

## ***Critical Examination of High-Performance Liquid Chromatography Methodology: Development and Validation***

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### ***Abstract***

*High-Performance Liquid Chromatography (HPLC) stands as the predominant separation technique for the detection, separation, and quantification of drugs. The versatility of HPLC makes it especially effective for analyzing multi-component dosage forms, owing to its advantages such as speed, specificity, accuracy, precision, and ease of automation. The application of HPLC extends to pivotal roles in the discovery, development, and manufacturing of pharmaceutical drugs, as well as in various studies related to both human and animal subjects. This review provides insights into the different stages involved in the development and validation of HPLC methods. The validation process, aligned with ICH guidelines, encompasses key performance characteristics including accuracy, precision, specificity, linearity, range, limit of detection, limit of quantification, robustness, and system suitability testing.*

***Keywords:*** *Pharmaceutical drugs, HPLC (high pressure liquid chromatography) method development, validation.*

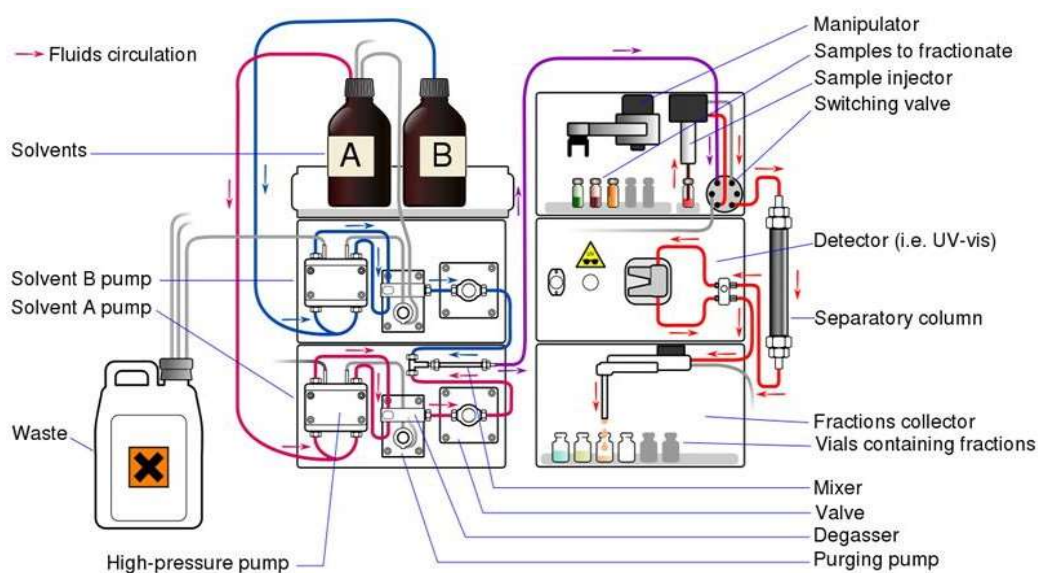
### **INTRODUCTION**

High 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 dissolving in liquid. High performance liquid chromatography

(HPLC) is the most accurate analytical method widely used for the quantitative as well as qualitative analysis of drug product.[1] The high-performance liquid chromatography is more versatile than gas chromatography since it is not limited to volatile and thermally stable samples, the choice of mobile and stationary phase is wider.[2]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 product.[3]The principle of followed is the adsorption of solute on stationary phase based on its affinity towards stationary phase.(Figure\_1)

The technique of HPLC has following features.[4]

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



**Figure:-1 flow diagram of HPLC**

## CLASSIFICATION

### Classification of HPLC can be done as following:

- Affinity chromatography
- Adsorption chromatography
- Size exclusion chromatography
- Ion exchange chromatography
- Chiral phase chromatography (based on principle of separation)
- Gradient separation and isocratic separation (based on elution technique)

### Normal phase chromatography and reverse phase chromatography (based on operation)

#### A. A Normal phase chromatography:

In normal phase mode of the stationary phase is polar and the mobile phase is non polar in nature .in this technique, non-polar compounds travel faster and are eluted first. This is because of the lower affinity between the non-polar compounds and the stationary phase. Polar components are retained for longer times because of higher affinity with the stationary phase.[6] for example. A typical column has an internal diameter of around 4.6 mm, and Lenth in the range of 150 to 250 mm. polar compound in the mixture that are passed through the column will stick longer to the polar silica than non-polar compounds. Therefore, the non-polar ones will pass more quickly through the column.[7]

