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THE SECRETS REVIEW OF RAPID HPLC METHOD DEVELOPMENT AND VALIDATION

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ABSTRACT

Analytical method development and validation assume fundamental part in the drug discovery, Drug advancement and assembling the pharmaceutical products. It include identification of the purity and toxicity of a drug substance. High performance liquid chromatography might be used as the reason of choices and identifying with controlling the medication to patients, play important roles and functions in new revelation, advancement, production of drugs and different examinations identified with humans and animals. Various chromatographic parameters have been assessed so as to improve the techniques in the examination of strategy advancement in HPLC. An appropriate mobile phase, column, column temperature, wavelength, and gradient are developed. This review gives data with respect to different stages engaged with advancement and approval of HPLC technique. Approval of HPLC method according to ICH Guidelines covers all the exhibition qualities of validation like Accuracy, precision, specificity, linearity, range and limit of detection, limit of quantification, robustness and system suitability testing.

KEYWORDS:- HPLC, Chromatography, Method Development, Method Validation, ICH, Regulatory Requirment.

INTRODUCTION

Analytical chemistry is used to decide qualitative and quantitative composition of material under study. Both these aspects are important to understand the sample material. Analytical chemistry is divided into two branches quantitative and qualitative. A qualitative analysis gives us the data about the idea of test by thinking about the presence or absence of certain components. A quantitative examination gives mathematical data with respect to the overall measure of at least one of this part for analyzing the drug samples in bulk, pharmaceutical

formulations and biological liquid, different analytical methods are routinely being used.[1] (Figure-1)

The technique of HPLC has following features[2]

1. High resolution
2. Small diameter, Stainless steel, Glass column
3. Rapid analysis
4. Relatively higher mobile phase pressure
5. Controlled flow rate of mobile phase

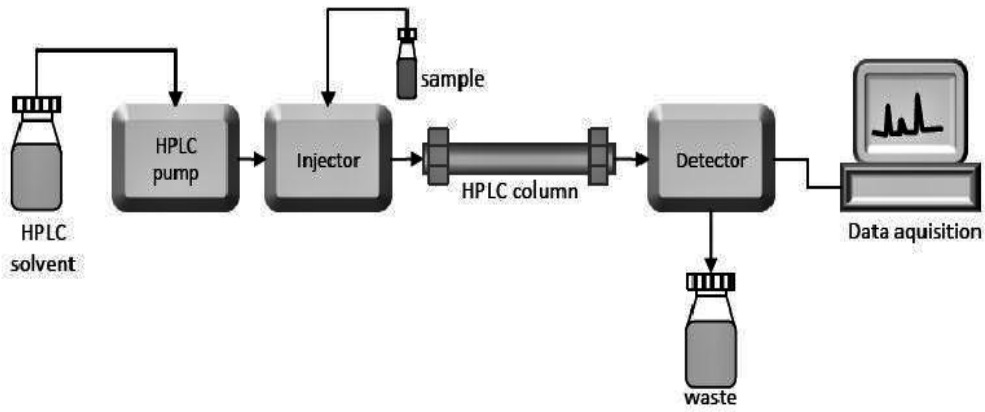

Fig. 1: Flow diagram of HPLC.

Chromatography

Chromatography is a strategy utilized for partition of the segments of phase by continuous appropriation of the segment between two phase. One phase moves mobile phase) over the other phase (stationary phase) in a consistent way Chromatography according to USP can be defined as a procedure by which solute are separated by a differential migration process in a system consisting of two or more phases, one of which move continuously in a given direction.[1]

Types of HPLC

Following types of HPLC generally used in analysis.

Normal phase chromatography

Additionaly realized Normal stage HPLC (NP-HPLC), this technique isolates analytes dependent on polarity. This kind of chromatography utilizes a polar stationary phase and a nonpolar mobile phase. The polar analyte cooperated with and is held by the polar stationary

phase. The expansion of adsorption strength with expanding analyte polarity and the association between the polar analyte and the polar stationary phase builds the elution time.[3,4]

