the drug's molecule's physical characteristics, such as its solubility, polarity, pKa, and pH. Polarity is a physical characteristic of a compound. It aids in figuring out the composition of the solvent and mobile phase. Molecular solubility can be explained in terms of a molecule's polarity. Nonpolar solvents like benzene and polar solvents like water do not combine. The choice of mobile phase or diluents is influenced by the analyte's solubility. When creating HPLC procedures, the pKa and pH values are crucial. Sharp and symmetrical peaks in HPLC are usually produced by selecting an optimum pH for ionizable analytes. Sharp, symmetrical peaks are necessary in quantitative analysis to achieve low detection limits, small relative standard deviations between injections, and repeatable retention times.

2. Choosing chromatographic conditions

The first "scouting" chromatograms of the sample are produced using a set of basic conditions (detector, column, mobile phase) during the early stages of technique development. Reversed-phase separations on a C18 column with UV detection are frequently employed. Either an isocratic approach or a gradient method should be developed at this point.

Selection of Column:

Of course, the first and most crucial part of a chromatograph is a column. A good chromatographic separation can be produced by a carefully selected column, leading to precise and trustworthy analysis. A poorly used column can commonly result in unclear, insufficient, and bad separations, which can lead to invalid or challenging-to-understand results. The stationary phase chemistry, retention capability, particle size, and column dimensions must all be considered when choosing the optimal column for a given application. The three primary parts of an HPLC column are hardware, matrix, and stationary phase. Some of the matrices used to sustain the stationary phase include silica, polymers, alumina, and zirconium. Silica is the most often used matrix for HPLC columns.[13]

The following is a list of the most popular reverse phase columns and their uses for them. Ion-pairing chromatography (C4) and peptides containing hydrophobic residues, as well as other big molecules, benefit from the employment of the propyl (C3), butyl (C4), and pentyl (C5) phases. Non-polar solutes are typically less well retained by C3-C5 columns as compared to C8 or C18 phases. Examples are the Zorbax SB-C3, YMC-Pack C4, and Luna C5. Compared to columns with longer alkyl chains, these columns are often less resistant to hydrolysis. There are many uses for octyl (C8, MOS) phases. Although this phase is less retention-enhancing than the C18 phases, it is nevertheless advantageous for drugs, nucleosides, and steroids.[18]

Selection of Chromatographic mode:

The molecular weight and polarity of the analyte determine the chromatographic modes. For tiny organic compounds, reversed-phase chromatography (RPC) is typically the method of choice. Ion-pairing reagents or buffered mobile phases (to prevent the analytes from getting ionized) are widely used in RPC to separate ionizable chemicals (acids and bases).

Optimization of Mobile phase:

Buffer Selection:

The system appropriateness criteria and overall chromatographic performance of several buffers, including potassium phosphate, sodium phosphate, and acetate, were examined. It was found that potassium dihydrogen

phosphate was suitable for the efficient separation of all peaks after a series of tests with different buffers. We investigated buffers with potassium dihydrogen phosphate concentrations of 0.02M, 0.05M, and 0.1M. The elution pattern or resolution was not significantly altered by the change in buffer concentration, although the technique became more sensitive at 0.05M.[19]

Effect of pH:

If the target analyte is ionizable, the proper mobile-phase pH must be selected based on the analyte pKa such that it is either ionized or neutral.

Effect of organic modifier:

The choice of the organic modifier type in reverse phase HPLC is rather straightforward. Acetonitrile and methanol are the most prevalent alternatives (THF is infrequent). Since it may be hard to elute all components with a single solvent strength between k (retention factor) 1 and 10 under isocratic conditions, gradient elution is often utilised with complex multicomponent samples.[20]

Selection of detector:

The target analyte is identified using the right detectors after the chromatographic separation. Commercial detectors used in LC include UV detectors, fluorescence detectors, electrochemical detectors, refractive index (RI) detectors, and mass spectrometry (MS) detectors.[15,17]

3. Creating an analytic approach.

The choice of several chromatographic parameters, including the mobile phase, column, flow rate of the mobile phase, and mobile phase pH, is the initial stage in establishing an analytical procedure for HPLC. These characteristics are all selected through testing, and they are then contrasted with the parameters for system suitability. Examples of typical system suitability parameters are a retention time greater than 5 minutes, a theoretical plate count greater than 2000, a tailing factor less than 2, a resolution greater than 5, and an R.S.D. of the area of analyte peaks in standard chromatograms no greater than 2.0%. The detection wavelength is typically an isobestic point when two components are estimated simultaneously.[21]