#### B. RP-HPLC (Reverse phase HPLC):

It is the most popular mode of analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is non polar hydrophobic packing with octyl or octa decyl function group bonded to silica gel and the mobile phase is polar solvent. The polar compounds get eluted first in this mode and non-polar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster.[8]

## INSTRUMENTATION OF HPLC

### A. Solvent delivery system:

Reciprocating pumps are the most favored solvent delivery system in microcolumn LC , because of their rapid flow equilibrium and stability , large column back pressure

compensation abilities and good possibilities for micro gradient delivery[11] minute flows in the low low  $\mu\text{L}$  and nL ranges, for columns with I.D.s  $< 500 \mu\text{m}$ , cannot be readily provided by direct pumping. For such small flows, the use of split-flow techniques is an alternative. These systems are based on the application of packed restrictor columns [22] or flow splitting devices based on a microflow processor concept. The latter system can be used in both isocratic and gradient mode, because it compensates for viscosity changes of the eluent, e.g. in gradient analysis. In gradient analysis, the microflow processor can be connected to a conventional gradient device, by means of which, part of the flow is split in a constant adjustable ratio to the microcolumn.

### **B. Injection systems:**

For 50-100  $\mu\text{m}$  I.D. columns, injection volumes range between a few nL up to approximately 1  $\mu\text{L}$  for 1.0 mm I.D. columns. Injection valves are the most favored injection systems in HPLC. Manual injections in the  $\mu\text{L}$  range down to approximately 20 nL can be performed with micro-injection valves equipped with a replaceable internal loop. Below 20 nL, manual valve injections can be performed by placing a split vent between the injector and the column [17]. The moving injection technique [15], the static split [6] and the pressure pulse-driven stopped-flow injection technique [37] can also be used for such small volumes. In all the aforementioned techniques, only a small part of the injection plug is injected on the top of the column, by controlling the injection time and flow through the injector. Automated injection in the  $\mu\text{L}$  range can easily be performed by commercial auto samplers, which however, require a thorough adjustment for use in the nL range. Modifications of conventional auto samplers for use in capillary LC have been described in the literature [18]. The small injected volumes or masses constitute a major problem in microcolumn LC, causing loss of detection sensitivity. This problem can be solved by the application of on-column focusing techniques [39,8], in which the sample solvent has a considerably lower eluent strength compared to the actual eluent. After arrival of the sample plug on the column top, the analytes are focused in a small plug, leading to enrichment factors of several hundreds and thus significantly increased detectability in microcolumn LC.

### **C. Tubing and connections:**

The tubing which is used to connect the various parts of an HPLC equipment contributes linearly to its length and to the power 4 of its radius to the extracolumn band-broadening

variance. For columns of 500  $\mu\text{m}$  – 1.0 mm I.D., specially designed stainless steel or polyetheretherketone or fused silica tubing of 0.25 or 0.125 mm I.D. can be employed. For smaller I.D. columns, the use of tubing is as much as possible avoided and direct connections of the column to injector and detector are preferred. Alternatively, connecting tubing with I.D.s of  $\leq 50 \mu\text{m}$  is used. The length of these tubes should also be as short as possible to prevent loss in resolution.[11]

#### **D. Detectors:**

All the conventional detectors can be applied in microcolumn and capillary LC. Refractive index (RI) detection has in general received only small attention in microcolumn and capillary LC, because of the intrinsic difficulty arising from the fact that RI differences have to be measured in extremely small volumes. However, with the high collimating power of lasers, nL to pL volumes can easily be probed. Laser-based RI detection has, among others, been applied by Bornhop et al. [21] and Bruno et al. [32]. The UV absorbance detector is the most commonly used detector even in microcolumn and capillary LC with the on-column detection being the first approach to prevent extra-column band broadening. Fiber optics have been introduced to collimate the excitation light onto the flow cell and for the collection of the UV light that has passed through the flow cell [83], however the sensitivity obtained was inferior to on-column detection. The introduction of longitudinal flow cells with an optical path length up to 3-8 mm [84] has also been tested. Photodiode array (PDA) detection has been used extensively in microcolumn LC [15-17]. Despite the small optical length of the PDA flow cell, ten times lower detection limits were reported for capillary LC, compared to conventional LC.

