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A REVIEW - ANALYTICAL METHOD DEVELOPMENT AND VALIDATION BY RP-HPLC

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ABSTRACT

RP-HPLC method is use for the determination of drugs in single or in combination with other component. RP-HPLC technique is widely use for the separation and analysis of drugs that is present in combined formulation. This technique is also used for the separation of drugs from the excipients that are present in the formulation. Analytical method is developed to carry out the determination of drugs or component present in the formulation. Validation studies are performed in order to assess the validation parameters for analytical method development in accordance to ICH guideline. In this article we can understand steps of method development and validation for the new drug or component by using RP-HPLC technique.

KEYWORDS

Analytical Method Development, Validation and RP-HPLC.

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INTRODUCTON

The objective of this review article is to understand basics of reverse phase high performance liquid chromatography method development an validation parameter and their limits. Chromatography is a separation technique by which molecules can be separated by the difference in their structure or composition. It involves moving a sample through the system over a stationary phase. The molecules in the sample will have different affinities and interactions with the stationary support, leading to separation of molecules. Sample components that have stronger interactions with the stationary phase will move more slowly through the column than components with

weaker interactions. Chromatographic separations can be carried out using a variety of stationary phases, including immobilized silica on glass plates (thin-layer chromatography), volatile gases (gas chromatography), paper (paper chromatography) and liquids (liquid chromatography). The differences in interaction with the column can help separate different sample components from each other[1].

HPLC technique is used to separate the components present in the mixture for analysis or purification. Components in a mixture get separated on the column by pumping mobile phase through the column. Depending on the affinity of each component between the mobile phase and the stationary phase, each analyte migrates along the column at different speeds and emerges from the column at different times, thus establishing a separation of the mixture. Analytes with higher affinity for the mobile phase migrate faster down the column, whereas those with higher affinity for the stationary phase migrate slower.

This migration time (retention time) is unique for each analyte and can be used in its identification. With the appropriate use of a detection method after the column, each analyte can also be quantified for analysis. Smaller column particle size can improve chromatographic resolution, but increased solvent delivery pressure is needed. Further reduction of column particle size can allow for higher solvent flow rates, reducing analysis time without sacrificing resolution. This is what gives Ultra High Pressure Liquid Chromatography its advantage over other LC techniques[2,3].

Phases of chromatography[4]

Normal phase chromatography

In this stationary phase is polar and the mobile phase is nonpolar in nature. In this technique, nonpolar compounds have lower affinity with the stationary phase so non polar compound travel faster and are eluted first. Polar compounds have higher affinity with stationary phase so they retained for longer times therefore take more times 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.

Reversed phase mode chromatography

This mode is generally used for the separation of drug molecules. In this mode, the stationary phase is nonpolar hydrophobic packing with octyl or octadecyl functional group bonded to silica gel and the mobile phase is polar solvent. The polar compound gets eluted first in this mode and nonpolar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are octadecylsilane (ODS) or C18, C8, C4, etc., (in the order of increasing polarity of the stationary phase).

Ion exchange chromatography

The stationary phase made up with ionic groups like NR3+ SO3-, which interact with the ionic groups of the sample molecules. It is suitable technique for the separation of charged molecules only. Changing the pH and salt concentration can change the retention.

Ion pair chromatography

It is 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 reversed phase mode by forming ion pairs (columbic association species formed between two ions of opposite electric charge) with suitable counter ions. This technique is referred to as reversed phase ion pair chromatography or soap chromatography.

Affinity chromatography

In this separation is done by using highly specific biochemical interactions. The stationary phase made up with 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 mixtures.

Size exclusion chromatography

Separation of molecules based on 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 solvents) and gel filtration chromatography (with aqueous solvents).

