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REVIEW ARTICLE

A Review on Method Development and Validation using HPLC

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ABSTRACT

High performance liquid chromatography (HPLC) is an analytical technique which is used to separate, detect and quantify various drugs and its related degradants. It is employed to separate manufactured drugs from drug related impurities, to detect and quantify synthesized drug and to reduce other impurities at the time of separation. A number of chromatographic parameters were evaluated in order to optimize the method. An appropriate mobile phase, column, column temperature, wavelength and gradient must be found that affords suitable compatibility and stability of drug as well as degradants and impurities. Validation of HPLC as per ICH guidelines covers all the performance characteristics of validation, likeaccuracy, precision, specificity, linearity, range, limit of detection, limit of quantification, robustness and system suitability testing.

KEYWORDS

HPLC, Validation, Method Development, Degradants

INTRODUCTION

Chromatography is an analytical technique based on the separation of molecules due to differences in their structure and/or composition. In general, chromatography involves moving a sample through the system over a stationary phase. The molecules in the sample will have different affinities and interactions with the stationary support, leading to separation of molecules. Sample components that display stronger interactions with the stationary phase will move more slowly through the column than components with weaker interactions. Different compounds can be separated from each other as they move through the column. Chromatographic separations can be carried out using a variety of Stationary phases, including immobilized silica on glass plates (thin-layer chromatography), volatile gases (gas chromatography), paper (paper chromatography) and liquids (liquid chromatography).

High-performance liquid chromatography (HPLC) is a type of liquid chromatography used to separate and quantify compounds that have been dissolved in solution. HPLC is used to determine the amount of a specific compound in a solution. For example, HPLC can be used to determine the amount of morphine in a compounded solution. In HPLC and liquid chromatography, where the sample solution is in contact with a second solid or liquid phase, the different solutes in the sample solution will interact with the stationary phase as described. The differences in interaction with the column can help separate different sample components from each other[1].

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Types of HPLC

There are following variants of HPLC, depending upon the phase system (stationary) in the process:

1. Normal Phase HPLC

This method separates analytes on the basis of polarity. Normal phase- HPLC uses polar stationary phase and non- polar mobile phase. Therefore, the stationary phase is usually silica and typical mobile phases are hexane, methylene chloride, chloroform, diethyl ether and mixtures of these. Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

2. Reverse Phase HPLC

The stationary phase is nonpolar in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. It works on the principle of hydrophobic interactions hence the more nonpolar the material is, the longer it will be retained.

3. Size-exclusion HPLC

The column is filled with material having precisely controlled pore sizes, and the particles are separated according to it's their molecular size. Larger molecules are rapidly washed through the column, smaller molecules penetrate inside the porous of the packing particles and elute later.

4. Ion-Exchange HPLC

The stationary phase has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionisable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time[2].

Instrumentation of HPLC

Mobile Phase and Reservoir

The type and composition of the mobile phase affects the separation of the components. Different solvents are used for different types of HPLC. For normal-phase HPLC, the solvent is usually nonpolar, and, in reverse-phase HPLC, the solvent is normally a mixture of water and a polar organic solvent. The purity of solvents and inorganic salts used to make the mobile phase is paramount. A general rule of thumb is to use the highest purity of solvent that is available and practical depending on the particular application. The most common solvent reservoirs are as simple as glass bottles with tubing connecting them to the pump inlet.

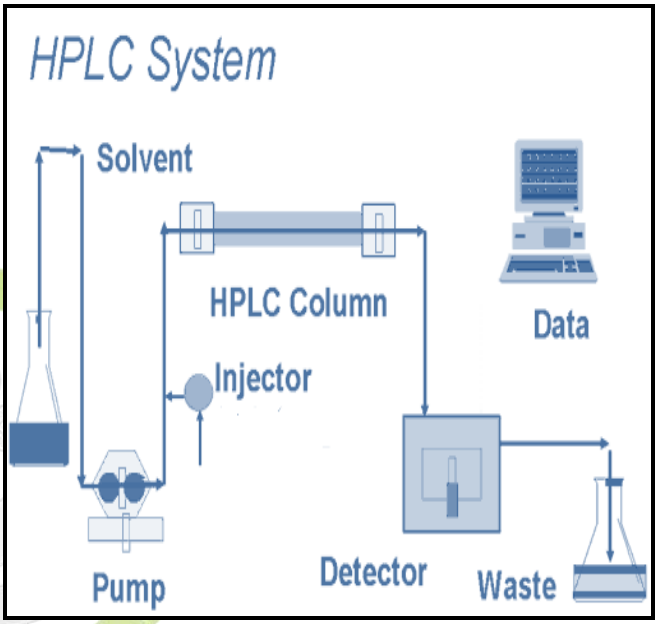

Figure 1: HPLC Instrument

Pumps

High-pressure pumps are needed to push the mobile phase through the packed stationary phase. A steady pump pressure (usually about 1000–2000 psi) is needed to ensure reproducibility and accuracy. Pumps are typically known to be robust, but adequate maintenance must be performed to maintain that characteristic. Inability to build pressure, high pressures or leakage could indicate that the pump is not functioning correctly. Proper maintenance of the pump system will minimize down time.

Injectors

The injector can be a single injection or an automated injection system. An injector for an HPLC system should provide injection of the liquid sample within the range of 0.1-100 mL of

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volume with high reproducibility and under high pressure (up to 4000 psi). For liquid chromatography, liquid samples can be directly injected and solid samples need only to be diluted in the appropriate solvent.

