REVIEWS ARTICLE ON HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD DEVELOPMENT AND VALIDATION

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ABSTRACT
Chromatographers face the need to develop a high-performance liquid chromatography (HPLC) method everyday. In this review systematic procedures for developing an HPLC method, based on the best information available. HPLC method development involves sample pre-treatment, choosing separation conditions, detection of sample bands, quantitation, and method validation. HPLC is an analytical tool which is able to detect, separate and quantify the drug, its various impurities and drug related degrades that can form on synthesis or storage. Validation of HPLC method gives information regarding various stages like Accuracy, specificity, linearity limit of detection, limit of quantification.

KEYWORDS: HPLC, mobile phase, stationary phase, method development, validation.

INTRODUCTION
Analytical chemistry is used to determine qualitative and quantitative composition of material under study by using chromatographic methods. A qualitative analysis gives information about the nature of the material. On the other hand quantitative analysis give information about the amount of the sample present in the sample. HPLC, GC and HPTLC has wide application for the qualitative and quantitative assurance of the raw material.[1]

HPLC (HIGH PERFORMANCE LIQUID CHROMATOGRAPHY)
High Performance Liquid Chromatography is now one of the most powerful tools in analytical chemistry.[2] In HPLC methodology the solutes are resolved by differential rates of elution as they pass through a chromatographic column. The separation by this instrument is governed by distribution of components between the mobile phase. The sample to be
analysed is introduced in a small volume to the stream of mobile phase through injector and is retarded by specific chemical or physical interactions with the stationary phase as it move to the length of the column. The amount of retardation depends on the interactions between the mobile phase and stationary phase.\textsuperscript{[3]} The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time and is considered a reasonably unique identifying characteristic of a given analyte.\textsuperscript{[4]} The instrumentational components include solvent reservoir, solvent delivery system(pump)\textsuperscript{[5]}, sample injection system, column, detector, integrator and waste reservoir (Figure.1).

![Figure 1: Components of HPLC.](image)

**TYPES OF HPLC**

**It includes**

1. Normal phase chromatography: In which mobile phase is non-polar and stationary phase is polar.
2. Reversed phase chromatography: Stationary phase is non polar and mobile phase is polar.
3. Ion pair chromatography: In this ion pair reagents is used for interaction between analyte and stationary phase.
4. Ion exchange chromatography: ion exchange resins are used in it for the separation of the highly polar analyte.
**Table 1: Types of HPLC and with their stationary phase mobile phase and uses.**

<table>
<thead>
<tr>
<th>Types</th>
<th>Stationary phase</th>
<th>Mobile phase</th>
<th>use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal phase HPLC</td>
<td>Polar(silanol)</td>
<td>Non polar (hexane, heptane, chloroform, dioxane)</td>
<td>To separate non polar compounds</td>
</tr>
<tr>
<td>Reverse phase HPLC</td>
<td>Non polar</td>
<td>Polar (methanol, acetonitrile, water) + Buffer for ionic compounds</td>
<td>To separate non-polar to medium polar</td>
</tr>
<tr>
<td>Ion pair HPLC</td>
<td>Same as reverse phase</td>
<td>Methanol, acetonitrile + Buffer + Ion pair reagents (tetra alkyl ammonium salts)</td>
<td>To separate ionic compounds</td>
</tr>
<tr>
<td>Ion exchange HPLC</td>
<td>1. Negatively charges cation exchange resins.</td>
<td>Acids, alkali, buffers</td>
<td>To separate ionic compounds</td>
</tr>
<tr>
<td></td>
<td>2. Positively charged anion exchange resins.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**METHOD DEVELOPMENT**

Analytical method development and validation play important role in discovery development and manufacturing of pharmaceuticals. These methods are used to ensure identity, purity, potency and performance of the drug. Best column, best mobile phase, best detection wavelength efforts in their selection makes a world of difference while developing HPLC method for routine analysis. Determining the ideal combination of these factors assures faster delivery of desired results.[5] The goal of HPLC method to separate and quantify the main
active ingredient from the reaction mixture and all available synthetic intermediates and degradants. Before starting the development procedure one should gain the knowledge about the sample to be analysed and need to set various HPLC conditions such as type of solvent, column, mobile phase, buffers, detection wavelength etc to be used. So there is step wise description of the various parameters one should consider while the method development for a drug.