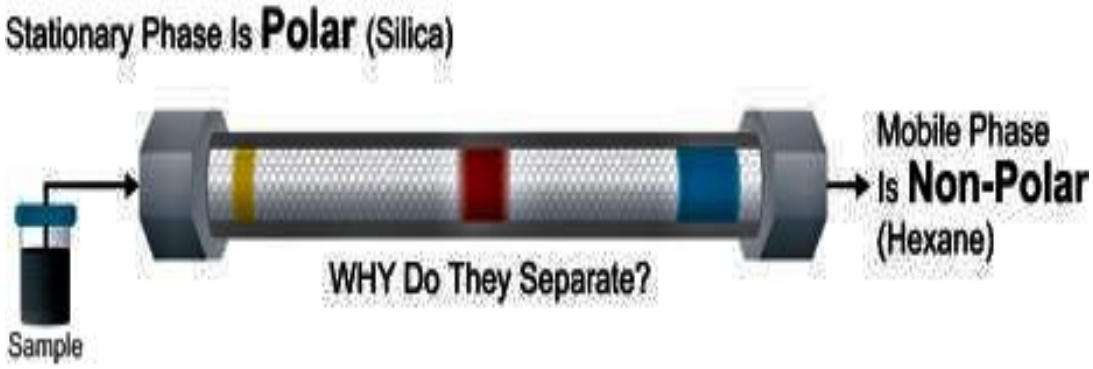

Fig. 2: Normal phase chromatography.

Reversed phase chromatography

Reversed Phase Chromatography has a non-polar stationary phase and polar or moderately polar mobile phase[5] RP-HPLC depends on the rule of hydrophobic interaction. In a combination of components those analytes which are moderately less polar will be held by the non-polar stationary phase longer than those which are generally more polar. In this way the most polar part will elute first.[6]

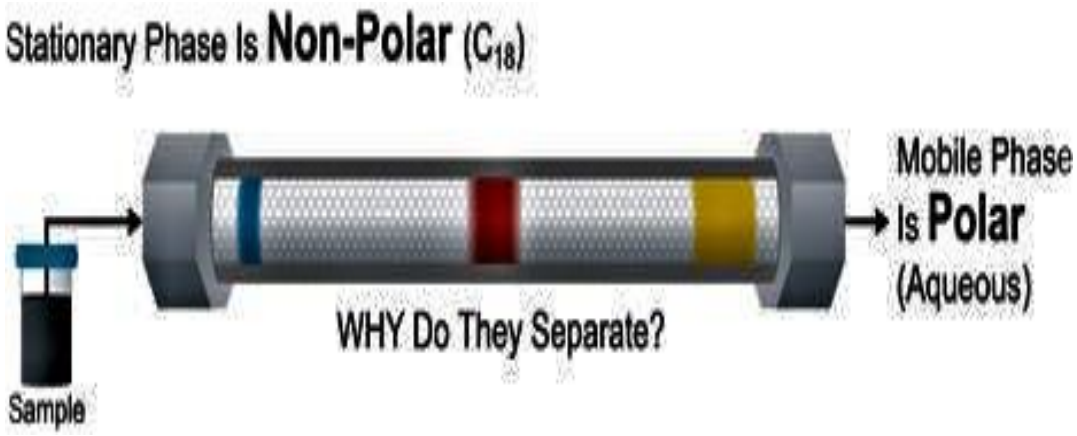

Fig. 3: Reversed phase chromatography.

Steps in method development

- Physicochemical Properties of drug molecule
- Selection of Chromatographic condition
- Preparation of sample
- Method Optimization

- Method validation

1) Physiochemical properties of drug molecule

Physicochemical properties of a medication particle assume a significant function in method development For Method development one needs to examine the actual properties like solubility, polarity, pKa and pH of the drug molecule. Polarity is an actual property of a compound. It helps an analyst to choose the dissolvable and composition of the mobile phase. In a nonpolar covalent bond, the electrons are shared similarly between two particles. A polar covalent bond is one in which one molecule has a more bigger attraction for the electrons than the other particle.[7,8]

a) Chemical properties

Compound structure of the known and expected product compound structures are acceptable contributions for starting the strategy improvement and it will give logical methodology for the method development. Draw the near distinction between impurities, beginning materials, results moderate and by-products intermediate and end products. Dissolvability study at various pH esteems data for all focused on atoms is ideal inputes for choosing the basic diluent for all particles. Selecting the polar/non polar HPLC column the data of polarity of particles is significant.[9]

b) pH and pKa value of compound

pH and pKa assumes a significant part in HPLC method development. The pH value is characterized as the negative of the logarithm to base 10 of the concentration of the hydrogen ion.

