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REVIEW ARTICLE

A Review on Method Development and Validation by Using HPLC

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ABSTRACT

Chromatography, although primarily a separation technique, is mostly employed in chemical analysis, in which High-performance liquid chromatography (HPLC) is an extremely versatile technique where analytes are separated by passage through a column packed with micrometer-sized particles. Now a day reversed-phase chromatography is the most commonly used separation technique in HPLC. This article involves the strategies and the issues for designing HPLC method development and validation. HPLC methods should be able to separate, detect and quantify the various drug-related impurities that may be introduced during synthesis. The method development often follows the well established steps, like selection of buffer, selection mobile phase, selection of column. Method validation includes the parameters like accuracy, precision, linearity, LOQ and LOD, specificity etc. A number of chromatographic parameters were evaluated in order to optimize the method.

KEYWORDS

HPLC, Method development, Validation, Impurity, Chromatography

INTRODUCTION

Chromatography[1]

It is a separation technique and the separated units can be measured by any analytical technique as UV-visible, Infrared, Mass spectroscopy, NMR etc.

Chromatography represents from two words:

"Chromo"- color and "graphy" - writing.

Principle of Chromatography

Principle

Mobile phase-liquid, Stationary phase- stable column

The samples are subjected to flow by a mobile liquid phase through the stable stationary phase.

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This results separation of sample compounds into individual components and this separation based on their relative affinity towards the two phases (mobile phase and stationary phase) during their travel.

The sample compound which show greater affinity towards the stationary phase will travel slower and for a shorter distance in comparison to compounds with less affinity (travel faster, longer distance).

Types of Chromatography

Adsorption based

- The stationary phase is a solid surface while the mobile phase is liquid. The compounds travel onto the solid surface under the influence of mobile liquid.
- The separation depends on the extent of physical adsorption of compounds to the solid surface.

Partition based

- In this method, both the stationary and mobile phases are liquids. So the compounds are separated because of affinity based on their partition coefficients into the individual liquid layers.
- The compound with greater partition coefficient to the mobile liquid has higher affinity to it so travels faster and vice versa.

Based on the type of stationary material used for the separation, it is of two types

Normal phase	Reverse phase
Stationary phase - polar Compounds- higher polarity elutes out last while non polar come out first.	Stationary phase- non-polar Compounds- lower polarity elute out last and vice-versa.(art19)
polar bed, non polar mobile phase (n-hexane, tetrahydrofuran)	non-polar bed with polar mobile phase (methanol, water, acetonitrile mixture)

High Pressure Liquid Chromatography[2]

It is regarded as high pressure / High performance liquid chromatography and also calling as High patience liquid chromatography based on the long human time requirement and patience needed in its operation.

- Applications of HPLC include detection, analysis, determination, and quantification, derivation of molecules from mixtures of biological, plant and medical importance.
- High performance liquid chromatography is basically a highly improved form of column chromatography.
- Faster process-Instead of allowing the solvent to drip through a column under just the force of gravity; it is externally forced through the column under high pressures of up to 400 atm.
- Better separation- It allows the use of very small particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing through it.
- Ability to identify, separate and quantitate the compounds that are present in any sample that can be dissolved in any liquid[2].
- 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 product[3].
- Aim- To try & separate, quantify the main drug, any reaction impurities, all available synthetic intermediates and any degradants[4].
- HPLC is the most accurate analytical methods widely used for the quantitative as well as qualitative analysis of drug product[5].

Chromatographic Principles[6]

Retention

- Retention or elution volume is the quantity of the mobile phase required to pull the sample through the column.
- Retention time is defined as how long a component is retained in the column by the stationary phase relative to the time it resides in the mobile phase.
- The retention is best described as a column capacity ratio (k′), which can be used to evaluate the efficiency of columns. The longer a component is retained by the column, the greater is the capacity factor.

The column capacity ratio of a compound (A) is defined by the following equation;

$$k' = T_A - T_O / T_O = V_A - V_O / V_O$$

Where, V_A is the elution volume of component A and

V_o- elution volume of a non retained compound. At constant flow rate, retention times (T_A and $T_{0)}$ can be used instead of retention or elution volumes.

Resolution

Resolution is the ability of the column to separate peaks on the chromatograph. Resolution (R) is expressed as the ratio of the distance between two peak maxima to the mean value of the peak width at the base line

$$R= (T_B–T_A)2/ w_A + w_B$$

Where, $T_B$is the retention time of component B,

T_A- is the retention time of component A,

w_A - peak width of component A and

w_B - peak width of component B. If R is equal to or more than 1, then components are completely separated, but if R is less than 1, then components overlap.

