New Method Development by HPLC and Validation as per ICH Guidelines

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Abstract

This Scientific paper consists of the information of HPLC new method development and Validation as per ICH Guidelines. It also explains importance of HPLC method development and types of HPLC columns. In this paper new binger clearly understand how new method development carried out and what are the ICH guidelines regarding any new method development by HPLC. In First step we have to check solubility of the APIs in same solvent and find out the absorption range and lambda Max. Then by dilution the API in range of Absorbance find out Linearity curve for both compounds single as well as in mixture. This method then applied on formulations as its application. The ICH guidelines explained in detail as validation parameters such as Selectivity, Linearity, Accuracy and precision, LOD and LOQ, Ruggedness and Robustness, Stability and system suitability and it's std values. It will help to budding students for new method development and it's validation.

Keywords: HPLC Method Development; Validation; ICH Guidelines; System Suitability

Introduction

Most of the drugs in multicomponent dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures. Some of the advantages are,

- Speed (analysis can be accomplished in 20 minutes or less),
- Greater sensitivity (various detectors can be employed),
- Improved resolution (wide variety of stationary phases)
- Reusable columns (expensive columns but can be used for many analysis),
- Ideal for substances of low volatility,
- Easy sample recovery, handling and maintenance,
- Instrumentation lends itself to automation and quantitation (less time and labour),
- Precise and reproducible,
- Calculations are done by integrator itself and
- Suitable for preparative liquid chromatography on a much larger scale.

There are different modes of separation in HPLC. They are normal phase mode, reverse phase mode, reverse phase ion pair chromatography, ion exchange chromatography, affinity chromatography and size exclusion chromatography (gel permeation and gel filtration chromatography).

In the normal phase mode, the stationary phase is polar and the mobile phase is nonpolar in nature. In this technique, nonpolar compounds travel faster and are eluted first. This is because of the lower affinity between the nonpolar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore, take more time to elute. Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute.

Reverse phase mode is the most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is a non polar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity. The polar compound gets eluted first in this mode and non polar compounds are retained for longer time. As most of the drugs and pharmaceutical are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are octa decyl silane (ODS) or C₁₈, C₈, C₄ etc. (in the order of increasing polarity of the stationary phase).

In ion exchange chromatography, the stationary phase contains ionic group like NR_3^+ or SO_3^- , which interact with ionic groups of the sample molecules. This is suitable for the separation of charged molecules only. Changing the pH and salt concentration can modulate the retention.

Ion pair chromatography may be used for the separation of ionic compounds and this method can also substitute for ion exchange chromatography. Strong acidic and basic compounds may be separated by reverse phase mode by forming ion pairs (columbic association species formed between two ions of opposite electrical charge) with suitable counter ions. The technique is referred to as reverse phase ion pair chromatography or soap chromatography.

Affinity chromatography uses highly specific biochemical interactions for separations. The stationary phase contains specific groups of molecules which can absorb the sample if certain steric and charge related conditions are satisfied. This technique can be used to isolate proteins, enzymes, as well as antibodies from complex mixture.

Size exclusion chromatography separates molecules according to their molecular mass. Largest molecules are eluted first and the smallest molecules last. This method is generally used when a mixture contains compounds with a molecular mass difference of at least 10%. This mode can be further subdivided into gel permeation chromatography (with organic solvent) and gel filtration chromatography (with aqueous solvents).

Steps Involved in Method development by HPLC

The various components of HPLC are pumps (solvent delivery system), mixing unit, gradient controller and solvent degasser, injector (manual or auto), guard column, analytical columns, detectors, recorders and/or integrators. Recent models are equipped with computers and software for data acquisition and processing.

The choice of the column should be made after a careful consideration of the mode of the chromatographic technique. Three types of columns are available based upon the type of packing and particle size, namely, rigid solids, hard gels porous and pellicular layer beads. The columns of smaller particles (3 - 10 μ) are always preferred because they offer high efficiency (number of theoretical plates/meter) and speed of analysis.

The different types of detection used in HPLC methods are ultraviolet (UV) detection, fluorescence detection, refractive index detection, mass spectrophotometric detection and electrochemical detection. In most cases, method development in HPLC is carried out with UV detection using a variable wavelength spectrophotometric detector or a diode array detector (DAD).