Fluorescence detection provides higher selectivity and sensitivity compared to UV and RI absorption. Straightforward on-column fluorescence detection is not a common approach in microcolumn LC. The majority of the applications deal with laser-induced fluorescence or detection of fluorescence emission in the packing [33]. Amperometric detection, potentiometric detection and conductivity are the three basic electrochemical detection modes. Miniaturized electrochemical detection - or the use of micro-electrodes - was first reported for open tubular LC and it was later also applied in microcolumn LC [19]. Amperometric detection is the most commonly used detection mode in microcolumn LC. However, only compounds that are easily oxidized and reduced at the set potential are detected, because the

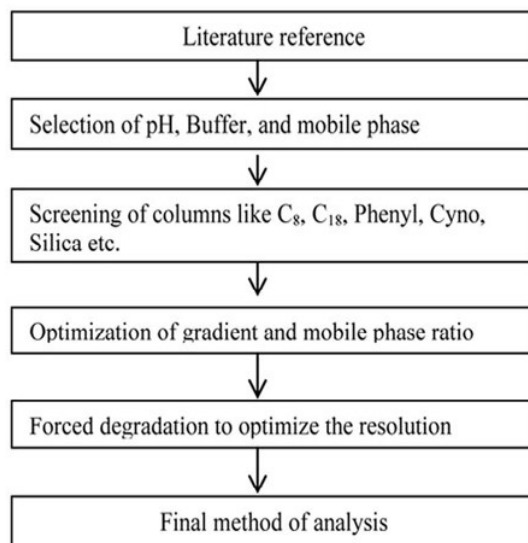
potential of the electrode is held at one value. By scanning the potential-or by applying triangular potential waveform to the electrode (voltammetric analysis), the number of detectable compounds that compounds that are electroactive in the potential range applied, is increased [40,41]. Voltammetric analysis has only limited applications in microcolumn LC. The main application area of electrochemical detection in microcounmn LC is bioanalysis. Microcolumns are more suited to work with the detectors currently used in gas chromatography (GC), because they are typically operated at volumetric flow- rates of a few  $\mu\text{L}$  per minute. After the introduction of microcolumn LC, various types of direct introduction- based GC detectors: flame-based and flameless thermionic- , flame photometric- and electron capture detectors were used by several research groups [22-25]. Other detection principles, have also been developed for microcolumn LC, including infrared spectrometry [96], chemiluminescence [7], inductively coupled plasma atomic emission spectrometry [98] or evaporative light scattering [9]. These detection techniques have been applied with limited success in microcolumn LC and definitely not for routine use, mainly because they are either too selective or not robust enough.

## **HPLC METHOD DEVELOPMENT**

Methods are developed for new product when no official methods are available. Alternate method for existing products is to reduce the cost and time for better precision and raggedness. When alternate method proposed is intended to replace the existing procedure comparative laboratory data including merit/demerits are made available. The goal of the HPLC method is try and separate quantify the main active drug, any reaction impurities, all available synthetic intermediates and any degradants.[9]

### **Steps involved in method of development are:**

- Understanding the physicochemical properties of drug molecule.
- Selection of chromatographic conditions.
- Developing the approach of analysis.
- Sample preparation.
- Method optimization.
- Method validation. (Figure-2)



**Figure: 2 steps involved in HPLC method development**

### 1. Understanding the physicochemical properties of drug molecule.

Physicochemical properties of a drug molecule play an important role in method development. Method development one has to study the physical properties like solubility, polarity, pKa, and PH of the drug molecule. Polarity is a physical property compound. It helps an analyst, to decide the solvent and composition of the mobile phase.

The solubility of molecule can be explained on the basis of the polarity of molecules. Polar e.g., Water, and nonpolar. Benzene, solvent do not mix. In general, like dissolves like ergometries with similar polarity are soluble in each other. The selection of mobile phase or diluents is based on the solubility of analyte. The analyte must be soluble in diluents and must not react with any of its component. PH and pKa plays an important role in HPLC method development. The PH value is defined as the negative of the logarithm to base 10 of the concentration of the hydrogen ion.

$$a. \text{ PH} = -\log_{10}[\text{H}^{+}].$$

Selecting a proper pH for ionizable analytes often leads to symmetrical and sharp peaks in HPLC. [10-11]

## 2. Selection of chromatographic conditions

During initial method development, a set of initial condition (detector, column, mobile phase) is selected to obtain the first “scouting” chromatograms of the sample. In most cases, there are based on reversed phase separations on a C18 column with UV detection. A decision on a developing either an isocratic or a gradient method should be made at this point.

