Development and validation of HPLC method - a review
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ABSTRACT
Many different strategies of high performance liquid chromatographic method development are used today. This review describes a strategy for the systematic development of High performance liquid chromatographic (HPLC) methods. HPLC is an analytical tool which is able to detect, separate and quantify the drug, its various impurities and drug related degradants that can form on synthesis or storage. It involves the understanding of chemistry of drug substance and facilitates the development of analytical method. A number of chromatographic parameters were evaluated in order to optimize the method. An appropriate mobile phase, column, column temperature, wavelength and gradient must be found that affords suitable compatibility and stability of drug as well as degradants and impurities. Forced degradation or alternatively referred as stress testing and it demonstrates specificity when developing stability indicating methods, especially when little is known about potential degradation products. Force degradation studies are helpful in development and validation of stability-indicating methodology, determination of degradation pathways of drug substances and drug products, discernment of degradation products in formulations that are related to drug substances versus those that are related to non-drug substances (e.g. excipients).

Key Words: HPLC, method development, validation, force degradation studies.

INTRODUCTION
High Performance Liquid Chromatography (HPLC) was derived from the classical column chromatography and, is one of the most important tools of analytical chemistry today. The principle is that a solution of the sample is injected into a column of a porous material (stationary phase) and a liquid (mobile phase) is pumped at high pressure through the column. The separation of sample is based on the differences in the rates of migration through the column arising from different partition of the sample between the stationary and mobile phase. Depending upon the partition behaviour of different components, elution at different time takes place. The technique, chromatography was originally developed by the Russian botanist M.S Tswett in 1903. High Performance Liquid Chromatography is more versatile than gas chromatography since (a) it is not limited to volatile and thermally stable samples, and (b) the choice of mobile and stationary phases is wider.

HPLC as compared with the classical LC technique is characterised by:
- High resolution.
- Small diameter (4.6 mm), stainless steel, glass or titanium columns.
- Column packing with very small (3, 5 and 10 μm) particles.
- Relatively high inlet pressures and controlled flow of the mobile phase.
- Continuous flow detectors capable of handling small flow rates and detecting very small amounts.
- Rapid analysis.

Method development
Analytical method development and validation play important roles in the discovery development and manufacture of pharmaceuticals. These methods used to ensure the identity, purity, potency, & performance of drug products. There are many factors to consider when developing methods. The initially collect the information about the analyte’s physicochemical properties (pKa, log P, solubility) and determining which mode of detection would be suitable for analysis (i.e., suitable wavelength in
case of UV detection). The majority of the analytical development effort goes into validating a stability indicating HPLC method. The goal of the HPLC-method is to try & separate quantify the main active drug, any reaction impurities, all available synthetic intermediates and any degradants.

Steps involve in method development are:

1. Understand the physicochemical properties of drug molecule.
2. Set up HPLC conditions.
3. Preparation of sample solution for method development.
5. Validation of method.

**Understand the physicochemical properties of drug molecule**

Physicochemical properties of a drug molecule play an important role in method development. For Method development one has to study the physical properties like solubility, polarity, pKa and pH of the drug molecule.

Polarity is a physical property of a compound. It helps an analyst, to decide the solvent and composition of the mobile phase. In a nonpolar 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 nonpolar, 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.

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.

\[
pH = -\log_{10}[H_3O^+]
\]

The acidity or basicity of a substance is defined most typically by the pH value. Selecting a proper pH for ionizable analytes often leads to symmetrical and sharp peaks in HPLC. Sharp, symmetrical peaks are necessary in quantitative analysis in order to achieve low detection limits, low relative standard deviations between injections, and reproducible retention times. The acidity of an aqueous solution is determined by the concentration of \([H_3O^+]\) ions. Thus, the pH of a solution indicates the concentration of hydrogen ions in the solution. The concentration of hydrogen ions can be indicated as \([H^+]\) or its solvated form in as \([H_2O^+]\) whose value normally lies between 0 and 14. The lower the pH, the more acidic is the solution. The pH of a solution can be changed simply by adding acid or base to the solution. The pKa is characteristic of a particular compound, and it tells how readily the compound gives up a proton.