Sensitivity

- Sensitivity is a measure of the smallest detectable level of a component in a chromatographic separation and is dependent on the signal-to-noise ratio in a given detector.
- Sensitivity can be increased by derivatization of the compound of interest, optimization of chromatographic system or minia- turization of the system.

HPLC SYSTEM[7, 8]

Typical HPLC system consists of the following main components:

Solvent Reservoirs

- Separation is commonly affected by the type and composition of the mobile phase.
- Different solvents are used. For normal-phase HPLC, the solvent is usually non-polar 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.
- The most common solvent reservoirs are as simple as glass bottles with tubing connecting them to the pump inlet.

Pump

- This provides the constant and continuous flow of the mobile phase through the system.
- Most modern pumps allow controlled mixing of different solvents from different reservoirs.
- A steady pump pressure (usually about 1000–2000 psi) is needed to ensure reproducibility and accuracy[6].

Injector

- This allows an introduction (injection) of the analytes mixture into the stream of the mobile phase before it enters the column
- Most modern injectors are auto samplers, which allow programmed injections of different volumes of samples that are withdrawn from the vials in the auto sampler tray[7, 8].
- Injector- provides injection within the range of 0.1-100 mL of volume under high pressure (up to 4000 psi)[6].

Column[7,8]

- This is the heart of HPLC system; it actually produces a separation of the analytes in the mixture.
- A column is the place where the mobile phase is in contact with the stationary phase, forming an interface with enormous surface.

Detector[7,8]

- This is a device for continuous registration of specific physical (sometimes chemical) properties of the column effluent.
- The most common detector used in pharmaceutical analysis is UV (ultraviolet).

Data Acquisition and Control System[7,8]

Computer-base system that controls all parameters of HPLC instrument (eluent composition (mixing of different solvents); temperature, injection sequence, etc.) and acquires data from the detector and monitors system performance (continuous monitoring of

the mobile-phase composition, temperature, backpressure, etc.).

Importance

HPLC is a versatile, reproducible chromatographic technique for the estimation of drug products. HPLC has many applications in the field of pharmaceutical, environmental, clinical, and forensic in food and flavor analysis[9].

The technique of HPLC has following features[10].

- High resolution
- Small diameter, Stainless steel, Glass column
- Rapid analysis
- Relatively higher mobile phase pressure
- Controlled flow rate of mobile phase

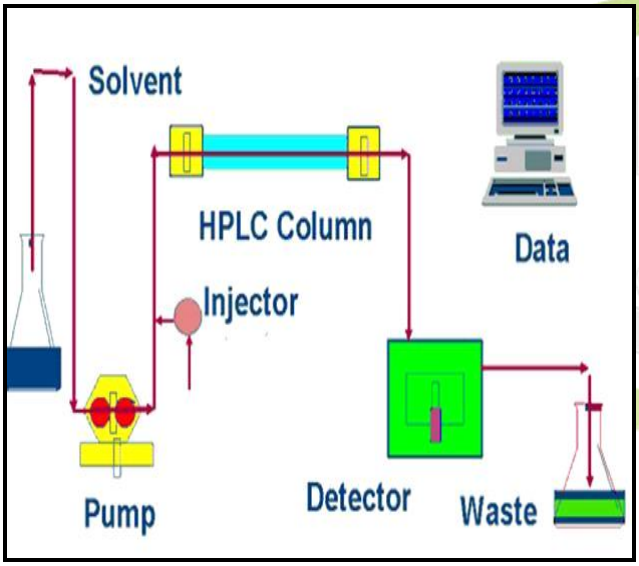
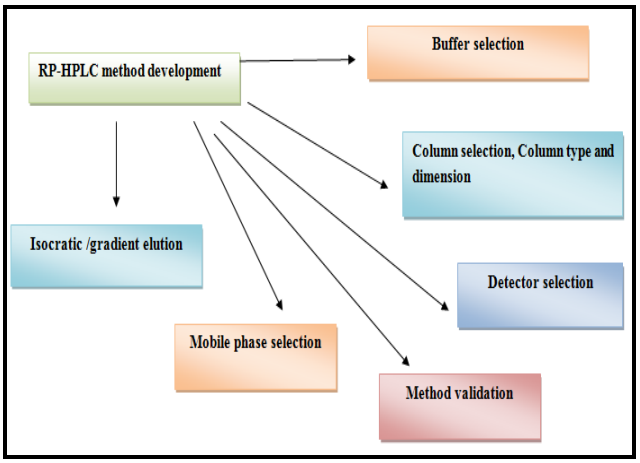