### Optimization of Mobile Phase:

- A. Selection of column:** selection of the stationary phase/ column is the first and the most important step in method development. The development of a rugged and reproducible method is impossible without the availability of a stable, high performance column. To avoid problems from irreproducible sample retention during method development, it is important that columns be stable and reproducible. A C8 or C18 column made from specially purified, less acidic silica and designed specifically for the separation of basic compounds is generally suitable for all samples and is strongly recommended.[12] column dimensions, silica Sunstate properties and bonded stationary phase characteristics are the main ones. The use of silica-based packing is favored in most of the present HPLC columns due to several physical characteristics.[13]
- B. Buffer selection:** choice of buffer is governed by the pH that is desired. The typical pH range for reversed phase on silica-based packing is pH 2 to 8. it is important that the buffer has a close to the desired pH since buffer controls pH best at their pKa. A rule is to choose a buffer with a pKa value <2 units of the desired mobile phase ph.

### General consideration for buffer selection:

1. Phosphate is more soluble I methanol /water than acetonitrile /water or THF/water.
2. Ammonium salts are generally more soluble in organic / water mobile phase.
3. Trifluoroacetic acid can degrade with time. It is volatile and absorbs at low UV wavelengths.
4. At pH greater than 7, phosphate buffer accelerates the dissolution of silica and severely shortens the lifetime of silica based HPLC columns. If possible, organic buffers should be used at pH greater than 7.
5. After buffers are prepared, they should be filtered through a filter.



6. Mobile phase should be degassed.[14]

**C. Effect of pH:** if analytes are ionizable, the proper mobile phase pH must be chosen based on the analyte pKa so the target analyte is in one predominate ionization state ionized or neutral. Alteration of the mobile phase pH is one of the greatest tools in the “chromatographers toolbox” allowing simultaneous change in retention and selectivity between critical pair of components.[15]

**D. internal diameter:** the internal diameter of a HPLC column is an important that influences the detection sensitivity and separation selectivity is gradient elution. It also determines the quality of analyte that can be loaded into a column.[16]

**E. Particle size:** particle size of column defines an ability of the analyte molecules to penetrate inside the particle and interact with its inner surface.[17]

**F. Selection of detector:** detector is a very important part of HPLC. Selection of detector depends on the chemical nature of analytes, potential interference, limit of detection required, availability cost of detector visible detector is versatile, dual wavelength absorbance detector for HPLC. this detector offers the high sensitivity required for routine UV based application to low level impurity identification and quantitative analysis.[18]

#### 4. Develoing The Approach For Analysis:

While developing the analytical method or RP HPLC the first step which is followed is the selection of the various chromatographic parameters like selection of mobile phase, selection of column , selection of flow rate of mobile pase, selection of pH of mobile phase . All of these parameters are selected on the basis of trails and followed by considering the system suitability parameters. [19]

**5. Sample Prepration :** sample preparation is essentiol part of HPLC analysis, intended to provide areproducibile and homogeneous solution that is suitsble for injection onto the column.the . The aim of sample prepration is sample aliquot that, is relatively free of interferences, will not damage the counn, and is compatible with the intended HPLC

method that is, the sample solvent will dissolve in the mobile phase without affecting sample retention or resolution. sample preparation begins at the point of collection, extends to sample injection on to the HPLC column.[20]

| Dector                                                            | Types of compound can be detected                                                                   |
|-------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|
| UV visible & photodiode array                                     | Compound with chromophores, such as aromatic rings or multiple alternating double bonds.            |
| Fluorescence detector                                             | Fluorescent compounds, such as fused rings or highly conjugated planar system.                      |
| Conductivity detector                                             | Charged compounds, such as inorganic ions and organic acids.                                        |
| Electrochemical detector                                          | For easily oxidised compounds like quinines or amines                                               |
| Refractive index detector & evaporative light scattering detector | Compound that do not show characteristics usable by the other detectors, eg. polymers, saccharides. |

6. **Method Optimization:** most of the optimization of HPLC method development has been focused on the optimization of hplc conditions.[21] the mobile phase and stationary phase compositions need to be taken into account. Optimization of mobile phase parameters is always considered first as this is much easier and convenient than stationary phase optimization. To minimize the number of trial chromatograms involved, only the parameters that are likely to have a significant effect on selectivity in the optimization must be examined.[22]
7. **Method Validation:** validation is the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled. A process of evaluation method performance and demonstrating that it meets particular requirements. In essence, it knows what your method is capable of delivering, particularly at low concentrations.[23]

