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METHOD DEVELOPMENT AND VALIDATION BY HPLC

Agari Nisha*, Chaudhary N. Alka and Kothiyal Preeti

India.

***Author for Correspondence: Agari Nisha**
India.

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INTRODUCTION

Analytical techniques hold the key to the design, development, standardization and quality control of medical products. They are equally important in pharmacokinetics and in drug metabolism studies. Both of which are fundamental to the assesment of bioavailability and the duration of clinical response. Analytical instrumentation placed an important role in the production and evaluation of new products, and in the production of consumers and the environment.[1] From a more applied point of view,analytical chemistry is the basis of chemical analysis,which corresponds to the study of the methods and their deiverse techniques applied to solving problems of analysis. Drug analysis means identification, characterization and determination of drugs in mixtures such as dosage forms and biological fluids.[2]

Chemical analysis is generally applied in two areas

(i) Quantitative Analysis (ii) Qualitative Analysis

Highly specific sensitive analytical techniques hold the key to the design, development, standardization and quality control of medicinal products. Modern physical methods of analysis are extremely sensitive, providing precise and accurate information about the standards of chemicals (or) drugs up to a nanogram level.[3,4]

CHROMATOGRAPHIC METHODS

Chromatography is the powerful techniques in which differential migration of components take place between two phases, one is stable which is known as stationary phase and another is movable which is known as a mobile phase. Species in the sample undergo repeated interactions (partitions) between the mobile phase and stationary phase. The stationary phase may be solid or a liquid (supported on a solid or a gel), and packed in a column, spread as a layer or film. The mobile phase may be gaseous or liquid. Those solutes, distributed preferentially in the mobile phase, will move rapidly through the system than those distributed preferentially in the stationary phase. This forms the basis of separation of component present in a sample.[5] The distribution of a solute between two phases results from the balance of forces between solute molecules and the molecule of each phase. It reflects the relative attraction or repulsion that molecule or ions of the competing phase shown for the solute and for them. These forces can be polar in nature arising from permanent on induced and dipole moment. In ion exchange chromatography, the forces on the solute molecules are substantially ionic in nature but include polar and non-polar forces as well.

Chromatographic method must having essentially,

- Stationary phase
- Mobile phase,
- Sample injection system,
- Solvent delivery system ,
- Column (support for stationary phase),
- Detection by detecting agent.[6,7]

High Performance liquid Chromatography [HPLC]

The technique, chromatography was originally developed by the Russian botanist M.S Tswett in 1903.HPLC is an analytical technique in which solutes are resolved by differential rates of elution as they pass through a chromatographic column. The method of separation by this instrument is governed by distribution between the mobile phase and stationary phase. The instrumentation is made-up of eight basic components, mobile phase reservoir, solvent delivery system, sample introduction device, column, detector, waste reservoir, connective tubing and computer, integrator or recorder. The successful use of HPLC for the possible problem requires the right combination of variety of operating conditions such as the type of column packing and mobile phase, column length and diameter, mobile phase flow rate , column temperature and sample size.[7] Now a day reversed-phase chromatography is the most commonly used separation technique in HPLC due to its broad application range. It is estimated that over 65% (possibly up to 90%) of all HPLC separations are carried out in the reversed phase mode. The reasons for this include the simplicity, versatility and scope of the reversed-phase method as it is able to handle compounds of a diverse polarity and molecular mass.[8]