Chromatographic Principles[1,4]

Retention

The retention of a drug with a given packing material and eluent can be expressed as a retention time or retention volume. Retention or elution volume is the quantity of the mobile phase required to pull the sample through the column. Retention time is defined as how long a component is retained in the column by the stationary phase relative to the time it resides in the mobile phase. The retention is best described as a column capacity ratio (k´), which can be used to evaluate the efficiency of columns. The longer a component is retained by the column, the greater is the capacity factor. The column capacity ratio of a compound (A) is defined by the following equation,

K= TA-T0/T0 = VA-V0/V0

Where, VA is the elution volume of component A and V0 is the elution volume of a non-retained compound. At constant flow rate, retention times (TA and T0) can be used instead of retention or elution volumes.

Resolution

Resolution is the ability of the column to separate peaks on the chromatograph. Resolution (R) is expressed as the ratio of the distance between two peak maxima to the mean value of the peak width at the base line.

$R = (TB-TA)^2 / WA+WB$

Where TB is the retention time of component B, TA is the retention time of component A, WA is the peak width of component A and WB is the peak width of component B. If R is equal to or more than 1, then components are completely separated, but if R is less than 1, then components overlap.

Sensitivity

Sensitivity is a measure of the smallest detectable level of a component in a chromatographic separation and is dependent on the signal-to-noise ratio in a given detector.

Quantitative analysis[5]

The quantification methods incorporated in HPLC are borrowed mostly from gas chromatography methods. The basic theory for quantitation involves the measurement of peak height or peak area. To determine the concentration (conc.) of a compound, the peak area or height is plotted versus the concentration of the substance. For peaks that are well resolved, both peak height and area are proportional to the concentration. Three different calibration methods, each with its own benefits and limitations, can be utilized in quantitative analysis: external standard (std.), internal standard and the standard addition method.

External Standard

The external standard method is the simplest of the three methods. The accuracy of this method is dependent on the reproducibility of the injection volume. To perform this method, standard solutions of known concentrations of the compound of interest are prepared with one standard that is similar in concentration to the unknown. A fixed amount of sample is injected. Peak height or area is then plotted versus the concentration for each compound. The plot should be linear and go through the origin. The concentration of the unknown is then determined according to the following formula.

$Conc_{unknown}$ = (Area Unknown/ Area Known) $Conc_{known}$

Internal Standard

Although each method is effective, the internal standard method tends to yield the most accurate and precise results. In this method, an equal amount of an internal standard, a component that is not present in the sample, is added to both the sample and standard solutions. The internal standard selected should be chemically similar to, have similar retention time and derivatize similarly to the analyte. Additionally, it is important to ensure that the internal standard is stable and does not interfere with any of the sample components. The internal standard should be added before any preparation of the sample so that extraction efficiency can be evaluated. Quantification is achieved by using ratios of peak height or area of the component to the internal standard.

Analytical method development

Strategy for method development in RP-HPLC[5,6]

It is defined as the procedure through which we approach for a new method for the analysis of drug or other substance by changing the parameters of existing method or develop a unique separate method[5]. 'Best

column, best Mobile phase, best detection wavelength efforts in their selection can make a world of difference while developing HPLC method for routine analysis. Determining ideal combination of these factors assures faster delivery of desired results.

HPLC follows a series of steps which are summarized as below[6],

On sample properties as much knowledge as possible should be collected.

Chromatographic method should be selected according to the sample property.

The sample is chromate graphed with HPLC condition where all compound elute in a reasonable time.

The HPLC method is optimized with regard to analysis time, resolution, selectivity, sensitivity. Knowledge about sample,

Origin (especially important with biological sample)
History (storage, sampling, sample preparation)
Component
Amount
Physical and chemical properties
Matrix

Important Physical and chemical property of sample compound:-

Molecular weight
Formula
Acid base property (pKa value)
Solubility (in organic or in water)
UV spectrum

METHOD VALIDATION

Analytical method validation is just one type of validation required during drug development and manufacturing. To comply with the requirements of current good manufacturing practice (GMP)[7,8]. 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 a simple testing alone[9].

