AMERICAN JOURNAL OF PHARMTECH RESEARCH

**Journal home page:** http://www.ajptr.com/

# High Performance Liquid Chromatography and Its Validation – Review

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## ABSTRACT

HPLC is the dominant separation technique in modern pharmaceutical and biomedical analysis because it results in highly efficient separations and in most cases provides high detection sensitivity. Most of the drugs in multi component dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC methods development and validation play important roles in new discovery, development, manufacture of pharmaceutical drugs and various other studies related to humans and animals. It is the most versatile, safest, dependable and fastest chromatographic technique for the quality control of drug components. This article was prepared with an aim to review different aspects of HPLC, such as principle, types, instrumentation, validation of HPLC and application.

**Keywords:** High performance liquid chromatography, instrumentation, validation of HPLC, applications.

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Received 28 March 2017, Accepted 04 April 2018

Please cite this article as: Kaushik K *et al.*, High Performance Liquid Chromatography and Its Validation – Review American Journal of PharmTech Research 2018.

# INTRODUCTION

Chromatography is probably the most powerful analytical technique available to the modern chemist. Its power arises from its capacity to determine quantitatively many individual components present in mixture by single analytical procedure.[1,2]High Performance Liquid Chromatography (HPLC) was derived from the classical column chromatography and, is one of the most important tools of analytical chemistry today.[3]In the modern pharmaceutical industry, high performance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development, and production.[4] HPLC is the method of choice for checking peak purity of new chemical entities, monitoring reaction changes is in synthetic procedures or scale up, evaluating new formulations and carrying out quality control / assurance of the final drug products.[5] The Goal of HPLC method is to try & separate, quantify the main drug, any reaction impurities, all available synthetic intermediates and any degradants.[6] 6High Performance Liquid Chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid. HPLC is the most accurate analytical methods widely used for the quantitative as well as qualitative analysis of drug product and used for determining drug product stability.[7]

**Principle**

HPLC is an advanced technique of column liquid chromatography. The solvent usually flows through column with the help of gravity but in HPLC technique the solvent will be forced under high pressures up to 400 atmospheres so that sample can be separated into different constituents with the help of difference in relative affinities. In HPLC, pumps will be used to pass pressurized liquid solvent including the sample mixture which is allowed to enter into a column filled with solid adsorbent material. The interaction of each sample component will be varies and this causes difference in flow rates of each component and finally leads to separation of components of column.[8-14]

**Types of HPLC**

1. Normal Phase HPLC- In this method the separation is based on polarity. The stationary phase is polar, mostly silica is used and the non-polar phase used is hexane, chloroform and diethyl ether. The polar samples are retained on column.
2. Reverse Phase HPLC- It is reverse to normal phase HPLC. The mobile phase is polar and the stationary phase is non polar or hydrophobic. The more is the non-polar nature the more it will be retained.

3. Size-exclusion HPLC- The column will be incorporating with precisely controlled substrate molecules. Based on the difference in molecular sizes the separation of constituents will occur.
4. Ion-exchange HPLC- The stationary phase is having ironically charged surface opposite to the sample charge. The mobile phase used is aqueous buffer which will control pH and ionic strength.

**Instrumentation**

The HPLC instrumentation involves pump, injector, column, detector, and integrator and display system. In the column the separation occurs. The parts include:

1. Solvent Reservoir: The contents of mobile phase are present in glass container. In HPLC the mobile phase or solvent is a mixture of polar and non-polar liquid components. Depending on the composition of sample, the polar and non-polar solvents will be varied.
2. Pump: The pump suctions the mobile phase from solvent reservoir and forces it to column and then passes to detector. 42000 K Pa is the operating pressure of the pump. This operating pressure depends on column dimensions, particle size, flow rate and composition of mobile phase.
3. Sample Injector: The injector can be a solitary infusion or a computerized infusion framework. An injector for a HPLC framework should give infusion of the fluid specimen inside the scope of 0.1 mL to 100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).
4. Columns: Columns are typically made of cleaned stainless steel, are somewhere around 50 mm and 300 mm long and have an inward distance across of somewhere around 2 and 5 mm. They are generally loaded with a stationary phase with a molecule size of 3 µm to 10 µm. Columns with inner diameters of <2 mm are regularly alluded to as micro bore segments. Preferably the temperature of the mobile phase and the column should be kept consistent during investigation.
5. Detector: The HPLC detector, situated toward the end of the column distinguishes the analytes as they elute from the chromatographic column. Regularly utilized detectors are UV-spectroscopy, fluorescence, mass spectrometric and electrochemical identifiers.
6. Data Collection Devices or Integrator: Signals from the detector might be gathered on graph recorders or electronic integrators that fluctuate in many-sided quality and in their capacity to process, store and reprocess chromatographic information. The PC coordinates

the reaction of the indicator to every part and places it into a chromatograph that is anything but difficult to interpret.