1. Physiochemical properties of Sample
2. Sample preparation methods
3. Set up HPLC initial condition
4. Method validation

PHYSIOCHEMICAL PROPERTIES OF SAMPLE
Physiological properties have an important role in method development. For method development one need to study the properties like pH, solubility, polarity etc. For better understanding one by one study of these properties is necessary.

$pka$ is the negative logarithm of the acid dissociation constant. It tells about the extent of the dissociation of the compound. Pka play very important role in the resolution of the hplc method. It generally determines the extent of the ionization of the compound. This is generally explain by the Henderson-Hasselbalch equation. Amount of drug that exist in the form of ionized and unionized form may be calculated by the equation equation. According to the equation amount of the ionized and unionized drug is the function of $p^{ka}$ of the drug and $p^H$ of the mobile phase. At half concentration the unionized drug will be equal to ionized drug i.e $[HA]/[A^-]=1$ So the value of the log 1 is =0. At this point the $p^H$ will be equal to the $p^{ka}$, so we can say that $pka$ is the value of $pH$ at which the drug is half dissociated\(^5\). When the pH is less then the $pka$ for acidic compound ,it will be less ionized and if the pH is more then the $pka$ it will be more ionized and vice-versa for the basic compounds. Less the ionized compound better will be the resolution due to less interatction with the stationary phase silanol groups.

$$pka = pH + \log \frac{\text{united drug concentration}}{\text{ionized drug concentration}}$$

Polarity: It is a physical property of a compound. It helps an analyst, to decide the solvent and composition of the mobile phase. In a non-polar covalent bond, the electrons are shared equally between two atoms. A polar covalent bond is one in which one atom has a greater
attraction for the electrons than the other atom. The solubility of molecules can be explained on the basis of the polarity of molecules. Polar e.g. water and non-polar e.g. benzene, solvents do not mix. In general, like dissolves like i.e., materials with similar polarity are soluble in each other. Selection of diluents is based on the solubility of analyte. The analyte must be soluble in the diluents and must not react with any of the diluent components. The diluent should match to the starting eluent composition of the assay to ensure that no peak distortion will occur, especially for early eluting components.

UV absorption: Before method development starts the UV spectra of the sample component should be taken to the know wavelength at which it can be detect by the UV detector in the Hplc on specific wavelength.

SAMPLE PREPARATION METHODS

Sample preparation is an essential part of HPLC analysis, intended to provide a reproducible and homogeneous solution that is suitable for injection onto the column. The aim of sample preparation is a sample aliquot that (A) is relatively free of interferences, (B) will not damage the column, (C) Compatible with the intended HPLC method; that is, the sample solvent will dissolve in the mobile phase without affecting sample retention or resolution. (D) to concentrate the analytes and/or derivatize them for improved detection or better separation. The sample should ideally be dissolved in the initial mobile phase. If this is not possible due to stability or solubility problems, formic acid, acetic acid or salt can be added to the sample to increase solubility. These additives do not usually affect the separation so long as the volume of the sample loaded is small compared to the column volume. Sample preparation begins at the point of collection, extends to sample injection onto the HPLC column, and encompasses the various operations. Types of Samples can be classified as organic (including biological) or inorganic and may be further subdivided into solids, semi-solids (including creams, gels, suspensions, colloids), liquids, and gases. Sample treatments are illustrated below.

1. Preliminary processing
   a) Reducing particle size

   It can be done by Blending, chopping, crushing, grinding, pulverizing, sieving.
b) Drying
Drying by using method direct heating (inorganic samples), vacuum heating (hygroscopic), vacuum & nitrogen drying (oxidizable).

c) Filtration
Method: By cellulose filter paper, polyester and nylon membrane filters, SPE cartilage, SPE disks.

2. Sample pretreatment
a) Sample: Volatile, organics, gases
Method: Solid phase trapping and liquid phase trapping

b) Sample: Liquid
Method: solid phase extraction (SPE), liquid phase extraction (LPE), Dilution, evaporation, distillation, Microdialysis, Lyophilization.

c) Sample: Suspension
Method: Filtration, centrifugation, Sedimentation

d) Sample: Solid
Method: Solid-solid extraction, Soxhlet extraction, Homogenization, force flow leaching, sonication, dissolution, ASE, microwave assisted extraction.