Columns

The column or stationary phase is the core of any chromatographic system. Columns are commercially available in different lengths, bore sizes and packing materials. The use of the correct combination of length and packing material in correlation with the appropriate mobile phase can assist in the most effective separation of a sample compound. A variety of column dimensions are available including preparative, normal- bore, micro- and mini-bore and capillary columns. Different column dimensions can be used for different types of separations and can utilize different packing materials and flow rates. The most widely used packing materials for HPLC separations are silica-based. The most popular material is octadecyl- silica (ODS-silica), which contains C18 coating, but materials with C1, C2, C4, C6, C8 and C22 coatings are also available. Miscellaneous chemical moieties bound to silica, as well as polymeric packing, are designed for purification of specific compounds. Other types of column packing materials include zirconia, polymer-based and monolithic columns.

Theoretical plates relate chromatographic separation to the theory of distillation and are a measure of column efficiency.

Detectors

There are many different types of detectors that can be used for HPLC. The detector is used to sense the presence of a compound passing through and to provide an electronic signal to a data-acquisition device. The main types of detectors used in HPLC are refractive index (RI), ultraviolet (UV-Vis) and fluorescence, but there are also diode array, electrochemical and conductivity detectors. Each detector has its assets, limitations and sample types for which it is most effective. Most applications in drug analysis use detectors that respond to the absorption of ultraviolet radiation (or visible light) by the solute as it passes through the flow-cell inside the detector. The recent development of the so-called hyphenated techniques has improved the ability to separate and identify multiple entities within a mixture. These techniques include liquid chromatography-mass spectrometry (LC-MS), liquid chromatography-mass spectrometry-mass spectrometry (LC-MSMS), liquid chromatography-infrared spectroscopy (LC-IR) and liquid chromatography-nuclear magnetic resonance (LC-NMR). These techniques usually involve chromatographic separation followed by peak identification with a traditional detector such as UV, combined with further identification of the compound with the MS, IR or NMR.

Data Acquisition/Display Systems

Since the detector signal is electronic, use of modern data acquisition techniques can aid in the signal analysis. The data acquisition system of most HPLC systems is a computer. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret. Other more advanced features can also be applied to a chromatographic system. These features include computer-controlled automatic injectors, multi-pump gradient controllers and sample fraction collectors[1].

Applications of HPLC

- ***Pharmaceutical Applications***
    1. To control drug stability
    2. Pharmaceutical quality control
    3. Tablet dissolution study of pharmaceutical dosages form
- ***Environmental Applications***
    1. Detection of phenolic compounds in drinking water
    2. Bio- monitoring of pollutants
- ***Applications in Forensics***
    1. Qualification of drugs in biological samples

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2. Identification of steroids in blood, urine etc.
3. Determination of cocaine and other drugs of abuse in blood, urine etc.

- ***Food and Flavour***
  1. Measurement of quality of soft drinks and water
  2. Sugar analysis in fruit juices
  3. Preservative analysis
  4. Analysis of polycyclic compounds in vegetables
- ***Application in Clinical Tests***
  1. Urine analysis, antibiotics analysis in blood
  2. Analysis of bilirubin, biliverdin in hepatic disorders
  3. Detection of endogenous neuropeptides in extracellular fluid of brain etc2.

Method Development

It is a process of selecting an accurate assay procedure to determine the composition of a formulation. It is the process of proving that an analytical method is acceptable for use in laboratory to measure the concentration of subsequent samples. Analytical methods should be used within GMP and GLP environments and must be developed using the protocols and acceptance criteria set out in the ICH guidelines Q2 (R1).

An analytical procedure is developed to test a defined characteristics of the substance against established acceptance criteria for that characteristic. In the development of a new analytical procedure, the choice of analytical instrumentation and methodology should be based on the intended purpose and scope of the analytical method.

The common steps followed in the method development are as follows:

1) Standard analyte characterization
2) Method requirements
3) Literature search
4) Selecting the method
5) Instrumental setup and preliminary studies
6) Optimization of parameters
7) Documentation of analytical figure
8) Evaluation of the method development with the sample
9) Determination of percent recovery of the sample[3].

Method Validation

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice. It is the process of defining an analytical requirement, and confirms that the method under consideration has performance capabilities consistent with what the application requires. Use of equipment that is within specification, working correctly and adequately calibrated is fundamental to the method validation process. Likewise the operator carrying out the studies must be competent in the analysis under study and have sufficient knowledge of the method/analysis to draw conclusions from the observations as the validation work proceeds. Quite often method validation evolves from method development and so the two activities are often closely tied, with the validation study employing the techniques and steps in the analysis as defined by the method development[4].

Validation parameters recommended by FDA, USP and ICH are as follows:

1 Specificity

2 Linearity

3 Range

4 Precision

a) Repeatability

b) Intermediate precision(Ruggedness)

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c) Reproducibility

5 Accuracy

6 Robustness

7 Limit of Detection(LOD)

8 Limit of Quantification(LOQ)

1. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

2. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. A linear relationship should be evaluated across the range of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighing of synthetic mixtures of the drug product components, using the proposed procedure. The latter aspect can be studied during investigation of the range. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data may need to be subjected to a mathematical transformation prior to the regression analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity.

3. Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) 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.

4. 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.

a) ***Repeatability:*** Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

b) ***Intermediate Precision:*** Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

c) ***Reproducibility:*** Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

5. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

6. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

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7. Limit of Detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

8. Limit of Quantification

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantification limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products[5].

CONCLUSION

Analytical method development plays important role in the discovery, development and manufacture of pharmaceuticals. The general approach for the method development for the separation of pharmaceutical compound was discussed. Final optimization can be performed by changing the temperature, gradient slope, flow rate and concentration of mobile phase.

Optimized method is validated with various parameters (accuracy, precision, specificity, linearity, limit of detection, limit of quantification etc.) as per ICH guidelines.

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