Digital electronic integrators are widely used today in HPLC for measuring peak areas. These devices automatically sense peaks and print out the areas in numerical form. Computing integrators

are even more sophisticated and offer a number of features in addition to basic digital integration because these devices have both memory and computing capabilities to upgrade integrating parameters to maintain accuracy as the separation progress and eluting peaks become broader. Many of these devices print out a complete report, including names of the compounds, retention times, peak areas correction factors. With the help of peak area and height values, the peak width can be used for the calculation of number of theoretical plates.

Method development and design of separation method by HPLC

Methods for analyzing drugs in multicomponent dosage forms can be developed, provided one has knowledge about the nature of the sample, namely, its molecular weight, polarity, ionic character and the solubility parameter. An exact recipe for HPLC, however, cannot be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase. In general one being with reverse phase chromatography when the compounds are hydrophilic in nature with many polar groups and are water soluble.

The organic phase concentration required for the mobile phase can be estimated by gradient elution method. For aqueous sample mixtures, the best way to start is with gradient reverse phase chromatography. Gradient can be started with 5 - 10% organic phase in the mobile phase and the organic phase concentration (methanol or acetonitrile) can be increased up to 100% within 30 - 45 min. Separation can be optimized by changing the initial mobile phase composition and slope of the gradient according to the chromatogram obtained from the preliminary run. The initial mobile phase composition can be estimated on the basis of where the compounds of interest were eluted, namely, at what mobile phase composition.

Elution of drug molecules can be altered by changing the polarity of the mobile phase. The elution strength of a mobile phase depends upon its polarity, the stronger the polarity, higher is the elution. Ionic samples (acidic or basic) can be separated, if they are present in undissociated form. Dissociation of ionic samples may be suppressed by the proper selection of pH.

The pH of the mobile phase has to be selected in such a way that the compounds are not ionized. If the retention times are too short, the decrease of the organic phase concentration in the mobile phase can be in steps of 5%. If the retention times are too long, an increase of the organic phase concentration is needed.

In UV detection, good analytical results are obtained only when the wavelength is selected carefully. This requires knowledge of the UV spectra of the individual components present in the sample. If analyte standards are available, their UV spectra can be measured prior to HPLC method development.

The molar absorbance at the detection wavelength is also an important parameter. When peaks are not detected in the chromatograms, it is possible that the sample quantity is not enough for the detection. An injection of a volume of 20 μ l from a solution of 1 mg/ml concentration normally provides good signals for UV active compounds around 220 nm. Even if the compounds exhibit higher λ_{max} , they absorb strongly at lower wavelength. It is not always necessary to detect compounds at their maximum absorbance. It is, however, advantageous to avoid the detection at the sloppy part of the spectrum for precise quantization. When acceptable peaks are detected on the chromatogram, the investigation of the peak shapes can help further method development.

The addition of peak modifier to the mobile phase can affect the separation of ionic samples. For example; the retention of the basic compounds can be influenced by the addition of small amounts of triethylamine (a peak modifier) to the mobile phase. Similarly for acidic compounds small amount of acetic acid can be used. This can lead to useful changes in selectivity.

When tailing or fronting is observed, it means that the mobile phase is not totally compatible with the solutes. In most cases the pH is not properly selected and hence partial dissociation or protonation takes place. If peak shape does not improve by using lower (1 - 2) or higher (8 - 9) pH, then ion-pair chromatography can be used. For acidic compounds, cationic ion pair molecules at higher pH and for basic compounds, anionic ion pair molecules at lower pH can be used. For amphoteric solutes or a mixture of acidic and basic compounds, ion-pair chromatography is the method of choice.

The low solubility of the sample in the mobile phase can also cause bad peak shapes. It is always advisable to use the same solvent for preparation of sample solution as the mobile phase to avoid precipitation of the compounds in the column or injector.

Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that all the compounds are detected by more or less symmetrical peaks on the chromatogram. By a slight change of the mobile phase composition, the shifting of the peaks can be expected. From few experimental measurements, the position of the peak can be predicted within the range of investigated changes. An optimized chromatogram is the one in which all the peaks are symmetrical and are well separated in less run time.

The peak resolution can be increased by using a more efficient column (column with higher theoretical plate number, N) which can be achieved by using a column of smaller particle size, or a longer column. These factors, however, will increase the analysis time. Flow rate does not influence resolution, but it has a strong effect on the analysis time.

The parameters that are affected by the changes in chromatographic conditions are,

- Retention time (R_t)
- Resolution (R_s),
- Capacity factor (k'),
- Selectivity (α),
- Column efficiency (N) and
- Peak asymmetry factor (A_s).