pH = - log10[H3O+]

The acidity or basicity of a substance is characterized most normally by the pH value. Choosing a proper pH for ionizable analytes regularly prompts even and sharp peaks in HPLC. Sharp, symmetrical peaks are necessary in quantitative analysis in order to achieve low detection limits.[7,8]

2) Selection of chromatographic conditions

During introductory method development, a set of beginning conditions (column, mobile phase and detectors) is chosen. In many cases, these depend on reversed phase mode on a C18 column with UV detection. A choice on developing either an isocratic or a gradient method should be made at this point.[10]

a) Selection of column

Column is the important part of HPLC instrument columns are constructed of heavy-wall, glass-metal tubing or stainless teel tubing to withstand high pressures (up to 680 tam) and the chemical action of the mobile phase. Column end fittings and connector must be designed with zero void volume to avoid upswept corners or stagnant pockets of mobile phase that can be contribute significantly to extra-column band broadening. Most column lengths range from 10 to 30 cm; Short, fast columns are 3 to 8 cm long. For exclusion chromatography, column are 50 to 100 cm long. Many HPLC separation are done on column with an internal diameter of 4 to 5 mm. Such columns provide a good compromise between efficiency, sample capacity, and the amount of packing and solvent required. Column packing feature is that the particles are uniformly sized and mechanically stable. Particle diameter lie in the range 3.5um, occasionally up to 10 um or higher for preparative chromatography. The column are classified into various categories depending on their carbon loading.

Columns used in our instrument

- Phenomenex
- Bondapak
- Nucleosil

❖ **Columns selection criteria**

The large number of available HPLC column and column type often makes it difficult to choose the right column for a particular separation. Column selection is based on the knowledge of sample, interaction behaviour of analyte with the stationary phase and goals of the separation. The approach has got the following benefits:

- Small initial investment
- Big time saving in the HPLC laboratory
- More efficient than the trial and error approach
- More informed approach to column selection

The knowledge of sample, which influences column selection, is:

- Structure of sample components
- Number of compound present
- Sample matrix
- The pKa values of sample components
- Concentration range

- Molecular weight range
- Solubility

Column length

- Choose longer column for enhance resolution
- Choose shorter column for shorter analysis time, lower backpressure and fast equilibration and less solvent consumption.

❖ **Column internal diameter**

- Choose wider diameter column for greater sample loading
- Choose narrow column for more sensitive and reduced mobile phase consumption.

❖ **Particle shape**

- Choose spherical particles for lower backpressure, column stability and greater stability.
- Choose irregular particles when high surface area and high capacity is required.

❖ **Particle size**

- Choose smaller particle (3-4µm) for complex mixture with similar component.
- Choose large particle (5-10µm) for sample with structurally different compounds.
- Choose very large particle (15-20µm) for preparative separation.

❖ **Pore size**

- Choose a pore size of 150A˚ or less for sample with molecular weight less than 2000.
- Choose a pore size of 300A˚ or less for sample with molecular weight greater than 2000.

❖ **Surface area**

- Choose end capped packing to eliminate unpredictable secondary interaction with the base material.
- Choose non-end capped phase for selectivity differences for polar compounds by controlling secondary interaction.[11]

b) Chromatographic condition

Choice of suitable chromatographic conditions is the next step in the method development. This includes choice of temperature, choice of composition of mobile phase, pH of mobile phase and flow rate and so on A choice of growing either an isocratic or a gradient method should be made now. In most cases, these depend on reversed-phase separations on a C18 section with UV detection.[12]

c) Optimization of mobile phase

The choice of suitable mobile phase is vital in HPLC as a successful chromatographic separation depends upon differences in the interaction of the solutes with the mobile phase and the stationary phase. The eluting power of the mobile phase is determined by its overall polarity, the polarity of the stationary phase and the nature of the sample components. For normal phase separation, eluting power increases with increasing the polarity of the solvent; for reverse phase separation, eluting power decrease with increasing solvent polarity. Many of the common solvents used in the HPLC are flammable and some are toxic, so it is advisable for HPLC instrumentation to be used in well-ventilated laboratory. Special grades of solvents are available for HPLC, which have been carefully purified to remove UV-absorbing impurities and any particulate matter. It is also important to remove dissolved air or suspended air bubbles, which can cause problems effecting the detector and pump. There is on sample solution the choose the most suitable mobile phase for given separation however, following factors should be taken in to account while selecting the mobile phase.[13] Increasing Polarity Order: Acetonitrile > Methanol > Ethanol > Pyridine > 2-propanol > Acetone > Ethyl acetate > Diethyl ether > t-Butyl ether > Chloroform > Toluene > Benzene > n-hexane > Cyclohexane.[14]