An acid dissociation constant is a particular example of equilibrium constant. For the specific equilibrium between a monoprotic acid, HA and its conjugate base \(A^-\):

\[
HA + H_2O \rightleftharpoons A^- + H_3O^+
\]

The position of equilibrium is measured by the equilibrium constant, \(K_{eq}\).

\[
K_{eq} = \frac{[H_2O^+][A^-]}{[HA][H_2O]}
\]

Now in dilute solutions of acid, \([H_2O]\) stays roughly constant. Therefore define a new equilibrium constant- the acidity constant \(K_a\).

\[
K_a = \frac{[H_2O^+][A^-]}{[HA]}
\]

This is also in logarithmic form are follows:

\[
pK_a = -\log_{10} K_a
\]

It turns that the pKa of an acid is the pH at which it is exactly half dissociated. This can be shown by rearranging the expression for \(K_a\):

\[
pH = pK_a - \log([AH]/[A^-])
\]

At half-neutralization \([A^-]/[HA] = 1\); since \(\log(1) = 0\), the pH at half-neutralization is numerically equal to pKa. Conversely, when \(pH = pK_a\), the concentration of HA is equal to the concentration of \(A^-\).

The buffer region extends over the approximate range pKa ± 2, though buffering is weak outside the range pKa ± 1. At pKa ± 1, \([A^-]/[HA] = 10\) or 1/10.

If the pH is known, the ratio may be calculated. This ratio is independent of the analytical concentration of the acid.
When the pKa and analytical concentration of the acid are known, the extent of dissociation and pH of a solution of a monoprotic acid can be easily calculated\(^6\).

**Set up HPLC conditions**

A buffer is a partially neutralised acid which resists changes in pH. Salts such as Sodium Citrate or Sodium Lactate are normally used to partially neutralise the acid. **Buffering Capacity** is the ability of the buffer to resist changes in pH (i) Buffering Capacity increases as the molar concentration (molarity) of the buffer salt/acid solution increases. (ii) The closer the buffered pH is to the pKa, the greater the Buffering Capacity. (iii) Buffering Capacity is expressed as the molarity of Sodium Hydroxide required to increase pH by 1.0.

Consideration of the affect of pH on analyte retention, type of buffer to use, and its concentration, solubility in the organic modifier and its affect on detection are important in reversed-phase chromatography (RPC) method development of ionic analytes. An improper choice of buffer, in terms of buffering species, ionic strength and pH, can result in poor or irreproducible retention and tailing in reverse-phase separation of polar and ionizable compounds\(^6\).

**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 <2 units of the desired mobile phase pH (see Table).

General considerations during buffer selection:

1. Phosphate is more soluble in methanol/water than in acetonitrile/water or THF/water.
2. Some salt buffers are hygroscopic. This may lead to changes in the chromatography (increased tailing of basic compounds, and possibly selectivity differences).
3. Ammonium salts are generally more soluble in organic/water mobile phases.
4. TFA can degrade with time, is volatile, absorbs at low UV wavelengths.
5. Microbial growth can quickly occur in buffered mobile phases that contain little or no organic modifier. This growth will accumulate on column inlets and can damage chromatographic performance.
6. 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.
7. Ammonium bicarbonate buffers usually are prone to pH changes and 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.
8. After buffers are prepared, they should be filtered through a 0.2-µm filter.
9. Mobile phases should be degassed.

**Buffer concentration**: 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. 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\(^10\).

**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 and/or cost of detector. UV-Visible detector is versatile, dual-wavelength absorbance detector for HPLC. This detector offers the high sensitivity required for routine UV-based applications to 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. Its integrated software and optics innovations deliver high chromatographic and spectral sensitivity. Refractive Index (RI) Detector offers high sensitivity, stability and reproducibility, which make this detector the ideal solution for analysis of components with limited or no UV absorption. Multi-Wavelength Fluorescence Detector offers high sensitivity and selectivity fluorescence detection for quantitating low concentrations of target compounds\(^12\).
Table: HPLC Buffers, pKa Values and Useful pH Range