Quantitative analysis in HPLC

Three methods are generally used for quantitative analysis. They are the external standard method, the internal standard method and the standard addition method.

External standard method

The external method involves the use of single standard or up to three standard solutions. The peak area or the height of the sample and the standard used are compared directly. One can also use the slope of the calibration curve based on standards that contain known concentrations of the compounds of interest.

Internal standard method

A widely used technique of quantitation involves the addition of an internal standard to compensate for various analytical errors. In this approach, a known compound of a fixed concentration is added to the known amount of samples to give separate peaks in the chromatograms to compensate for the losses of the compounds of interest during sample pretreatment steps. Any loss of the component of interest will be accompanied by the loss of an equivalent fraction of internal standard. The accuracy of this approach obviously depends on the structural equivalence of the compounds of interest and the internal standard. The requirements for an internal standard are,

- It must have a completely resolved peak with no interferences,
- It must elute close to the compound of interest,
- It must behave equivalent to the compounds of interest for Analysis like pretreatments, derivative formations, etc.
- It must be added at a concentration that will produce a peak Area or peak height ratio of about unity with the compounds of interest,
- It must not be present in the original sample,
- It must be stable, unreactive with sample components, column Packing and the mobile phase and
- It is desirable that compound is commercially available in high Purity.

The internal standard should be added to the sample prior to sample preparation procedure and homogenized with it. To be able to recalculate the concentration of a sample component in the original sample, one has to determine first the response factor. The response factor (RF) is the ratio of peak areas of sample component (A_x) and the internal standard (A_{ISTD}) obtained by injecting the same quantity. It can be calculated using the formula,

$$RF = A_x/A_{ISTD}$$

When more than one component is to be analyzed from the same sample, the response factor of each component should be determined.

Standard addition method

In the standard addition method a known amount of the standard compound is added to the sample solution to be estimated. This method is suitable if sufficient amount of the sample is available and is more realistic in the sense that it allows calibration in the presence of excipients or other components.

Validation as per ICH guidelines

Validation is a process that confirmation or establishment by laboratory studies that a method developed is accurate, precise and rugged. In simple terms, validation of an analytical procedure is to demonstrate that the procedure developed is suitable for its intended purpose and it works in a reproducible manner when carried out by the same or different persons, in the same or different laboratories, using different brands of reagents and equipment's, etc.

The various validation performance parameters are,

- Accuracy,
- Precision (repeatability and reproducibility),
- Specificity,
- Linearity and range,
- Limit of detection (LOD)/limit of quantization (LOQ),
- Selectivity/specificity,
- Ruggedness/robustness
- Stability and
- System suitability.

Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of an analytical method should be established across its range. Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with confidence intervals.

Accuracy is calculated from the test results as the percentage of analyte recovered by the assay. Dosage form assays commonly provide accuracy within 3 - 5% of the true value.

Figure 1: Accuracy and Precision.

Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. The precision of an analytical method is determined by assaying a sufficient number of aliquots of a homogeneous sample to be able to calculate statistically valid estimate of standard deviation or relative standard deviation. The precision determinations permit an estimate of the reliability of single determination and are commonly in the range of 0.3 to 3% for dosage form assays.

Figure 2: Accuracy and Precision.

Specificity

The International Conference of Harmonization (ICH) documents define specificity as the ability to assess unequivocally the analyte into the presence of components that may be expected to be present, such as impurities, degradation products and matrix components.

In case of assay, demonstration of specificity requires that the procedure is unaffected by the presence of impurities or excipients. In practice, it can be done by spiking the substance or product with appropriate levels of impurity or recipients and demonstrating

that the assay results are unaffected by the presence of this extra-neous material.

If impurity or degradation product standards are unavailable specificity may be demonstrated by comparing test results of sam- ples containing impurities or degradation product to a well charac- terized procedure. This comparison should include sample stored under relevant stress conditions e.g., light, heat, humidity, acid/ base hydrolysis and oxidation.

Selectivity

The selectivity of an analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of components that may be expected to be present in the same matrix. Selectivity in HPLC is usually expressed by the minimum resolution factor (Rs) of two neighboring peaks and peak purity. The peak purity can be checked by subtracting two chromatograms of the sample obtained at two different wavelengths. If the peak is pure, the absorption ratio at the two wavelengths should be exactly same from the beginning to end of the peak.