SET UP HPLC INITIAL CONDITIONS
When developing an HPLC method, the first step is always to consult the literature to ascertain whether the separation has been previously performed and if so, under what conditions - this will save time doing unnecessary experimental work. Based on a knowledge of sample composition and the goals of separation, choose Which chromatographic method is most promising for this particular sample.[8] The next step is to classify the sample as regular or special. We define regular samples as typical of small molecules (<2000 Da) that can be separated using more-or-less standardized starting conditions. Exceptions or special samples are usually better separated with a different column and customized conditions. Regular samples can be further classified as neutral or ionic. If the sample is neutral, buffers or additives are generally not required in the mobile phase. Acids or bases usually require the addition of a buffer to the mobile phase, for basic or cationic, samples, "less acidic" reversed
Phase columns are recommended, and amine additives for the mobile phase may be beneficial.

1. The column
2. Mobile phase
3. pH of mobile phase
4. Buffer
5. Detectors
6. Separation techniques
7. Method validation

THE COLUMN
Column is the heart of the HPLC separation method. The availability of a stable and high performance column is necessary for the rugged and best separation. Reverse phase HPLC columns are made by packing the column with spherical silica gel beads which are coated with the hydrophobic stationary phases. There are several types of matrices for support of the stationary phase, including silica, polymers, and alumina. Silica is the most common matrix for HPLC columns. Silica is chemically stable to most organic solvent and to low pH systems. It will dissolve above pH 7. To create stationary phase for reverse phase chromatography, nonpolar layer is coated on silica and the free silanol are react with a chlorosilane to introduce the non-polar surface.\(^9\) Due to steric constraints, only about 1/3 of the surface Silanol are derivatized. It further reacted with chlorotrimethylsilane to end cap the remaining free silanol and improve the column efficiency analytes. In the same way in normal phase chromatography in which polar phase is layered on silica. CN or cyano columns are polar and can be used for normal phase applications. Propyl (C3), Butyl (C4), and Pentyl (C5) columns are useful for ion-pairing chromatography. Selection of the column based on the type the the sample to be eluted. If the sample is non ionic or less ionic (polar) then reversed phase and noraml phase column should be used but if the sample is higly polar (ionic) thean ion pair column should be used.\(^10\) Table 1 shows the different type of column and their use in different type of chromatography.

Table 2: Different types of columns used in HPLC.

<table>
<thead>
<tr>
<th>Type of chromatography</th>
<th>Types of column with their stationary phase on matrix</th>
<th>use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reversed phase chromatography</td>
<td>C4 (butyl) C18 (octadecyl)</td>
<td>Non polar compounds to medium polar (ionic &amp; non</td>
</tr>
</tbody>
</table>
MOBILE PHASE

Methanol, acetonitrile and tetrahydrofuran are the commonly used mobile phases in HPLC. They affect the method selectively. Selection of the mobile-phase and gradient conditions is dependent on the ionogenic nature of the analyte and the hydrophobicity of the analytes in the mixture respectively. Methanol is a polar protic solvent while acetonitrile is a polar aprotic solvent possesses a stronger dipole moment. Binary and tertiary system of solvent may be used as per requirement. If the analyte is neutral then no buffer required but if analyte is polar and ionic then buffer must be used as one part of mobile phase with controlled pH of mobile phase. It is best to start with initial gradient run 5% to 100% methanol. This will suggest you the initial mobile phase strength for isocratic run if possible. If K’ will lie between 0.5<k<20 then isocratic run possible otherwise gradient elution should be done[8]. Triethylamine is the main modifier used in mobile phase to mask the free silanol groups of stationary phase. It will improve the the peak and reduce tailing. Ion pair reagents (alkyl sulfonate) are also used while separation of ionic compounds on lower ph in reverse phase chromatography.

P\text{H} OF MOBILE PHASE

It has great influence on the method development of the drug. P\text{H} is defined as the negative logarithm to base ten of hydrogen ion concentration. Acidity and basicity of the substance mostly defined by the p\text{H} value. Since most of the drug are ionizable weak acids and weak bases, so extent of the ionization influence by the p\text{H} of mobile phase. Let us take example of weak acidic drug and weak base drug in different p\text{H}.

For weak acidic drug

\[
\begin{align*}
\text{HA} & \rightarrow \text{A}^- + \text{H}^+ \\
(\text{Un-ionized}) & \quad \text{(ionized)}
\end{align*}
\]

At high p\text{H}

At low p\text{H}
Reason: On increasing the pH common ion effect dominates and equilibrium shift to left side of reaction.