Figure 1: HPLC system

HPLC Method Development

Development involves selection of best mobile phase, best detector, best column, column length, stationary phase and best internal diameter for the column[12,13]

- Set up HPLC condition[14,15]
- Preparation of sample solution for method development.
- Method optimization.
- Validation of method.



Set up HPLC Condition[16,17,18]

Buffer Selection

- Choice of buffer is typically governed by the desired pH. The typical pH range for reversed- phase on silica-based packing is pH 2 to 8.
- It is important that the buffer has a pKa close to the desired pH since buffer controls pH best at their pKa. A rule is to choose a buffer with a pKa value greater than 2 units of the desired mobile phase pH.
- General considerations during buffer selection:
- Phosphate is more soluble in methanol/water than in acetonitrile/water or THF/water.
- Some salt buffers are hygroscopic. This may lead to changes in the chromatography (increased tailing of basic compounds, and possibly selectivity differences).
- Ammonium salts are generally more soluble in organic/water mobile phases.
- TFA can degrade with time, is volatile, absorbs at low UV wavelengths.
- Microbial growth can damage chromatographic performance.
- At pH < 7, phosphate buffer accelerates the dissolution of silica and severely shortens the lifetime of silica-based HPLC columns.
- Ammonium bicarbonate buffers are usually stable for only 24 to 48 hours. The pH of this

mobile phase tends to become more basic due to the release of carbon dioxide.

Buffer (art 8)	pKa	Useful pH range
Ammonium acetate	4.8 9.2	3.8-5.8 8.2-10.2
Ammonium formate	3.8 9.2	2.8-4.8 8.2-10.2
KH_2pO_4/phosphoric acid	2.1	1.1-3.1
KH_2pO_4/K_2pO4	7.2	6.2-8.2
Potassium acetate/acetic acid	4.8	3.8-5.8
Borate($H_3BO_3/Na_2B_4O_7.10H_2O$)	9.2	8.2-10.2
Ammonium hydroxide/ammonia	9.2	8.2-10.2
Trifluroacetic acid	<2	1.5-2.5
Potassium formate/formic acid	3.8	2.8-4.8

Buffer Concentration

- Generally, a buffer concentration of 10-50mm is adequate for small molecules. Generally, no more than 50% organic should be used with a buffer. This will depend on the specific buffer as well as its concentration.
- Phosphoric acid and its sodium or potassium salts are the most common buffer systems for reversed-phase HPLC.
- Phosphonate buffers can be replaced with sulfonate buffers when analyzing organophosphate compounds

Selection of Detector

Selection of detector depends on the chemical nature of analytes, potential interference, limit of detection required, availability and/or cost of detector.

Detectors	Description
UV-Visible detector	• Versatile, dual-wavelength absorbance detector for HPLC. • Offers the high sensitivity, low-level impurity identification and quantitative analysis.
Photodiode Array (PDA) Detector	• Offers advanced optical detection for Waters analytical HPLC, preparative HPLC, or LC/MS system solutions. • Deliver high chromatographic and spectral sensitivity (integrated software and optics innovations)
Refractive Index (RI) Detector	• Offers high sensitivity, stability and reproducibility.
Multi-Wavelength Fluorescence Detector	• Offers high sensitivity and selectivity for quantitating low concentrations of target compounds.[11](art20)

Selection of Column[15]

- Column parameter like internal diameter, particle size, surface area, carbon load to be checked to verify system suitability criteria.
- Column- C8, C18, cyano, phenyl & amino can be use against a more polar mobile phase.
- Normal phase- column is more polar Cyano, phenyl, silica column are used.

Selection of Column Oven Temperature[15]

Column oven temperature can be selected from the elution of the analytes & peak shapes.

Selection of Injection Volume[15]

In general case 20l is used & if the samples have less absorbance then it may change to 100l & if the samples have high absorbance at the selected detector condition then researcher can use the less micro liter also.

Selection of Runtime[15]

Samples analysis time can be fixed from the late elution of the peak.