Typical validation characteristics which should be considered are listed below[10,11],

Specificity
Accuracy
Precision
Intermediate Precision
Detection Limit
Quantitation Limit
Linearity
Range

Specificity[11,12]

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Suitable identification tests should be able to discriminate between compounds of closely related structures which are likely to be present. The discrimination of a procedure may be confirmed by obtaining positive results (perhaps by comparison with a known reference material) from samples containing the analyte, coupled with negative results from samples which do not contain the analyte. In addition, the identification test may be applied to materials structurally similar to or closely related to the analyte to confirm that a positive response is not obtained. The choice of such potentially interfering materials should be based on sound scientific judgment with a consideration of the interferences that could occur.

Linearity[11,12]

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. A linear relationship should be evaluated across the range of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighing of synthetic mixtures of the drug product components, using the proposed procedure. The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity. For the establishment of linearity, a minimum of 5 concentrations is recommended.

Range[11,12]

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these

concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

The following minimum specified ranges should be considered

For the assay of a drug substance or a finished (drug) product, normally from 80 to 120 percent of the test concentration,

For content uniformity, covering a minimum of 70 to 130 percent of the test concentration, unless a wider more appropriate range, based on the nature of the dosage form (e.g., metered dose inhalers) is justified,

For dissolution testing: +/-20 % over the specified range,

e.g., if the specifications for a controlled released product cover a region from 20%, after 1 hour, up to 90%, after 24 hours, the validated range would be 0-110% of the label claim.

For the determination of an impurity, from the reporting level of an impurity to 120% of the specification,

For impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects, the detection/quantitation limit should be commensurate with the level at which the impurities must be controlled,

Note:

For validation of impurity test procedures carried out during development, it may be necessary to consider the range around a suggested (probable) limit.

If assay and purity are performed together as one test and only a 100% standard is used, linearity should cover the range from the reporting level of the impurities to 120% of the assay specification.

Accuracy[11,12]

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. Accuracy should be established across the specified range of the analytical procedure. Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations/3 replicates each of the total analytical procedure). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

Precision[11,12]

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Repeatability

Repeatability should be assessed using:

A minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each) or

A minimum of 6 determinations at 100% of the test concentration.

Intermediate Precision

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc. It is not considered necessary to study these effects individually. The use of an experimental design (matrix) is encouraged.

Reproducibility

Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias. These data are not part of the marketing authorization dossier. The standard deviation, relative standard deviation (coefficient of variation) and confidence interval should be reported for each type of precision investigated.

Detection limit[11,12]

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

The detection limit (DL) may be expressed as:

$DL = 3.3\sigma / s$

Where σ = the standard deviation of the response
S = the slope of the calibration curve
The detection limit and the method used for determining the detection limit should be presented. If DL is determined based on visual evaluation or based on signal to noise ratio, the presentation of the relevant chromatograms is considered acceptable for justification. In cases where an estimated value for the detection limit is obtained by calculation or extrapolation, this estimate may subsequently be validated by the independent analysis of a suitable number of samples known to be near or prepared at the detection limit.

Quantitation limit[11,12]

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.
The quantitation limit (QL) may be expressed as,

$$QL = 10\sigma / s$$

Where σ = the standard deviation of the response
S = the slope of the calibration curve
The quantitation limit and the method used for determining the quantitation limit should be presented. The limit should be subsequently validated by the analysis of a suitable number of samples known to be near or prepared at the quantitation limit.

Robustness[11,12]

The robustness of an analytical procedure is a measure of its capacity to remain un affected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.
In the case of liquid chromatography, examples of typical variations are,
Influence of variations of pH in a mobile phase.
Influence of variations in mobile phase composition.
Different columns (different lots and/or suppliers).
Temperature.
Flow rate.