d) Column temperature

The utilization of temperature in HPLC method development presents a test since it can affect selectivity. The utilization of raised temperatures will reduce mobile phase viscosity and back-pressure. This can permit you to work at higher flow rates or to utilize longer columns more modest particle sizes. Decrease elution time.

Improve method reproducibility (instead of working at room temperature). However, it is difficult to decide whether the utilization of raised temperatures will help or thwart a particular separation. For complex separation, enhancements in one part of the chromatogram are nearly continuously joined by diminishes in another part of a similar chromatogram.[15]

e) Selection of detector

Method of detection used for HPLC include UV absorption fluorescence, refractive index, electrical conductivity, mass spectroscopy, electrochemical and radiochemical. Many modern LC instruments use diode array UV detectors. As well as the traditional two-dimensional presentation of detector signal against time. It is possible to display the UV spectra of the components giving rise to different chromatographic peaks.[11]

Table 1: Type of detector.

Detectors	Working
UV/Visible detector	A versatile dual-wavelength absorbance detector for HPLC. This detector offers the high sensitive required for routine UV-based applications to low-level impurity identification and quantitative analysis.
Photodiode Array (PDA) Detector	Offers advanced optical detection for analytical HPLC, Preparative HPLC, or LC.MS system solution. Its integrated software and optics innovation deliver high chromatographic and special sensitivity.
Refractive index (RI) Detector	Offers high sensitivity, stability and reproducibility making this detector the ideal solution for analysis of components with limited or no UV absorption.
Multi-wavelength florescence Detector	Offers high sensitivity and selectivity fluorescence detection for quantitating low concentrations of target compounds.
Conductivity Detector	Feature a multi-electrode flow cell that offers exceptional sensitivity and stability for single-column or suppressor-based ion chromatography.
Evaporative light scattering (ELS) Detector	It is a compact detector that controls temperature in the nebulization and evaporation stages maintaining low-dispersion characteristics for dependable HPLC/ELS results.
Evaporative	Provides sensitivity for a variety of application needs. It is configurable with a variety of flow cells, variable volume, reference electrode, working electrodes and working electrode.

3) Prepration of sample

Sample preparation is a basic advance of method development that the analyst must examine. For example, the analyst ought to examine if centrifugation (deciding the ideal rpm and time) shaking or potentially filtration of the sample is required, particularly if there are insoluble components present in the sample. The goal is to show that the sample filtration doesn't influence the analytical result because of adsorption as well as extraction of leachable. The adequacy of the syringe filters is to a great extent determined by their ability to eliminate contaminants/insoluble components without leaching undesirable artifacts (i.e., extractable)

into the filtrate. The sample preparation procedure should be satisfactorily depicted in the respective analytical technique that is applied to a genuine in-process sample or a dosage form for ensuing HPLC analysis. The analytical procedure must indicate the producer, kind of filter, and pore size of the filter media.[16] The reason for sample preparation is to make a handled sample that prompts better analytical results compared with the initial sample. The prepared sample should be an aliquot relatively free of interferences that is compatible with the HPLC method and not harmful the column.[17,18]

4) Method optimization

The experimental conditions should be optimized to get desired separations and sensitivity after getting appropriate separations. Stability showing measure experimental conditions will be accomplished through planned/systemic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, sample amounts, Injection volume and diluents solvent type.[19]

5) Method validation

Analytical method validation is "A Documented proof, which gives a high level of confirmation that a particular process will reliably produce, A product meeting its predetermined specifications and quality attributes". The validation of analytical methods is done as per ICH guidelines.