HPLC as compared with the classical LC technique is characterised by

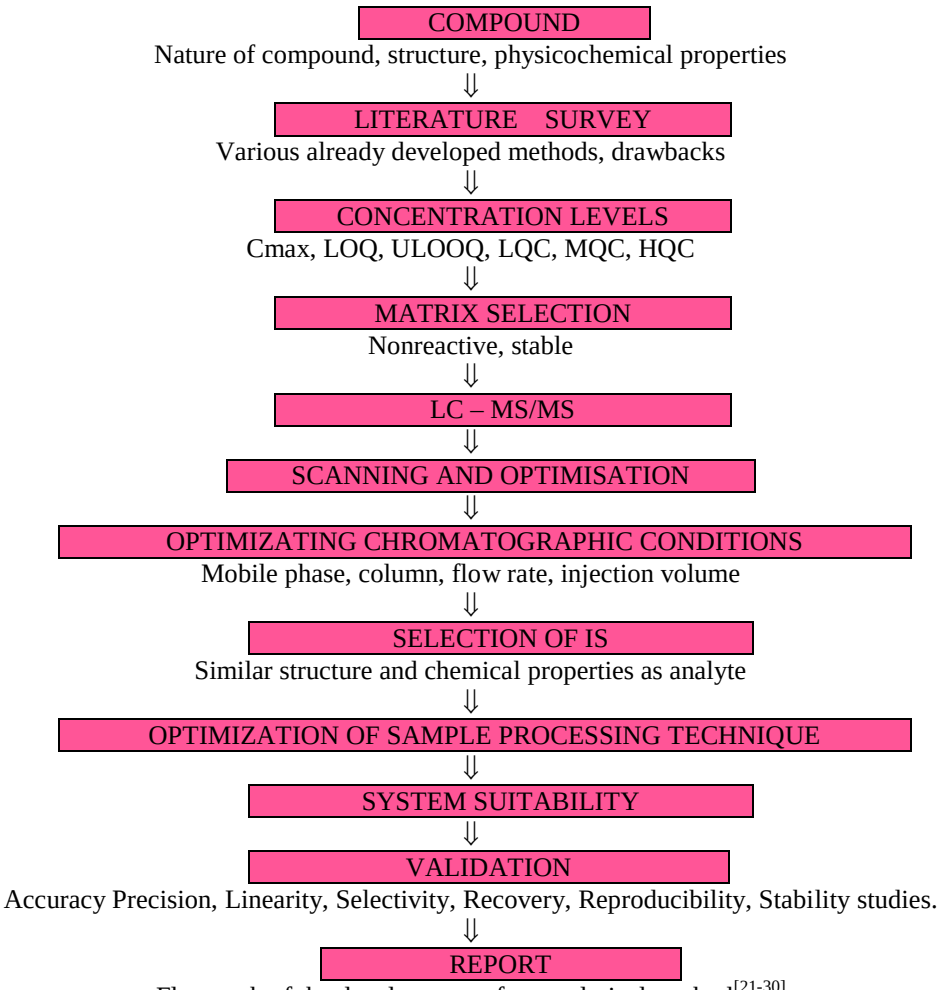
. High resolution.
. Small diameter (4.6 mm), stainless steel, glass or titanium columns.
. Column packing with very small (3, 5 and 10 µm) particles.
. Relatively high inlet pressures and controlled flow of the mobile phase.
. Continuous flow detectors capable of handling small flow rates and detecting very small amounts.[9]

Types of HPLC methods

1. Reverse Phase-HPLC Reversed phase chromatography has found both analytical and preparative applications in thearea of biochemical separation and purification. Molecules that possess some degree of hydrophobic character can be separated by reversed phase chromatography with excellent recovery and resolution.[10] Uses water-organic as mobile phase, columns may be C18 (ODS), C8, phenyl, Trimethyl Silane (TMS), cyano as a stationary phase. It is first choice for most samples especially neutral or non ionized compounds, that dissolve in water organic mi.
2. Normal Phase HPLC- In this the mixtures of organic solvents for mobile phase and columns i.e. cyano, diol and amino silica can be used as stationary phase. It is first choice for mixtures of isomers and for preparative scale HPLC and second choice for lipophilic samples that cannot dissolve well in water-organic mixtures.[11]

Steps for HPLC Method Development

The wide variety of equipment, columns, eluant and operational parameters involved makes high performance liquid chromatography (HPLC) method development seem complex The process is influenced by the nature of the analytes and generally follows the following steps:



Flow path of the development of an analytical method[21-30]

Step 1- Selection of the HPLC method and initial system
Step 2- Selection of initial conditions
Step 3- Method optimization
Step 4- Method validation[12,13]

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.[14] 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.[15]

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.[16] UV-Visible detector is versatile, dualwavelength absorbance detector for HPLC. This detector offers the high sensitivity required for routine UV-based applications to low-level impurity identification and quantitative analysis.[17] Photodiode Array (PDA) Detector offers advanced optical detection for Waters analytical HPLC, preparative HPLC, or LC/MS system solutions. Its integrated software and optics innovations deliver high chromatographic and spectral sensitivity.[18] Refractive Index (RI) Detector offers high sensitivity, stability and reproducibility, which make this detector the ideal solution for analysis of components with limited or no UV absorption. Multi-Wavelength Fluorescence Detector offers high sensitivity and selectivity fluorescence detection for quantitating low concentrations of target compounds.[19]