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pKa</th>
<th>Useful pH Range</th>
<th>UV cutoff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium acetate</td>
<td>4.8</td>
<td>3.8-5.8</td>
<td>205(10mM)</td>
</tr>
<tr>
<td></td>
<td>9.2</td>
<td>8.2-10.2</td>
<td></td>
</tr>
<tr>
<td>Ammonium formate</td>
<td>3.8</td>
<td>2.8-4.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.2</td>
<td>8.2-10.2</td>
<td></td>
</tr>
<tr>
<td>Ammonium hydroxide/ammonia</td>
<td>9.2</td>
<td>8.2-10.2</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄/K₂PO₄</td>
<td>7.2</td>
<td>6.2-8.2</td>
<td>&lt;200nm (0.1%)</td>
</tr>
<tr>
<td>KH₂PO₄/phosphoric acid</td>
<td>2.1</td>
<td>1.1-3.1</td>
<td>&lt;200nm (0.1%)</td>
</tr>
<tr>
<td>Potassium Acetate/ acetic acid</td>
<td>4.8</td>
<td>3.8-5.8</td>
<td>210nm (10mM)</td>
</tr>
<tr>
<td>Potassium formate/formic acid</td>
<td>3.8</td>
<td>2.8-4.8</td>
<td>210nm (10mM)</td>
</tr>
<tr>
<td>Trifluoroacetic acid</td>
<td>&lt;2</td>
<td>1.5-2.5</td>
<td>210nm (0.1%)</td>
</tr>
<tr>
<td>Borate (H₃BO₃/Na₂B₄O₇·10H₂O)</td>
<td>9.2</td>
<td>8.2-10.2</td>
<td></td>
</tr>
<tr>
<td>Tri-K-Citrate/hydrochloric acid 1</td>
<td>3.1</td>
<td>2.1-4.1</td>
<td>230nm (10mM)</td>
</tr>
<tr>
<td>Tri-K-Citrate/hydrochloric acid 2</td>
<td>4.7</td>
<td>3.7-5.7</td>
<td>230nm (10mM)</td>
</tr>
<tr>
<td>Tri-K-Citrate/hydrochloric acid 3</td>
<td>5.4</td>
<td>4.4-6.4</td>
<td>230nm (10mM)</td>
</tr>
</tbody>
</table>

**Column selection**

The heart of a HPLC system is the column. Changing a column will have the greatest effect on the resolution of analytes during method development. Generally, modern reverse phase HPLC columns are made by packing the column housing with spherical silica gel beads which are coated with the hydrophobic stationary phase. The stationary phase is introduced to the matrix by reacting a chlorosilane with the hydroxyl groups present on the silica gel surface. In general, the nature of stationary phase has the greatest effect on capacity factor, selectivity, efficiency and elution. 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 matrices are robust, easily derivatized, manufactured to consistent sphere size, and does not tend to compress under pressure. Silica is chemically stable to most organic solvents and to low pH systems. One shortcoming of a silica solid support is that it will dissolve above pH 7. In recent years, silica supported columns have been developed for use at high pH.

The nature, shape and particle size of the silica support effects separation. Smaller particle results in a greater number of theoretical plates, or increased separation efficiency. However, the use of smaller particles also results in increased
backpressure during chromatography and the column more easily becomes plugged.