Parameters of analytical method validation

1. Accuracy
2. Precision
   - ❖ Repeatability
   - ❖ Intermediate Precision
   - ❖ Reproducibility
3. Specificity
4. Detection and Quantitation limit
5. Linearity
6. Range
7. Stability
8. Robustness/ Ruggedness
9. System Suitability

1) Accuracy

It is the nearness of obtained value to the true or accepted value. It differentiates between the mean value found and the accepted value It separates between the mean value found and the acknowledged value. These should be checked by comparing with standard and blank solutions for guarantee that no interference exists. The accuracy is then determined from the test results as a level of the analyte recovered by the assay. It might regularly be communicated as the recovery by the assay of known, added measures of analyte.[20]

2) Precision

It is characterized as the closeness of agreement between a series of measurements got from multiple sampling of a similar homogeneous sample. Precision may be considered at three levels, such as repeatability, intermediate precision and reproducibility.

- **Repeatability-** It is the precision under similar working conditions throughout a short time. Repeatability is additionally named intra-assay precision.
- **Intermediate precision-**It is performed inside laboratories variations for example, different analysts, different equipments, different days, etc.
- **Reproducibility**-It is performed between laboratories for collaborative studies.[21]

3) Specificity

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. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure.

This definition has the following implications

- Identification: To ensure the identity of an analyte.
- Purity Tests: Ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, ie. related substances test, heavy metals, residual solvents content, etc.
- Assay (content or potency): To provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.[22]

4) Limit of detection (lod) and limit of quantitation (loq): Limit of detection (lod)

The detection limit of procedure is the little amount of analyte in a sample that can be detected however not really quantitated as a 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.

The signal-to-noise ratio is determined by: s = H/h Where 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.[23]

LOD= 3.3σ s

Where σ-Standard deviation

s-Average of slope

Limit of quantitation (LOQ)

The limit of Quantitation is the little 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.[22]

LOQ= 10σ s

Where σ-Standard deviation

s-Average of slope

5) Linearity

The linearity of an analytical technique is its capacity (inside an offered range) to acquire test results which are directly proportional to the concentration (amount) of analyte in the sample Linearity is determined by a progression of five to six injections of at least five guidelines whose concentrations span 80–120 percent of the expected concentration range. The reaction should be directly proportional to the concentrations of the analytes or proportional by methods for an well-defined characterized numerical computation. A linear regression equation applied to the outcomes ought to intercept not significantly different from zero. If a significant non-zero intercept is acquired, it should be shown that this has no impact on the accuracy of the strategy.[24]

6) Range

The range of an analytical procedure is the interval between the upper and lower concentration of an analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable precision, accuracy and linearity.[25]

7) **Stability:** 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.[26] 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. 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.

8) **Ruggedness/Robustness:** Ruggedness, according to the USP, is the degree of reproducibility of the results obtained under a variety of condition, expressed as percentage relative standard deviation (RSD). These condition, include difference in laboratories, analyst, instruments, reagents, and experimental periods. To evaluate the ruggedness of the method, run on the batch of precision and accuracy using different column by a different analyst employing the same instrument or another instrument. The robustness of an analytical procedure is a measure of the capacity to remain unaffected by small, but deliberate variation in method parameters. The robustness of a method is evaluated by varying method parameters such as percent organic solvent, pH, ionic strength, or temperature and determining the effect (if any) on the result of the method.[27,28]

9) **System stability:** System suitability testing is an internal part of many analytical procedure. The tests are based on the concept that the equipment, electronics, analytical operation and sample to be analysed constitute an integral system that can be evaluated as such. System suitability test parameters to be established foe a particular procedure depend on the type of procedure being validated.[29]

The parameters used in the system suitability tests (SST) report are as follows

1. Number of theoretical plates or Efficiency (N).
2. Capacity factor (K).
3. Separation or Relative retention (α).
4. Resolution (Rs).
5. Tailing factor (T).

6. Relative Standard Deviation (RSD).

CONCLUSION

HPLC method development and validation of optimized method is described in this review article. The study of the pKa, pH and solubility of the compound is important in the HPLC method development. Selection of buffer and mobile phase composition plays a important role in the separation. The information about method validation parameters are well explained. n as per ICH guidelines. Optimized method is validated with various parameters (e.g) accuracy, precision, linearity robustness, detection limit, quantitation limit). It is an important procedure in the pharmaceutical industry and it is utilized to ensure that quality is built in to the processes supporting drug development and manufacture.

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