*For weak basic drug:*

\[
\begin{align*}
B + \text{H}_2\text{O} & \rightarrow \text{BH}^+ + \text{OH}^- \\
\text{(Unionized)} & \quad \text{(ionized)}
\end{align*}
\]

At high $p^H$  
At low $p^H$

Reason: On decreasing the $p^H$ (high hydrogen ion concentration) leads to the Protonation of base and the more ionized form will be there.

We can see that acidic drugs are non-ionized at lower pH. Which is good for separation while basic analytes at lower pH will be ionized and retain strongly, So buffer should be used for basic analytes at lower pH because reversed phase is mostly used and it is most stable at lower pH.

**BUFFER**

Buffers are widely used for pH control of chemical processes. In reversed-phase high-performance liquid chromatography (RP HPLC) separations of compounds with acid/base properties, appropriate buffered solutions are needed\[(11)\]. When we inject our analyte/diluent tends to change in pH at the column inlet. We have already seen the effect of pH on the analyte ionization. To prevent this pH change buffer necessary for reverse phase chromatography. A buffer consist of a weak acid and salt of weak acid (conjugate base) or weak base and salt of weak base (conjugate acid). It resists the minor change in pH. Methanol-water mobile phases provide high solubility then acetonitrile or THF water solution. So methanol may be first choice as solvent. pH of the buffer should adjusted before adding organic phase.

*Buffer capacity:* Buffering Capacity is the ability of the buffer to resist changes in pH when acidic or basic sample were introduced. Buffering Capacity is expressed as the molarity of Sodium hydroxide required to increase pH by 1. For best results and for proper function of buffer one should work within ±1 pH unit of the buffer pKa value for good pH control of the mobile phase. Commonly used buffers are given in table 2. When control at a lower pH (2-3) is desired, phosphate, or stronger organic acids such as TFA or acetic acid or when volatility
is of concern, are commonly. If control at pH 4-5 is desired, an organic acid buffer such as acetate or citrate should be considered in place of phosphate.[12]

**Concentration of buffer:** Generally, a buffer concentration of 10-50 mM is adequate for small molecules. Generally, no more than 50% organic should be used with a buffer. Phosphoric acid and its sodium or potassium salts are the most common buffer systems for reversed-phase HPLC.

**Table 2: Commonly used buffer and their pka value.**

<table>
<thead>
<tr>
<th>Common Buffers</th>
<th>$\text{pK}_a$</th>
<th>Useful pH Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td>$\text{pK}_1$</td>
<td>1.1-3.1</td>
</tr>
<tr>
<td></td>
<td>$\text{pK}_2$</td>
<td>6.2-8.2</td>
</tr>
<tr>
<td></td>
<td>$\text{pK}_3$</td>
<td>11.3-13.3</td>
</tr>
<tr>
<td>Citrate</td>
<td>$\text{pK}_1$</td>
<td>2.1-4.1</td>
</tr>
<tr>
<td></td>
<td>$\text{pK}_2$</td>
<td>3.7-5.7</td>
</tr>
<tr>
<td></td>
<td>$\text{pK}_3$</td>
<td>4.4-6.4</td>
</tr>
<tr>
<td>Formate</td>
<td>3.8</td>
<td>2.8-4.8</td>
</tr>
<tr>
<td>Acetate</td>
<td>4.8</td>
<td>3.8-5.8</td>
</tr>
<tr>
<td>Tris</td>
<td>8.3</td>
<td>7.3-9.3</td>
</tr>
<tr>
<td>Ammonia</td>
<td>9.2</td>
<td>8.2-10.2</td>
</tr>
<tr>
<td>Borate</td>
<td>9.2</td>
<td>8.2-10.2</td>
</tr>
<tr>
<td>Diethylamine</td>
<td>10.5</td>
<td>9.5-11.5</td>
</tr>
</tbody>
</table>

**DETECTORS**

A detector in HPLC is placed at the end of the system. Its work is to analyse the solution which is eluting from the column. Concentration of the analyte is proportional to the electronic signal coming out of the component of the mixture. Detectors for HPLC are designed to take advantage of some physical or chemical attribute of either the solute or mobile phase in one of four ways:

1. A bulk property or differential measurement
2. Analyte specific properties
3. Other

1. **Bulk property**
These detectors are the most universal detectors for HPLC as they measure properties common to all analytes. They measure the changes in solute and mobile phase in
combination. Example: refractive index detectors and conductivity detectors. show fluctuation with slight change in mobile phase combination. Due to poor sensitivity and limited range, they are used less despite being universally applicable.[5,6]

- **Refractive Index HPLC detectors**: They are also one of the bulk property detectors and are based on the change of the refractive index of the eluent from the column with respect to pure mobile phase. There are different types of Refractive index detectors: Christiansen effect detector, interferometer detector, thermal lens detector and the dielectric constant detector.

