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REVIEW ARTICLE

Method development and validation using HPLC technique – a review

Suman Sood*, Rajni Bala

*Department of Pharmaceutics, Rayat Institute of Pharmacy, Rail Majra S.B.S Nagar (Punjab).

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ABSTRACT

Analytic method development and validation are key elements of any pharmaceutical development program. HPLC analysis method is developed to identify, quantity or purifying compounds of interest. This article mainly focuses on the optimization of HPLC conditions and other important perspectives during method development and validation. Various critical steps related to analytical method development and validation is discussed. A sequence of events required for method development and analytical validation are described. The steps involved in developing a stability-indicating HPLC method influences the analysis of degradation products/impurities in stability study and its validation demonstrate the suitability for its intended purpose.

Key words: HPLC, method development, method validation.

INTRODUCTION:

Validation is defined as 'finding or testing the truth of something'. When analytical methods are used to generate results about the characteristics of drug related samples it is vital that the results are trustworthy: they may be used as the basis for decisions relating to administering the drug to patients. A validation study is performed on an analytical method to ensure that reliable results are always obtained. Analytical method validation is just one type of validation required during drug development and manufacturing. To comply with the requirements of current Good Manufacturing Practices (GMP) pharmaceutical companies should have an overall validation policy which documents how validation will be performed. This will include the validation of: production processes, cleaning procedures, analytical methods, in-process control test procedures, and computerized systems. The purpose of this validation is to show that processes involved in the development and manufacture of drugs, such as production, cleaning and analytical testing, can be performed in an effective and reproducible manner.[1-3]

The reason that validation is included in cGMP in this way is to ensure that quality is built in at every step, and not just tested for at the end. 'Validation is intended to provide assurance of the quality of a system or process through a quality methodology for the design, manufacture and use of that system or process, that cannot be found by simple testing alone.[4]

The life cycle of an analytical method:

Once a method has been developed and validated it may then be used for routine analysis, as shown in Figure 1. However changes may occur which make it necessary to evaluate whether the method is still suitable for its intended use. The change may be covered by the existing validation, in which case no further validation is required or the change may result in revalidation, and in some cases, redevelopment of the method followed by validation of the new method.

