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Research Article

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A Quick and Standard Pharmaceutical Industrial Approach to Develop a Robust Analytical Method by HPLC

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Abstract High-performance liquid chromatography, often known as HPLC, is a type of chromatography that uses columns and is used to separate compounds, identify them, and quantify them. The stationary phase of HPLC is termed a column, and it is responsible for separating compounds according to their polarity. The mobile phase of HPLC is characterized by a pump and detector that detects and displays the response of the compounds that have been eluted from the stationary phase. The pharmaceutical and biopharmaceutical sectors, the food business, forensic applications, and environmental studies are some of the many fields that make use of HPLC. This article focuses on the role that HPLC plays in the development of analytical methods. It emphasizes fundamental methodologies and important chromatographic parameters for effective optimization.

Keywords HPLC, analytical method development, column chemistry, diluent selection, robust method

1. Introduction

HPLC is a very useful analytical method, especially in analytical chemistry, that can be used in many different ways. It is a very important part of the research process in the pharmaceutical, biopharmaceutical, environmental, food, and taste industries because it can separate, identify, and measure compounds of interest in samples [1]. HPLC is a very important tool for making sure that a drug substance or drug product is of good quality and that it has the right amount of impurities. When paired with different detectors, like UV-visible (UV-Vis), Photodiode-Array Detector (PDA), and Mass spectrometry (MS), it can be used in many ways. The HPLC instrument has eight parts: 1) the mobile phase storage, 2) the solvent delivery system or gradient pump, 3) the sample entry device, 4) the column, 5) the detector, 6) the computer or recorder, 7) the connecting tubes, and 8) the waste solvent collector. The successful HPLC analytical method is the right choice of working conditions like the type of column packing and mobile phase, column length and thickness, mobile phase flow rate, column temperature, and sample load. In reverse phase HPLC, the stationary phase is not polar, and the mobile phase is polar or mostly polar. The idea behind RP-HPLC is that the molecule of interest in the mobile phase interacts with the stationary phase in a way that does not involve water [2]. In the sample mix, the parts that are less polar will stay on the nonpolar fixed phase longer than the parts that are more polar. So, the component with the most polarity will come out of the motionless phase first [1,2].

2. Methods and Discussions

Quick steps to method development on HPLC:

Developing a robust analytical method as quickly as possible with a minimum number of experiments is possible with the following development strategy [3-5].

1) Familiarity with the drug's fundamental physiochemical characteristics
2) Mobile phase selection
3) Stationary phase / Column selection
4) Selection of diluent
5) Selection of sample concentration, injection volume
6) Detector and wavelength selection
7) Solvent delivery system selection
8) Flow rate selection
9) Column temperature selection
10) Checking for specificity and stability of the test procedure
11) Selection of run time
12) Setting up system suitability requirements

Familiarity with the drug's fundamental physiochemical characteristics:

Method development is greatly aided by knowledge of a drug molecule's physical characteristics. Selecting diluents, extraction solvents, and optimizing elution patterns all benefit from knowledge of solubility, pKa, pH, dissociation, and polarity. The resolution of tightly eluting peaks is aided by the fact that pH alters ionization properties. Impurities, their degradation profile, production paths, and spectral profiles must be understood in order to ensure compliance with ICH recommendations and patient safety. Appropriate detectors and wavelengths for analysis are decided based on the absorption properties of contaminants [3,4].

Mobile phase selection:

The mobile phase selection plays a vital role in achieving adequate peak separation. The mobile phase and stationary phase compositions should be compatible with the precise, sensitive, and robust analytical method. Mobile phases used in the HPLC method are usually buffers or organic solvents and aqueous mixtures.

Buffer selection:

The choice of buffer is regulated by the pH that is required. The classic pH range for reverse phase chromatography column packing (Silica) is pH 2 to 8. For pH values outside the range of 2.0 to 8.0, it is essential to select packing materials that can withstand those specific ranges. When the pH is higher than 7, using phosphate buffer can speed up the breakdown of silica, which can shorten the life of HPLC columns made of silica. For pH levels above 7, organic buffers should be used whenever possible. It is suitable that the buffer has a pKa close to the desired pH of the analyte compound since the buffer controls pH best at its pKa. A rule is to choose a buffer with a pKa value equal to two units higher than that of the desired mobile phase [1,3,6].