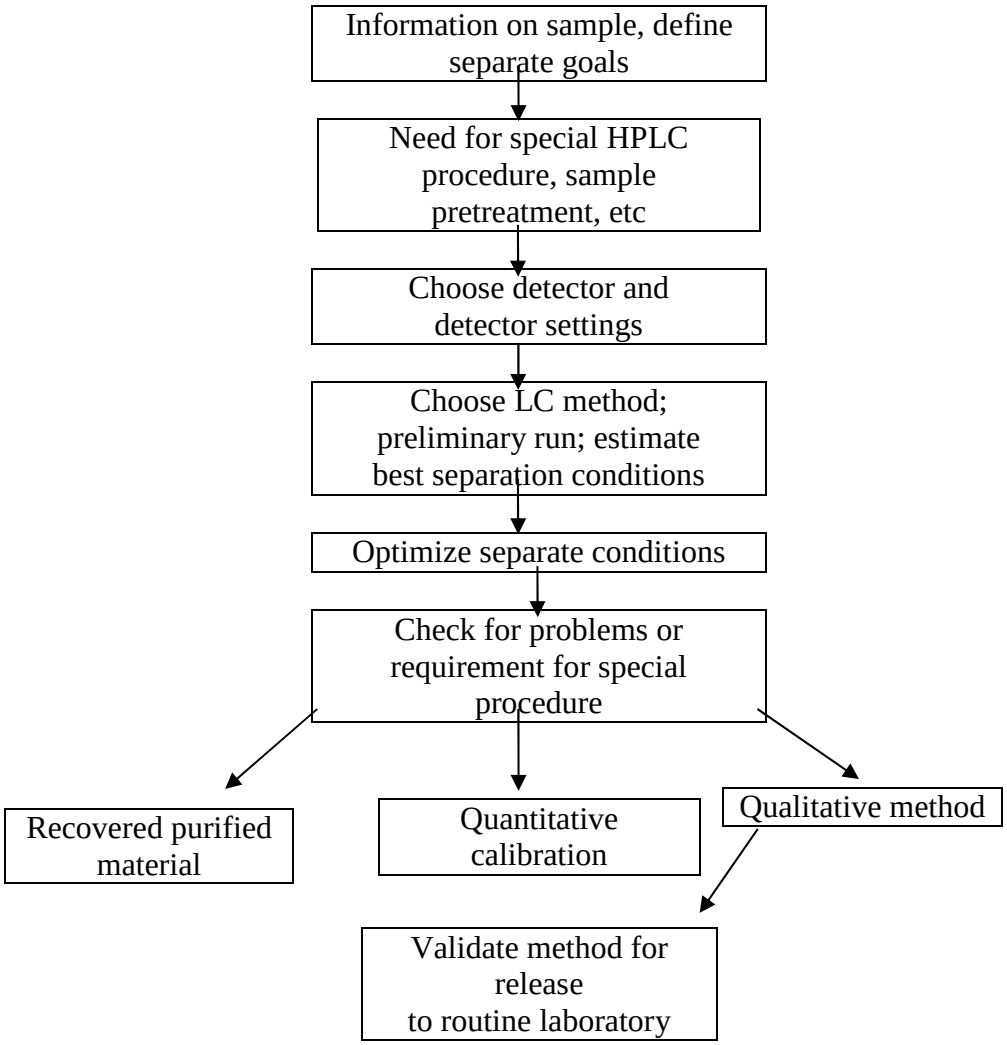

Figure No.1: HPLC follows a series of steps

CONCLUSION

HPLC is probably the most universal type of analytical procedure; its application areas include quality control, process control, forensic analysis, environmental monitoring and clinical testing. In addition HPLC also ranks as one of the most sensitive analytical procedures and is unique in that it easily copes with multicomponent mixtures. It has achieved this position as a result of the constant evolution of the equipment used in LC to provide higher and higher efficiencies at faster and faster analysis times with a constant incorporation of new highly selective column packing. Analytical methods development plays important roles in the discovery, development and manufacture of pharmaceuticals. RP-HPLC is probably the most universal, most sensitive analytical procedure and is unique in that it easily copes with multi-component mixtures. While developing the analytical methods for pharmaceuticals by RP-HPLC, must have good practical understanding of chromatographic separation to know how it varies with the sample and with varying experimental conditions in order to achieve optimum separation. To develop a HPLC method effectively, most of the effort should be spent in method development and optimization as this will improve the final method performance.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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