#### Types of analytical procedure to be validated

The discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures

- Identification tests;
- Quantitative tests for impurities content;
- Limit tests for the control of impurities;
- Quantitative tests of the active moiety in sample of drug substance or drug product or other selected components in the drug product.[24]

**Components of method validation:** the following are typical analytical performance characteristics which may be tested during method validation:

1. Accuracy
2. Precision
3. Linearity
4. Detection limit
5. Quantitation limit
6. Specificity
7. Range
8. Robustness

**Accuracy:** accuracy is defined as the nearness of measured value to the true or accepted value. Practically accuracy indicates the deviation between the mean value found and the true value. It is determined by applying the method of sample to which known amounts of analyte have been added. These should be analyzed against standard and blank solutions to ensure that no interference exists.[25]

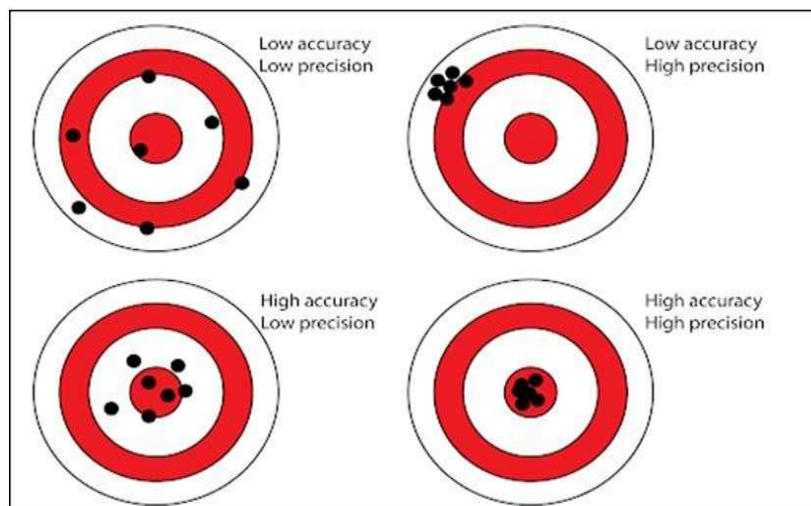
**Precision:** It expresses closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision is a measure of the reproducibility of the whole analytical method. [26] It consists of two components: repeatability and intermediate precision. 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. [27] The precision is then expressed as the relative standard deviation.

$$\%RSD = \frac{\text{Std dev.} * 100}{\text{MEAN}}$$

Accuracy and precision are not the same, as the diagram below indicates. A method can have good precision and yet not be accurate. See figure 3

**Linearity:** the linearity of an analytical procedure is its ability 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.[28]



**Figure: 3**

**Limit of quantification (LOQ):** the limit of quantitation is 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 standereds which give rhis S/N ratio and have an acceptable percent relative standerd deviation as well.[29]

**Specificity:** selectivity of an analytical method as its ability to measure accurately an analyte in the presence of interference, such as a synthetic precursors, excipients, enantiomers, and known degradation products that may be expected to be present in the sample matrix.[30]

**Range:** the range of the method is interval between the upper and lower levels of an analyte that have been determined with acceptable precision, accuracy, and linearity. It is determined on either a linear or nonlinear response curve and is normally expressed in the same units as the test results.[31]

**Robustness:** is defined as the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal use. Determination of robustness is a systematic process of varying a parameter and measuring the effect on the method by monitoring system suitability or the analysis of sample.[32]

### Applications of HPLC

|                                                                                    |
|------------------------------------------------------------------------------------|
| 1. HPLC is used for chemistry and biochemistry research analyzing complex mixture. |
| 2. Purifying chemical compounds.                                                   |
| 3. Quality control to ensure the purity of raw materials.                          |
| 4. Analyzing air and water pollution.                                              |
| 5. To control drug stability.                                                      |
| 6. Detection of phenolic compounds in drinking water.                              |
| 7. Quantification of drugs in biological samples.                                  |
| 8. Identification of steroids in blood, urine etc.                                 |
| 9. Forensic analysis of textile dyes.                                              |
| 10. Sugar analysis in fruit juices.                                                |

### CONCLUSION

This review describes about RP HPLC technique. The method development and validation are continuous and interrelated processes that measure a parameter as intended and establish the performance limits of the measurement. The selection of column, buffer, detector and wavelength and other conditions composition plays a dramatic role on the separation

selectivity the advantages of HPLC were high selectivity, sensitivity, economic, less time consuming and low limit of detection

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