In reverse phase chromatography the stationary phase is non-polar and the mobile phase is polar, causing polar peaks to generally elute earlier than non-polar peaks. To create a stationary phase for reverse phase chromatography on silica support, the free silanols are reacted with a chlorosilane with hydrophobic functionality to introduce the non-polar surface. Due to steric constraints, only about 1/3 of the surface silanols are derivatized. The remaining free silanols can interact with analytes, causing peak tailing. Typically, after the derivitization of a column with the desired stationary phase, the column is further reacted with chlorotrimethylsilane to end cap the remaining free silanols and improve the column efficiency. Common stationary phases are C₄ (butyl), C₈ (octyl), C₁₈ (octadecyl), nitrile (cyanopropyl), and phenyl (phenyl propyl) columns. In general, longer alkyl chains, higher phase loading, and higher carbon loads provide greater retention of non-polar analytes. Commonly used reverse phase columns and their uses are listed below. Propyl (C₃), Butyl (C₄), and Pentyl (C₅) columns are useful for ion-pairing chromatography. Examples include Zorbax SB-C₃, YMC-Pack C₄, and Luna C₅. These columns are generally less stable to hydrolysis than columns with longer alkyl chains. Octyl (C₈) columns have wide applicability. This phase is less retentive than the C₁₈ phases, but is still quite useful for pharmaceuticals. Examples include (Zorbax SB-C₈, Luna C₈ and YMC-Pack-MOS). Octadecyl (C₁₈, ODS) columns are the most widely used and tend to be the most retentive for non-polar analytes. Commonly used reverse phase columns include Zorbax SB-C₁₈, YMC-Pack ODS, and Luna C₁₈. Xterra RP-C₁₈ and Zorbax Extend-C₁₈ columns have been formulated to tolerate high pH systems (pH >7, normally up to pH 11). Varying the pH can affect selectivity and resolution of polar analytes, especially for ionizable compounds. Phenyl (Ph) columns offer unique selectivity from the alkyl phases and are generally less retentive than C₈ or C₁₈ phases. Phenyl columns are commonly used to resolve aromatic compounds. Examples include Zorbax SB-Phenyl, YMC-Pack Phenyl and Luna Phenyl-Hexyl. Nitrile (CN) or cyano) columns are polar and can be used for both reverse and normal phase applications. This phase is often used to increase retention of polar analytes. Examples include Zorbax SB-CN, Luna-CN, and YMC-Pack CN.

The type of column chosen for a particular separation depends on the compound and the aim of analysis.

Mobile phase

The mobile phase affects resolution, selectivity and efficiency. In reverse phase chromatography, the mobile phase consists of an aqueous buffer and a non-UV active water miscible organic solvent. The effect of the organic and aqueous phase and the proportions in which they are mixed will affect the analysis of the drug molecule. 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. The aqueous buffer serves several purposes. At low pH, the mobile phase protonates free silanols on the column and reduces peak tailing. At sufficiently low pH basic analytes are protonated; when ionized the analyte will elute more quickly but with improved peak shape. Acidic analytes in buffers of sufficiently low pH will remain uncharged, increasing retention. Conversely, at higher pH neutral basic compounds will be more retained, and ionized acidic compounds will elute earlier. Peak splitting may be observed if the pKa of a compound is similar to the pKa of the buffer, and the analyte elutes as both a charged and uncharged species. The pH of a buffer will not greatly affect the retention of non-ionizable sample components.

Typically a 10 – 50 mM solution of an aqueous buffer is used. The most commonly used aqueous phase is H₃PO₄ in water i.e. phosphate buffer. The pH of a phosphate buffer is easily adjusted by using mono-, di-, or tribasic phosphate salts. However, when phosphate salts are used the solution should be filtered to remove insoluble particles with 0.22µm filter paper. Other non-UV active acids and bases may also be used to effect differences in peak shape and retention.

Isocratic or gradient separations: Isocratic, constant eluent composition means equilibrium conditions in the column and the actual velocity of compounds moving through the column are constant; analyte-eluent and analyte-stationary-phase interactions are also constant throughout the whole run. This makes isocratic separations more predictable, although the separation power (the number of compounds which could be resolved) is not very high. The peak capacity is low; and the longer the component is retained on the column, the wider is the resultant peak.

Gradient separation significantly increases the separation power of a system mainly because of the dramatic increase of the apparent efficiency (decrease of the peak width). The condition where the tail of a chromatographic zone is always under the influence of a stronger eluent composition leads to the decrease of the peak width. Peak width
varies depending on the rate of the eluent composition variation (gradient slope).

Changing Gradient: Gradient elution is employed for complex multicomponent samples since it may not be possible to get all components eluted between k (retention factor) 1 and 10 using a single solvent strength under isocratic conditions. This leads to the general elution problem where no one set of conditions is effective in eluting all components from a column in a reasonable time period while still attaining resolution of each component. This necessitates the implementation of a gradient. Employing gradients shallow or steep allows for obtaining differences in the chromatographic selectivity. This would be attributed to the different slopes of the retention versus organic composition for each analyte in the mixture. When a gradient method is used, the column must be allowed to equilibrate at the starting mobile-phase conditions prior to the next sample injection and the start of the next gradient run.