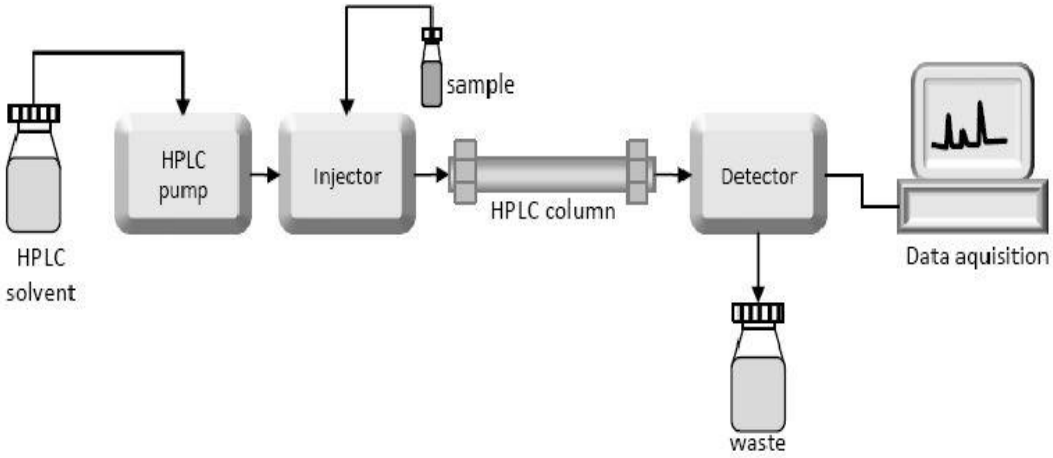


**Figure 1: HPLC PARTS**

## METHOD DEVELOPMENT

Before starting the method development, various physiochemical properties like pK a value, log P, solubility and absorption maximum of the drug must be known, for it lays a foundation for HPLC method development. Log P and solubility helps select mobile phase and sample solvent while pK value is important as it helps determine the pH of the buffer and pH related changes in retention occur at pH values within ±2 of pK value.[15]

Reverse phase column is a preferred choice to start the separation of sample components as the degradation is carried out in aqueous solution. Methanol, water and acetonitrile can be used as mobile phase in various ratios for the initial stages of separation. Selection between methanol and acetonitrile for organic phase is based on the solubility and properties of the analyte. Initially the water: organic phase ratio can be kept at 50:50 and suitable modifications can be made as trials proceed to obtain a good separation of peaks. Latter buffer can be added if it is required to obtain better peak separation and peak symmetry. Variation in column temperature affects the selectivity of the method as analytes respond differently to temperature changes. A temperature in the range of 30–40˚C is suitable to obtain good reproducibility. Also a sufficient run time after the drug peak is to be allowed to obtain the degradants peak eluting after the drug peak. During the method development it may happen that the drug peak may hide an impurity or degradants peak that co-elutes with the drug. This requires peak purity analysis which determines the specificity of the method. Direct analysis can be done online by using photodiode array (PDA) detection. PDA provides information of the homogeneity of the spectral peak but it is not applicable for the

degradants that have the similar UV spectrum to the drug. Indirect method involves change in the chromatographic conditions like mobile phase ratio, column, etc. which will affect the peak separation. The spectrum of altered chromatographic condition is then compared with the original spectra. If the degradants peaks and area percentage of the drug peak remain same, then it can be confirmed that the drug peak is homogeneous. The degradants that co-elutes with the drug would be acceptable if it is not found to be formed in accelerated and long term storage conditions. The method is then optimized for separating closely eluting peaks by changing flow rate, injection volume, column type and mobile phase ratio.[15, 16, 17]

## COMPONENTS OF HPLC METHOD VALIDATION

The following are typical analytical performance characteristics which may be tested during methods validation

1. Accuracy
2. Precision
3. Repeatability
4. Intermediate precision
5. Linearity
6. Detection limit
7. Quantitation limit
8. Specificity
9. Range
10. Robustness
11. System suitability determination

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. It 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. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay. It may often be expressed as the recovery by the assay of known, added amounts of analyte.