- **Conductivity HPLC Detectors**: These detectors senses all the ions, whether they are from a solute, or from the mobile phase. It measures the conductivity of mobile phase along with the solute which needs to be backed-off by suitable electronic adjustments. Thus it is a type of Electrical Conductivity Detector. The measured electronic resistance is directly proportional to the concentration of ions present in the solution.

2. **Analyte specific property detectors**

They respond to a characteristic that is unique to an analyte. Example UV detector, fluorescence, conductivity, and electrochemical. h. UV detectors are usually thought of as somewhat specific, responding only to compounds with chromophores, but at low UV wavelengths. These detectors can be used in gradient run.

- **Ultraviolet/Visible Detectors**: The most common HPLC detectors used are UV detectors because of the fact that most of the compounds absorb in UV or visible region. They give specific response to the class of compounds or particular compounds depending upon the functional group of eluting molecules.

- **Diode Array Detectors**: In diode array detector, the sample is subjected to light of all wavelengths generated by the lamp at once. The lights from emission source when collimated by an achromatic lens system, the total light passes through the detector cell onto a holographic grating and then falls onto a diode array which contains 100s of diodes. The chromatogram can be produced using the UV wavelength that was falling on that particular diode at the end of the run.

- **Fluorescent detectors**: These are the most selective, sensitive and specific than all other HPLC detectors. Specific wavelength is used to excite and then emit light signal in analyte atoms. They intensity of light is monitored continuously to quantify the analyte concentration.
3. Other detectors

- **Mobile phase modification detectors**: They change the mobile phase of post-column to induce a change in the properties of the analyte e.g. Evaporative light scattering and corona discharge detectors.

- **Hyphenated techniques**: It refer to the coupling of a separate independent analytical technology to an HPLC system e.g. LC-MS, LC-IR.

**SEPARATION TECHNIQUES**

There are generally two types of separation technique i.e. Isocratic and gradient.

- **Isocratic technique**: In isocratic techniques solvent composition or ratio remain same throughout the elution process. Example: 50% water-50% methanol.[15]

- **Gradient technique**: In gradient separation organic phase concentration continuously increases throughout the process.[16,17]

**METHOD VALIDATION**

Many HPLC procedures will be used for routine quantitative analysis. Accurate results require the use of standards and a calibration procedure. HPLC method is finalized, it should be validated. The following are typical analytical performance characteristics which may be tested during methods validation.[18]

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

- Accuracy
- Precision
- Repeatability
- Linearity
- Detection limit
- Quantitation limit
- Specificity
- Range
- Robustness

**Accuracy**

Accuracy is the nearness of a measured value to the true or accepted value. Practically accuracy indicates the deviation between the mean value found and the true value.
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.

**Precision**

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. 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 analysing 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. The precision is then expressed as the relative standard deviation.

![%RSD formula](image)

**Linearity**

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.

**Limits of detection and quantitation**

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantified. LOD is expressed as a concentration at a specified signal: noise ratio, usually 3:1. The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. For LOQ, ICH has
recommended a signal: noise ratio 10:1. LOD and LOQ may also be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve(s) at levels approximating the LOD according to the given below formulæ.

\[
\text{LOD} = 3.3 \times S / \text{SD} \quad \text{and} \\
\text{LOQ} = 10 \times S / \text{SD}
\]

**Specificity**

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.\textsuperscript{[24]}

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**Range**

The range of the method is the 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 (i.e. where more than one range is involved, as shown below) and is normally expressed in the same units as the test results.

**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.\textsuperscript{[25]}

**CONCLUSION**

This review lighten the introduction, instrumentation, method development and validation by high performance liquid chromatography. It also pointed out the advantages of hplc i.e convenient, fast and accurate for daily analysis. Stationary phase, mobile phase and sample are the main phases. The method development and validation are continuous and interrelated processes that are conducted throughout the drug development process. Reproducibility of the method depends upon the proper handling of the high performance liquid chromatography by
the expert analyst. It gives out the qualitative and quantitative information about the drug sample that remains same for same drug throughout with same conditions in a laboratory.

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