**Corresponding author:* Suman Sood *|E-mail:* sumansood.87@gmail.com

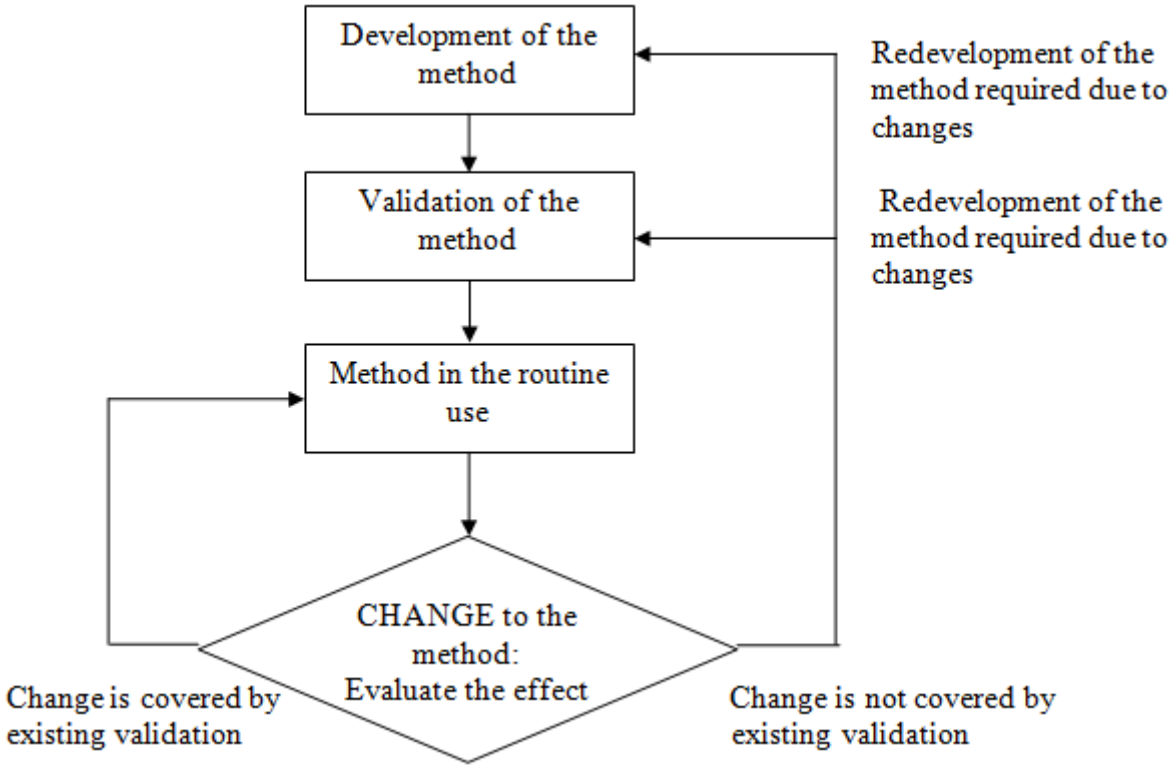

Figure 1: The life cycle of an analytical method

Method Development:

Methods are developed for new products when no official methods are available. Alternate method for existing (Non-Pharmacopoeial) products are to reduce the cost and time for better precision and ruggedness. When alternate method proposed is intended to replace the existing procedure comparative laboratory data including merit/demerits are made available. 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 inter-mediates and any degradants. [5]

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.
4. Method optimization.
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.

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.

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.

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 [H3O+] 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 [H3O+] 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 + H2O ⇌ A− + H3O+

The position of equilibrium is measured by the equilibrium constant, Keq.

$$Keq = \frac{[H3O+]\,[A-]}{[H2O]\,[HA]}$$

Now in dilute solutions of acid, [H2O] stays roughly constant. Therefore define a new equilibrium constant- the *acidity constant* Ka.

$$Ka = \frac{[H3O+]\,[A-]}{[HA]}$$

This is also in logarithmic form are follows:

pKa = −log10 Ka.

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 Ka: pH = pKa – 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 = pKa, 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[6.7].

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. 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[8, 9].

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:

- 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·
- 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.
- 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.
- 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.

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, 11].

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. 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.[12].

Table 1: HPLC Buffers, pKa Values and Useful pH Range

Buffer	pKa	Useful pH Range	UV cutoff
Ammonium acetate	4.8 9.2	3.8-5.8 8.2-10.2	205(10mM)
Ammonium formate	3.8	2.8-4.8	
	9.2	8.2-10.2	
Ammonium hydroxide/ ammonia	9.2	8.2-10.2	
KH2PO4/K2PO4	7.2	6.2-8.2	<200nm (0.1%)
KH2PO4/ phosphoric acid	2.1	1.1-3.1	<200nm (0.1%)
Potassium Acetate/ acetic acid	4.8	3.8-5.8	210nm
Potassium format / formic acid	3.8	2.8-4.8	210nm (10mM)
Trifluoroacetic acid	<2	1.5-2.5	210nm (0.1%)
Tri-K-Citrate/hydrochloric acid 1	3.1	2.1-4.1	230nm (10mM)
Tri-K-Citrate/hydrochloric acid 2	4.7	3.7-5.7	230nm (10mM)
Tri-K-Citrate/hydrochloric acid 3	5.4	4.4-6.4	230nm (10mM)

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 reacted 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. Silica due to smaller particle size 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 thenon-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. The type of column chosen for a particular separation depends on the compound and the aim of analysis[13-15].

Mobile phase:

The mobile phase effects resolution, selectivity and efficiency. In reverse phase chromatography, the mobile phase consists of an aqueous buffer and anon-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 themobile-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[16].

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-sizefilter is generally recommended for removal of particulates. Filtration is a preventive maintenance tool for HPLC analyses.[17, 18, 19, 20]

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[21]. 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 characterstics which may be tested during methods validation:

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

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. lt 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[22].

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[23]. 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.

Intermediate precision is the variation within a laboratory such as different days, with different instruments, and by different analysts.[24, 25]. The precision is then expressed as the relative standard deviation.

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.

Detection Limit or limit of detection (LOD) of an individual procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. In analytical procedures that exhibit baseline noise, the LOD can be based on a signal-to-noise (S/N) ratio (3:1), which is usually expressed as the concentration of analyte in the sample.(book) Thesignal-to-noise ratio is determined by: s = H/hWhere 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.

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

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

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

Robustness is defined as the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters (e.g. pH, mobile phase composition, temperature and instrumental settings) and provides an indication of its reliability during normal usage[25].

CONCLUSION:

The method development and validation are continuous and interrelated process. 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 analytical validation verifies that a given method measures a parameter as intended and establishes the performance limits of the

measurement. Reproducible quality HPLC results can only be obtained if proper attention has been paid to the method development, validation and system's suitability to carry out the analysis. 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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