The selectivity of analytical method is determined by comparing test result from the analyses of samples containing impurities or degradation products or placebo ingredients with those obtained from the analyses of samples without impurities or degradation products or placebo ingredients.

Linearity and range

The linearity of an analytical method is its ability to elicit test re- sults that are directly proportional to the concentration of analyte in sample within a given range. It should be established across the range of the analytical procedure. Linearity is usually expressed in terms of the variance around the slope of the regression line calcu- lated according to an established mathematical relationship from test results obtained by the analysis of samples with varying con- centrations of analyte, minimum 6 concentrations.

The range of an analytical method is the interval between the upper and lower levels of analytic (including these level) that have been demonstrated to be determined with a suitable level of pre- cision, accuracy, and, linearity using the method as written. The range is normally expressed in the same unit as test result (e.g. per- cent/ppm).

Figure 3: Linearity and Range of API.

Figure 4: Linearity and Range of API Spectra Overlay.

Limit of detection

Limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitative, under the stated experimental conditions. The detection limits is usually expressed as the concentration of analyte (e.g., percentage ppb) in the sample.

- Expressed as a concentration at a specified signal: noise ratio.
- Determination based on
 - Visual evaluation (non-instrumental and instrumental methods)
 - Signal to Noise (baseline noise)
 - Standard deviation of response (s) and slope (S).

$DL = 3.3s/S$, Estimation of S, from the calibration curve of the analyte.

Estimation of s, from the standard deviation of the blank, from the standard deviation (regression line or y-intercept) of a calibration curve in the range of the DL.

Limit of quantitation

Limit of quantitation (LOQ) is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. It is expressed as the concentration of analyte (e.g., percentage, ppb) in the sample.

- Determination based on
 - Visual evaluation (non-instrumental and instrumental methods)
 - Signal to Noise (baseline noise)
 - Standard deviation of response (s) and slope (S).

$QL = 10s/S$, Estimation of S, from the calibration curve of the analyte.

Estimation of s, from the standard deviation of the blank, from the standard deviation (regression line or y-intercept) of a calibration curve in the range of the QL.

Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperature, different days, etc. Ruggedness is normally expressed as the lack of influence on test results of operational and environmental variable of the analytical method.

Robustness

The robustness of analytical method 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. A good practice is to vary important parameters in method systematically and measure their effect on separation. Such parameters include mobile phase composition and pH, mobile phase additives, column temperature, flow rate etc.

Stability

Stability of sample, standard and reagents used in HPLC and UV method is required for a reasonable time to generate reproducible and reliable results. For example 24 hour stability is desired for solutions and reagents that need to be prepared for each analysis. Long term column stability is critical for method ruggedness since even a best HPLC column will eventually degrade and loose its initial performance.

Solutes may readily decompose prior to chromatographic investigations e.g. during sample preparation, extraction, cleanup, phase transfer or storage of prepared vials (refrigerators or automatic sampler). Method development should investigate the stability of the analytes and standards [1-5].

System stability

- Stability of the samples being analyzed in a sample solution.
- Measure of the bias in assay results generated during a pre-selected time interval e.g. 1 - 48 hours using a single solution
- Should be determined by replicate analysis of the sample solution.
- Considered appropriate when the RSD, calculated on the assay results obtained at different time intervals, Less than 20 percent of the corresponding value of the system precision.

System suitability tests

System suitability tests ensure that the method developed can generate results of acceptable accuracy and precision. The USP defines parameters that can be used to determine system suitability prior to analysis. These parameters include column efficiency (N),

peak asymmetry factor (As), resolution (Rs), capacity factor (k') and/or separation factor (α) and relative standard deviation (RSD) of peak area.

- The checking of a system, before or during analysis of unknowns, to ensure system performance.
- “No sample analysis is acceptable unless the requirements for system suitability have been met”. (USP Chapter 621)
- Plate Count, Tailing, Resolution
- Determination of reproducibility (%RSD)
- For %RSD < 2.0%, Five replicates
- For %RSD > 2.0%, Six replicates
- System Suitability "Sample" - A mixture of main components and expected by-products utilized to determine system suitability
- “Whenever there is a significant change in Equipment or Reagents System Suitability Testing Should be performed” (USP Chapter 621).

Conclusion

As per conclusion, this paper will help to the academic persons such as student as well as new Teaching staff for method development and use full for referring to industrial people also. In this paper explained in easy and understandable language the HPLC method development and validation of the developed method as per ICH guidelines.

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