General consideration for buffer selection:

In HPLC, peak shapes and separations depend a lot on which buffers are used and how strong they are. As buffers, phosphate, acetate buffer, and triethylamine/diethylamine are often used. Phosphate buffers, such as KH2PO4, K2HPO4, NaH2PO4, Na2HPO4, and H3PO4, dissolve better in a mixture of methanol and water than in a mixture of acetonitrile and water or THF and water. Some hygroscopic salt buffers, like ammonium acetate and sodium acetate, can change the way chromatography works by changing the tailing of important molecules and their selectivity [2]. Triethylamine, diethylamine, and trifluoroacetic acid tend to break down over time. It is volatile and absorbs UV light at low wavelengths. Using the right buffer strength helps control the injection load on the column, which reduces peak tailing caused by changes in the ionic form of the analytes. As the buffer strength goes up and the retention times go down, the molar strength and retention times go in the opposite direction. Small drugs usually work well with a buffer concentration between 10 and 50 mM. When choosing the makeup of the organic phase in the mobile phase, the choice of cushion and how strong it is should always be taken into account. If more separation is needed, the buffer strength can be adjusted. But it is important to make sure that a higher buffer strength does not cause precipitation or haze in the mobile phase, standard solutions, or test solutions when they are kept at room temperature or in a refrigerator. When there are few or no organic modifiers and the mobile phase is stabilized, bacteria grow quickly. If the growth builds up on the column inlets, it can hurt how well the chromatography works. To separate the individual impurity peaks from the active pharmaceutical ingredient (API) peak, you need to choose a buffer with a certain power. Adjusting it

within a range of about 10–20% of the original strength should be done to see what happens when the cushion strength changes. After looking at the results of these changes, a buffer and its strength should be picked that can handle at least a 2% change in strength while keeping its toughness and the performance wanted for separation. The pH of the buffer can be changed by up to 0.2 units from the chosen value, and the effects of these changes will also be studied. Experiments will be done with mobile phases that have buffers with different pH values and with different organic phases to see how well the impurities can be separated. A drug solution with all known impurities can be used to test how well separations work with different amounts of mobile phase. Alternately, solutions of stressed drug substances can be used to check for the separation of impurities. A mobile phase that separates all the impurities and degradation products from the API peak and can handle changes in both the water and organic phases that are at least 5% of the chosen mobile phase composition. In reverse-phase chromatography, the solvents most often used are methanol and acetonitrile. Tetrahydrofuran is used less often. After making buffers, it is best to run them through a 0.2-µm filter and degas them to get rid of any particles and gases that have dissolved [1,6,7].

Stationary phase / Column selection

Generally, the column is considered the heart of the HPLC method development. Columns are long, narrow tubes containing stationary phase at particle diameters of 25-µm or less, made of heavy glass, polished stainless steel, polyethylene, or poly ether ketone tubing to withstand pressure in the HPLC system. In the analytical method, the column plays an important role which determining the separation goals. The separation goals like resolution, separation time, pressure, peak height, and quantitation within a reasonable time make the enhanced method robust for testing. The selection of packing material and mobile phases depends on the physical properties of the compound of interest. Various properties such as column length, diameter, particle size, particle shape, pore size/pore volume, specific surface area, end capping, and percentage of carbon loading in the stationary phase are crucial factors in achieving the desired separation goals in chromatography. Silica-based columns are commonly used in normal-phase chromatography, where the mobile phase consists of nonpolar organic solvents, while the stationary phase is polar [2,5,6]. This polarity is attributed to the silanol groups present on the surface of the silica packing material. In reverse phase chromatography, a wide range of column options are available with varying polarities achieved by cross-linking the Si-OH groups with alkyl chains such as C6, C8, and C18, as well as other groups like CN (nitrile), C6H6 (phenyl), and NH2 (amino). These crosslinked silica-based columns can be arranged in increasing order of polarity as follows: C18 < C8 < C6 < Phenyl < Amino < Cyano < Silica [8]. The best separations can be achieved by designing multiple experiments and selecting the one that separates all impurities and degradants from the API peak while being robust to changes in the mobile phase. Columns and their bonded phases are shown in Table 1.