Preparation of sample solutions for method development

The drug substance being analyzed should be stable in solution (diluent). During initial method development, preparations of the solutions in amber flasks should be performed until it is determined that the active component is stable at room temperature and does not degrade under normal laboratory conditions. The sample solution should be filtered; the use of a 0.22 or 0.45 μm pore-size filter is generally recommended for removal of particulates. Filtration is a preventive maintenance tool for HPLC analyses.17, 18, 19, 20

Sample preparation is a critical step of method development that the analyst must investigate. The effectiveness of the syringe filters is largely determined by their ability to remove contaminants/insoluble components without leaching undesirable artifacts (i.e., extractables) into the filtrate. If any additional peaks are observed in the filtered samples, then the diluent must be filtered to determine if a leachable component is coming from the syringe filter housing/filter.

Method optimization

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.

Validation of method

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use. The methods validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to support analytical procedures. All analytical methods that are intended to be used for analyzing any clinical samples will need to be validated. The validation of analytical methods is done as per ICH guidelines.

Components of method validation

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

- Accuracy
- Precision
- Repeatability
- Intermediate precision
- Linearity
- Detection limit
- Quantitation limit
- Specificity
- Range
- Robustness
- System suitability determination
- Forced degradation studies
- Solution stability studies

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.21

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...
Interpretation of validation studies involves several aspects, including system suitability, intermediate precision, repeatability, and specific degradation studies. During validation, intermediate precision is achieved through testing within a laboratory using different instruments, analysts, and assay composites. The precision value is expressed as relative standard deviation (%RSD).

\[
\text{\%RSD} = \frac{\text{std dev.}}{\text{mean}} \times 100
\]

Accuracy and precision are distinct; a measurement can have high precision and yet not be accurate. Linearity is the analytical procedure's ability to obtain a response directly proportional to the concentration of analyte. The precision is usually expressed as the confidence limit around the regression line.

Quantitation Limit

The limit of quantitation (LOQ) or quantitation limit of a measurement is the lowest amount of analyte in a sample that can be determined with suitable precision and accuracy. For analytical procedures with baseline noise, the LOQ is usually determined from a signal-to-noise ratio (3:1) and is confirmed by injecting standards that meet this S/N ratio.

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

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

Robustness is defined as the measure of the method's ability 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.

System Suitability Determination

The evaluation of the components of an analytical system to show that the performance of a system meets the standards required by a method is achieved through a systematic process of varying parameters and measuring their effects on the method by monitoring system suitability and the analysis of samples. These parameters can be calculated experimentally to provide a 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.

Forced Degradation Studies

Forced degradation or stress studies are undertaken to deliberately degrade the sample. These studies are required to assess the analytical method's ability to measure an active ingredient and its degradation products, without interference, by generating potential degradation products. During validation of the method, drug substances are exposed to acid,
base, heat, light, and oxidizing agent to produce approximately 10% to 30% degradation of active substance. The studies can also provide information about the degradation pathways and degradation products that could form during storage. These studies may also help in the formulation development, manufacturing, and packaging to improve a drug product. Reasons for carrying out forced degradation studies include: development and validation of stability-indicating methodology, determination of degradation pathways of drug substances and drug products, discernment of degradation products in formulations that are related to drug substances versus those that are related to non–drug substances (e.g., excipients).24,25

Solution Stability Studies

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.27,28

CONCLUSION

This review describes the general technique of HPLC method development and validation of optimized method. The general approach for the method development for the separation of pharmaceutical compounds was discussed. The knowledge of the pKa, pH and solubility of the primary compound is of utmost importance prior to the HPLC method development. Knowledge of pH can help to discern the ionizable nature of the other impurities (i.e., synthetic byproducts, metabolites, degradation products, etc.) in the mixture. Selection of buffer and mobile phase composition (organic and pH) plays a dramatic role on the separation selectivity. Final optimization can be performed by changing the temperature, gradient slope, and flow rate as well as the type and concentration of mobile-phase modifiers. Optimized method is validated with various parameters (e.g. accuracy, precision, specificity, linearity, detection limit etc.) as per ICH guidelines.

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