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.[20] 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.[21] 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.[22] 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.[23]

The nature, shape and particle size of the silica support effects separation. Smaller particle results in a greater number of theoretical plates, or increased separation efficiency. However, the use of smaller particles also results in increased backpressure during chromatography and the column more easily becomes plugged.[24]

In reverse phase chromatography the stationary phase is non-polar and the mobile phase is polar, causing polar peaks to generally elute earlier than non-polar peaks. To create a stationary phase for reverse phase chromatography on silica support, the free silanols are reacted with a chlorosilane with hydrophobic functionality to introduce the non-polar surface.[25] 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[26]. Common stationary phases are C4 (butyl), C8 (octyl), C18 (octadecyl), nitrile (cyanopropyl), and phenyl (phenyl propyl) columns. In general, longer alkyl chains, higher phase loading, and higher carbon loads provide greater retention of non-polar analytes.[27] Commonly used reverse phase columns and their uses are listed below. Propyl (C3), Butyl (C4), and Pentyl (C5) columns are useful for ionpairing chromatography.

Examples include Zorbax SB-C3, YMC-Pack C4, and Luna C5. These columns are generally less stable to hydrolysis than columns with longer alkyl chains. Octyl (C8) columns have wide applicability.[28] This phase is less retentive than the C18 phases, but is still quite useful for pharmaceuticals. Examples include (Zorbax SB-C8, Luna C8 and YMC-Pack-MOS). Octadecyl (C18, ODS) columns are the most widely used and tend to be the most retentive for non-polar analytes. Examples include Zorbax SB-C18, YMCPack ODS and Luna C18. Xterra RP-C18 and Zorbax Extend-C18 columns have been formulated to tolerate high pH systems (pH >7, normally up to pH 11). Varying the pH can affect selectivity and resolution of polar analytes, especially for ionizable compounds. Phenyl (Ph) columns offer unique selectivity from the alkyl phases and are generally less retentive than C8 or C18 phases. Phenyl columns are commonly used to resolve aromatic compounds.[29] Examples include Zorbax SB-Phenyl, YMC-Pack Phenyl and Luna Phenyl-Hexyl. Nitrile (CN or cyano) columns are polar and can be used for both reverse and normal phase applications. This phase is often used to increase retention of polar analytes. Examples include Zorbax SB-CN, Luna-CN, and YMC-Pack CN[29]. The type of column chosen for a particular separation depends on the compound and the aim of analysis.[30]

Mobile phase

The mobile phase effects resolution, selectivity and efficiency. In reverse phase chromatography, the mobile phase consists of an aqueous buffer and a non-UV active water miscible organic solvent. The effect of the organic and aqueous phase and the proportions in which they are mixed will affect the analysis of the drug molecule. Selection of the mobile-phase and gradient conditions is dependent on the inorganic nature of the analyte and the hydrophobicity of the analytes in the mixture respectively.[31] 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.[32] 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.[33]

Typically a 10 – 50 mM solution of an aqueous buffer is used. The most commonly used aqueous phase is H3PO4 in water i.e. phosphate buffer. The pH of a phosphate buffer is easily adjusted by using mono-, di-, or tribasic phosphate salts. However, when phosphate salts are used the solution should be filtered to remove insoluble particles with 0.22µm filter paper. Other non-UV active acids and bases may also be used to effect differences in peak shape and retention.[34]

Isocratic or gradient separations

Isocratic, constant eluent composition means equilibrium conditions in the column and the actual velocity of compounds moving through the column are constant; analyte-eluent and analyte-stationaryphase interactions are also constant throughout the whole run. This makes isocratic separations more predictable, although the separation power (the number of compounds which could be resolved) is not very high. The peak capacity is low; and the longer the component is retained on the column, the wider is the resultant peak.[35]

Gradient separation significantly increases the separation power of a system mainly because of the dramatic increase of the apparent efficiency (decrease of the peak width). The condition where the tail of a chromatographic zone is always under the influence of a stronger eluent composition leads to the decrease of the peak width. Peak width varies depending on the rate of the eluent composition variation (gradient slope).[36]