Table 1: Different types of columns and their bonded phases.

Column	Phase	Application
C18	Octadecyl	General, nonpolar
C8	Octyl	General, nonpolar
Phenyl	Styryl	Fatty acids, double bond
Cyano	Cyanopropyl	Ketones, aldehydes
Amino	Aminopropyl	Sugars, anions
Diol	Dihydroxy hexyl	Proteins
SAX	Aromatic quaternary amine	Anions
SCX	Aromatic sulfonic acid	Cations
DEAE	Alkyl ether, ethyl 2-amine	Protein cations

Selection of diluent:

The solubility of the drug ingredient plays an important role in the decision-making process when choosing a diluent for the preparation of a test. However, the final choice of diluent is selected by taking into consideration of several criteria, including its extraction efficiency, peak symmetries, resolution of impurities from the API peak, and the lack of interference during diluent blank injections [3]. Choose a diluent in which the drug ingredient can dissolve entirely, and in which the extraction of the compound of interest may be carried out

without being impeded in any way and with no unwanted interference. Sonication, rotary shaking, or both processes combined are used in the extraction methods that are utilized. Heating is an option that can be utilized in situations where the required active pharmaceutical ingredient (API) cannot be properly extracted using the approaches that have been described previously. Nevertheless, it is of the utmost importance to rule out the possibility of precipitation after bringing the sample down to ambient temperature. The type of diluent used has a direct impact on the peak symmetry as well as the resolution between the API and any impurities. Checking the interference and resolution between peaks requires injecting a spiked sample into the chromatogram. The sample should contain all the known impurities. Next, determine the non-interference of the blank in the estimation of API, as well as the effect of diluent on the resolution of impurities from API peak and peak summary. Therefore, the diluent that is chosen ought to be able to meet the requirements that were discussed earlier. It is also important to verify whether or not the filter is compatible with the preparation of the sample that was centrifuged. It is also important to investigate the volume of the sample that is going to be discarded via the filter before obtaining the sample for analysis [6,9].

Selection of sample concentration and injection volume:

The sample concentration will be selected based on the absorbance or response of the compound of interest at the selected detector wavelength. The compound of interest absorbance should not go beyond the saturation point. The sample concentration should be finalized without compromising the complete extraction of the compound of interest and absorbance height. It is advised that an injection volume in the range of 10 to 20 µL be used; however, if the concentration of the substance being analyzed is low, the injection volume can be increased to 50 µL. It is viable to choose an injection volume of up to 70% of the injector's capacity. This allows for the possibility of column overload to be managed while maintaining peak symmetry and resolution between individual impurities and the compound of interest. After determining the concentration of the sample and the amount of diluent to use, the next step is to produce the test solution. After the solution has been filtered, it is required to be put into a flask that has a stopper and then stored on the bench in a closed environment. After 24 hours, the solution needs to be monitored for any indications of cloudiness or precipitation. It is important that the solution remains clear without any turbidity or precipitation [10,11].

Detector and wavelength selection:

A crucial step in completing the analytical technique is choosing the detector wavelength (Table 2). Injecting the API/Reference standard solution into the chromatographic system with a photodiode array detector and collecting the spectra is a useful method for choosing the wavelength. From the Standard chromatogram, select the wavelength where it has higher absorbance for the compound of interest. It is common to choose between wavelengths in the related compounds testing method as impurities might have maximum absorbance at different wavelengths [3,6,9].

Table 2: Different types of detectors and their detection compound types.

Detector type	Types of compounds can be detected
UV-Visible (UV)	Compounds with chromophores
Photodiode array (PDA)	Compounds with chromophores are required to determine the purity of the analyte
Refractive Index (RI)	Compounds with limited or no chromophore
Evaporative light scattering	Volatile compounds without chromophores
Fluorescence (FL)	Fluorescent compounds
Conductivity detector	Charged compounds
Electrochemical (EC)	Oxidized compounds

Solvent delivery system selection:

Gradient HPLC is an excellent way for initial sample analysis to separate the maximum number of peaks from complex samples, but chromatographic separation with isocratic elution is always preferable. Gradient dilution is a potent method for separating compounds with widely varying polarities because the polarity and ionic strength of the mobile phase can be altered during the run. Experiment with several mobile phase combinations and gradient programs to separate all the impurities and degradants from the API peak. Common practice, while running gradient, is two different compositions are kept in different channels. The two mobile phases can be

introduced into the column by low-pressure or high-pressure gradient approaches. The low-pressure gradient method was most suitable when not more than 80% organic phase is to be pumped, where a high-pressure gradient will be used in reverse. Mixing at least 10% of aqueous or the same buffer is a common practice to avoid pumping problems. Optimization of the gradient program can be done to check the overall system pressure of the system should not cross 300 bar at any point of time during the run, ruggedness of the gradient program up to ±10% variation in the organic phase, and up to ±0.2 mL in flow rate. The system was stabilized before the next injection by running the initial condition [9,10].

Flow rate selection:

The retention times of peaks were directly proportional to the flow rate of the analytical method. During the flow rate column selection, back pressure should be considered along with the separation of impurities, and peak symmetries should not be compromised. The flow rate should ideally not be greater than 2.5 mL/min. The analytical method's robustness will be tested with a flow variation of 0.2 mL from the chosen flow rate [9,12].

Column temperature selection:

Increasing the column temperature above ambient may be considered if peak symmetry cannot be achieved using any combination of column and mobile phase. Typically, increasing the column temperature can lead to improved peak symmetries and retention times. However, it is important to note that different columns have different temperature threshold limits. A column temperature ranging between 30°C and 80°C can be selected based on the specific requirements. Care should be taken to stay within the recommended temperature range of the chosen column [8,13].

Checking for specificity and stability of the test procedure:

Showing the specificity of the method has paramount importance before finalizing an analytical method. To prove the method specificity, conduct a force degradation study targeting about 20% of degradation in each stress condition (Acid, Base, H2O2, Heat, and Photostability). Analyze the sample with the presumed analytical method on HPLC equipped with a PDA detector. Review the chromatograms demonstrating the separation of degradants formed under stress conditions and the peak purity of the API. The method can be regarded as sufficiently specific and stable if the peak purity of the API peak satisfies the standards and is determined to be satisfactory, which means that the purity threshold is lower than the purity angle. This indicates that the method is suitable for its intended use, providing confidence in the reliability and accuracy of the results. For the solution stability study, the samples, the standard solution, will be analyzed at the initial time and after 12, 24, or 48 hours will be tested. After initial testing, the samples can be stored at both room temperature and refrigerated. If the solutions recoveries were found to be meeting the guideline's predefined limits, the samples were found to be stable for that amount of time in the specified storage condition [9,12].

Selection of run time:

The optimization of chromatographic run time is based on the separation of all impurities and placebo peaks without having any interference before injecting the next sample. Run time should be reasonable with minimal solvent consumption [6,9].

Setting up system suitability requirements:

The selection of system suitability parameters varies from method to method and purely depends on the criticality of separation between the peaks. The resolution factor should be considered in the system suitability parameter for closely eluting peaks in the chromatogram. USP tailing factor can also be added to check the peak symmetry. For some methods, plate counts will also be studied to look over the column performance [8,14]. System suitability criteria are mentioned in Table 3.

Table 3: General criteria for system suitability acceptance.

S. No.	Parameter Name	Acceptance Criteria
1	Number of theoretical plates of efficiency	>2000
2	Capacity Factor	<1
3	Separation or relative retention time (α)	>1
4	Resolution (Rs)	>1.5
5	Tailing factor or Asymmetry (T)	<2
6	Relative standard Deviation	<2

3. Conclusion:

This manuscript outlines the simple steps required to develop analytical methods for Related Substances (impurities), Assay of active and excipients, Dissolution, and Cleaning verification for Active Pharmaceutical Ingredients (API's), Tablets, Capsules, Oral suspensions/solutions, Topicals, Transdermal, and Injectable dosage forms in the pharmaceutical industry. Scientists are able to build a reliable way of analysis in a shorter amount of time when they use this methodology. When developing new procedures, it is important to decrease the amount of solvent used and save time as much as possible. This strategy can help with both of those goals.

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