Selection of Organic Modifier[15]

The most useful solvents are Acetonitrile, Methanol & THF.

Selection Isocratic & Gradient Elution[6]

- Elution techniques are methods of pumping mobile phase through a column.
- In the isocratic method, the composition of the mobile phase remains constant, whereas, in the gradient method the composition changes during the separation process.
- The isocratic method is the simplest technique and should be the first choice when developing a separation.

Selection of Mobile Phase pH Value[6]

pH selection is critical and based on the solubility, solution, stability, column stationary phase, elution of the analytes & peak shapes.

Sample Preparation[19,20]

- Sample preparation is a critical step.
- Purpose- to create a processed sample that leads to better analytical results compared with the initial sample.
- The prepared sample should be an aliquot relatively free of interferences that is compatible with the HPLC method and that will not damage the column.

Method Optimization[21]

- The experimental conditions should be optimized to get desired separations and sensitivity after getting appropriate separations.
- Stability indicating assay experimental conditions will be achieved through planned/systemic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, temperature, sample amounts, Injection volume and diluents solvent type.

Method Validation

Validation of an analytical method is the process by which it is established by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application.

Validation is required for any new or amended method to ensure that it is capable of giving reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories. The type of validation program required depends entirely on the particular method and its proposed applications[19].

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. Use of equipment that is within specification, working correctly and adequately calibrated is fundamental to the method validation process. Analytical methods need to be validated or revalidated.

Typical parameters recommended by FDA, USP, and ICH are as follow[22,23].

- Specificity
- Linearity & Range
- Precision
- Method precision (Repeatability)
- Intermediate precision (Reproducibility)
- Accuracy (Recovery)
- Solution stability
- Limit of Detection (LOD)
- Limit of Quantification (LOQ)
- Robustness
- System suitability

Specificity

Selectivity of an analytical method as its ability to measure accurately an analyte in the presence of interference, such as synthetic precursors, excipients, enantiomers, and known (or likely)

degradation products that may be expected to be present in the sample matrix[24].

Linearity and Range

- Its ability (within a given range) to obtain test results, which are directly proportional to the concentration of analyte in the sample.
- A linear relationship should be evaluated across the range of the analytical procedure.
- It is demonstrated directly on the drug substance by dilution of a standard stock solution of the drug product components, using the proposed procedure.
- Linearity is usually expressed as the confidence limit around the slope of the regression line[22,23]. For the establishment of linearity, minimum of five concentrations are recommended by ICH guideline[25].

Precision

- 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.
- Considered at three levels: repeatability, intermediate precision and reproducibility[25].
- Expressed as the standard deviation or relative standard deviation of series of measurements.
- Precision may be either the degree of reproducibility or of the repeatability of the analytical procedure under normal conditions.
- Intermediate precision (also known as ruggedness) expresses within laboratories variations, as on different days, or with different analysts or equipment within same laboratory[26].

Accuracy (Recovery)

- 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.
- 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[23,25].

Solution Stability

During validation the stability of standards and samples is established under normal conditions, normal storage conditions, and sometimes in the instrument to determine if special storage conditions are necessary, for instance, refrigeration or protection from light[23].

Limit of Detection (LOD)

- 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.
- 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[23,26].

Limit of Quantification (LOQ)

- Quantitation limit - 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[25, 26].

Robustness

- 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[23, 25].

System Suitability

- An integral part of liquid chromatographic methods.
- Used to verify that the detection sensitivity, resolution and reproducibility of the chromatographic system are adequate for the analysis.

The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Factors, such as the peak resolution, number of theoretical plates, peak tailing and capacity have been measured to determine the suitability of the used method[22, 26].

Application (Art 15 all appl. HPLC)

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

- Clean peak of the known sample is observed from the chromatograph and show a reasonable retention time and should be well separated from extraneous peaks at the detection levels which the assay will be performed.

Other Applications of HPLC

Pharmaceutical applications[27-30]	➢ 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[31-34]	➢ Detection of phenolic compounds in drinking water ➢ Identification of diphenhydramine in sedimented samples ➢ Bio-monitoring of pollutant
Forensics[35-37]	➢ 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
Clinical[38-41]	➢ 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.
Food and Flavor[42]	➢ Ensuring the quality of soft drink and drinking water. ➢ Analysis of beer. ➢ Sugar analysis in fruit juices. ➢ Analysis of polycyclic compounds in vegetables. ➢ Trace analysis of military high explosives in agricultural crops.

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