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. It consists of two components: repeatability and intermediate precision.[18]

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 analyzing multiple replicates of an assay composite sample by using the analytical method. The recovery value is calculated.

Intermediate precision is the variation within a laboratory such as different days, with different instruments, and by different analysts.[19, 20] the precision is then expressed as the relative standard deviation.

$$\%RSD = \frac{\text{Std dev.} \times 100}{\text{Mean}}$$

Linearity is the ability of analytical procedure to obtain a response that is directly proportional to the concentration (amount) of analyte in the sample. If the method is linear, the test results are directly or by well-defined mathematical transformation proportional to concentration of analyte in samples within a given range. Linearity is usually expressed as the confidence limit around the slope of the regression line.

The detection limit (DL) or limit of detection (LOD) of an individual procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. In analytical procedures that exhibit baseline noise, the LOD can be based on a signal-to-noise (S/N) ratio (3:1), which is usually expressed as the concentration of analyte in the sample.(book) The signal-to-noise ratio is determined by: s = H/h Where H = height of the peak corresponding to the component. h = absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.

The limit of Quantitation (LOQ) or Quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. For analytical procedures such as HPLC that exhibit baseline noise, the LOQ is generally estimated from a determination of S/N ratio (10:1) and is usually confirmed by injecting standards which give this S/N ratio and have an acceptable percent relative standard deviation as well.

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as impurities, degradation products, and excipients. Specificity measures only the desired component without interference from other species that might be present; separation is not necessarily required.

Range is defined as the interval between the upper and lower concentrations of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity.

Robustness is defined as the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters (e.g. pH, mobile phase composition, temperature and instrumental settings) and provides an indication of its reliability during normal usage.

Determination of robustness is a systematic process of varying a parameter and measuring the effect on the method by monitoring system suitability and/or the analysis of samples.

System Suitability Determination is the evaluation of the components of an analytical system to show that the performance of a system meets the standards required by a method. These parameters can be calculated experimentally to provide a quantitative system suitability test report: number of theoretical plates (efficiency), capacity factor, separation (relative retention), resolution, tailing factor, relative standard deviation (precision). These are measured on a peak or peaks of known retention time and peak width.[21]

**Applications of HPLC [22-37]**

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 are 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 include controlling of drug stability, dissolution studies and quality control, Tablet dissolution study of pharmaceutical dosages form, Shelf-life determinations of pharmaceutical products, Identification of active ingredients of dosage forms , Pharmaceutical quality control

**Environmental Applications**

Monitoring of pollutants and detecting components of drinking water, Identification of diphenhydramine in sediment samples, Bio-monitoring of pollutant

**Forensic Applications**

Analysis of textile dyes, quantification of drugs and steroids in biological samples. Quantification of the drug in biological samples. Identification of anabolic steroids in serum, urine, sweat, and hair .Forensic analysis of textile dyes. Determination of cocaine and metabolites in blood.

**Food and Flavour Applications**

Sugar analysis in fruit juices, detecting polycyclic compounds in vegetables, analysis of preservatives, ensuring the quality of soft drink and drinking water. Analysis of beer. Trace analysis of military high explosives in agricultural crops.

**Clinical applications**

Quantification of ions in human urine Analysis of antibiotics in blood plasma. Estimation of bilirubin and bilivirdin in blood plasma in case of hepatic disorders. Detection of endogenous neuropeptides in extracellular fluids of brain.

## CONCLUSION

Analytical methods development plays important roles in the discovery, development and manufacture of pharmaceuticals. The HPLC is mostly used analytical technique. It is having several advantages. With the use of HPLC one can produce extremely pure compounds. It can be used in both laboratory and clinical science. With the use of HPLC the accuracy, precision and specificity can be increased. The only disadvantage of HPLC is high cost. It can be concluded from the entire review that HPLC is a versatile, reproducible chromatographic technique for the estimation of drug products. It has wide applications in different fields in term of quantitative and qualitative estimation of active molecules. This review mainly focuses on the HPLC technique its principle, types, instrumentation, validation and applications.

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