4. Preparation of Samples

The solution must be consistent and homogeneous enough to be injected into the column, which is why sample preparation is a crucial step in HPLC analysis. Creating a sample aliquot that is largely devoid of interferences, will not damage the column, and is compatible with the desired HPLC method is the aim of sample preparation. To do this, the sample solvent must dissolve in the mobile phase without impacting sample retention or resolution.[22]

5. Method Improvement

Utilising experimental design, identify the method's "weaknesses" and improve it. Recognise how the approach operates in different scenarios, with various instrument configurations, and with various samples. [23]

VALIDATION OF METHODS

1. Selectivity / Specificity

   Specificity is one of the significant features of HPLC, and it refers to the ability of the analytical method to discriminate between the analyte and the other components in the complex mixture. The terms selectivity and specificity are often used interchangeably. It is the measure of the degree of interference from other active ingredients, excipients, impurities and degradation products, ensuring that a peak response is due to a single component only. [24]

2. Linearity and Range

   Analytical method linearity is defined as the ability of the method to obtain test results that are directly proportional to the analyte concentration, within a specific range

   Linearity is usually expressed in terms of variance around the slope of the regression line calculated according to an established mathematical relationship from the test results obtained by the analysis of the samples with varying concentration of analyte in it. The method is said to be linear when the regression coefficient (R^2) is ≥0.99.

   The range of an analytical method is the interval between the upper and lower level of the analyte that has been demonstrated to be determined with precision, accuracy, & linearity using the method as written.[25]

3. Precision

   The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.[26]

4. Accuracy

   The accuracy of an analytical method expresses the nearness between the expected value and the value found. It is obtained by calculating the percent recovery (R%) of the analyte recovered. For a drug substance, the common method of determining accuracy is to apply the analytical procedure to the drug substance and to quantitate it against a reference

standard of known purity. For the drug product, accuracy is usually determined by the application of the analytical procedure to synthetic mixtures of the drug product components or placebo dosage form to which known quantities of drug substance of known purity have been added. The range for the accuracy limit should be within the linear range. Typical accuracy of the recovery of the drug substance in the mixture is expected to be about 98 to 102%. Values of the accuracy of the recovery data beyond this range need to be investigated.[27]

5. Robustness

   The analytical technique's robustness is a measure of its ability to be unaffected by little but intentional changes in method parameters and offers a clue as to its dependability under typical conditions. By altering variables like temperature, buffer pH and ionic strength, and the amount of additives in the mobile phase, experiments can be carried out. The technique must be sturdy enough to survive minor adjustments and enable frequent analysis of the substance.[28,29]

6. Solution Stability

   Solubility of the standard, sample and reagents is required for a reasonable time to generate reproducible and reliable results. In solution stability test standard and sample solutions stored under suitable conditions such as at specific temperature over a specific period to evaluate the stability of the method. The stability of the solution is evaluated by comparing solution preparations at different time intervals to that initial.

7. Filter Compatibility

   In the filter Compatibility or Suitability test, the standard solution and sample solution are filtered through various or different filters to check the suitability or compatibility of filters to the analytical solutions. For standard and sample solutions, the results for filtered solutions (after discarding appropriate samples) to those for the unfiltered solutions can be compared.[30]

8. Limit of Detection & Limit of Quantitation:

   LOD is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. LOQ is the lowest concentration of analyte that can be determined with acceptable precision and accuracy. These two parameters were calculated using the formula LOD = 3.3 * S D/S and LOQ = 10 * SD/S, where SD = standard deviation of response (peak area) and S = slope of the calibration curve.[31,32]

APPLICATION

Pharmaceutical applications: Drug dissolution study of the pharmaceutical dosage form [33–36], to control drug stability, shelf-life determination[37–41], Identification of active ingredient [42–44], Pharmaceutical quality control [45].

Food applications: HPLC is widely using the food analysis of particular product research and quality control [46–50], for testing of the labile compound for a complex matrix[51,52] Analysis of natural compounds (sugar, fats, protein, amino acids) food additives and contaminants are determined, Multiresidues testing of contaminants and pesticides.

Forensics applications: drugs measurement in a biological sample, Anabolic steroid detection in serum, urine, sweat, and hair examination of textile colours for forensics identifying the presence of cocaine and its metabolites in blood.[53–57]

Clinical Applications: clinical analysis is performed regularly, Glycated hemoglobin measurement entails keeping an eye on diabetic patients' long-term plasma glucose management. It is frequently employed to determine the origin of poisoning and to pinpoint the drug metabolism that might play a role in drug toxicity.[58–62]

CONCLUSION

A broad overview of HPLC apparatus, along with the development and validation of methods, is provided in this article. It was explained how to create HPLC methods for compound separation in a general and straightforward manner.

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