Preparation of sample solutions for method development: Sample preparation is a critical step of method development that the analyst must investigate. The effectiveness of the syringe filters is largely determined by their ability to remove contaminants/insoluble components without leaching undesirable artifacts (i.e., extractables) into the filtrate. If any additional peaks are observed in the filtered samples, then the diluent must be filtered to determine if a leachable component is coming from the syringe filter housing/filter.[37] The drug substance being analyzed should be stable in solution (diluent). During initial method development, preparations of the solutions in amber flasks should be performed until it is determined that the active component is stable at room temperature and does not degrade under normal laboratory conditions. The sample solution should be filtered; the use of a 0.22 or 0.45 µm pore-size filter is generally recommended for removal of particulates. Filtration is a preventive maintenance tool for HPLC analyses.[38]

Step 2- Selection of initial conditions: This step determines the optimum conditions to adequately retain all analytes; that is, ensures no analyte has a capacity factor of less than 0.5 (poor retention could result in peak overlapping) and no analyte has a capacity factor greater than 10– 15 (excessive retention leads to long analysis time and broad peaks with poor detectability).[39]

Step 3- Method optimization the experimental conditions should be optimized to get desired separations and sensitivity after getting appropriate separations. Stability indicating assay experimental conditions will be achieved through planned/systemic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, temperature, sample amounts, Injection volume and diluents solvent type.[40]

Step 4 - Validation of method Validation of an analytical method is the process which is established by laboratory studies to evaluate the performance uniqueness of the procedure meet the requirements for its intended use. The methods validation process for analytical procedures begins with planned and systematic collection by the applicant of the validation data to support analytical procedures.[41] The following are typical analytical performance characteristics which may be tested during methods validation:

1. Specificity
2. Accuracy
3. Precision

3.1 System Precision
3.2 Method Precision
3.3 Intermediate Precision

4. Limitd Quantitation
5. Linearity
6. Range
7. Robustness
8. Ruggedness

Parameters used for Assay Validation[42]

The validation of the assay procedure was carried out as per ICH guidelines using the following parameters.

Specificity

Specificity is the ability to access unequivocally the analyte in the presence of the components which may be expected to be present lack of specificity of an individual analytical procedure may be compensated by other analytical procedures. For the chromatographic methods developing a separation involves demonstrating specificity, which is the ability of the method to accurately measure the analyte response in the presence of all potential sample components. The response of the analyte in test mixtures containing the analyte and all potential sample components (placebo formulation, process impurities etc) is compared with the response of

the solution containing only the analyte. A specificity criterion for an assay method is that the analyte peak will have baseline chromatographic resolution of at least 1.5 from all other sample components. If this cannot be achieved the unresolved components at their maximum expected level will not affect the final assay result by more than 0.5%.[43]

Linearity

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 the analyte in the sample. For assay methods, this study is generally performed by preparing standard solutions at five concentration levels. Five levels are required to allow detection of curvature in the plotted data acceptability of linearity data is often judged by examining the correlation and y-intercept of the linear regression line for the response verses concentration plot. A correlation coefficient of >0.999 is generally considered as evidence of the data to the regression line. The y-intercept should be less than a few percent responses obtained for the analyte at the target level.[44]

Accuracy

The accuracy of an analytical procedure expresses the closeness of the agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. The accuracy of the method is the closeness of the measured value to the true value for the sample. Accuracy is usually determined in one of four ways.[45]

Quantitation limit

The quantitation of an analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. Quantitation limit based on the standard deviation of the response and the slope. It can be expressed as,

$QL = [10\sigma/S]$

σ =standard deviation of the response S =slope of the calibration curve (of the analyte).

Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test result obtained, the analysis of conditions such as different laboratories, different analysis using different instrument, on different days. Different source of reagent, elapsed assay, times, assay temperature conditions. Ruggedness is the measure of reproducibility of test result under the variation in conditions normally expected from analyst to analyst. The criteria of the ruggedness is the RSD should be not more than 2%.

Robustness

The robustness of a method is its ability to remain unaffected by small changes in parameter such as percent organic content, pH of the mobile phase, buffer concentration, temperature and injection volume. The criteria for robustness are the RSD should be not more than 2%.

System suitability testing

System suitability testing is an integral part of many analytical procedures the tests are based on the concept that the equipment, electronics, analytical operation and samples to be analyzed constitute an integral system that can be evaluated as such. Typically the process involves making five injections of a standard solution and evaluating several chromatographic parameters such as resolution, area % reproducibility, number of theoretical plates and tailing factor.

Applications of HPLC method[46]

- the various applicability, speed, sensitivity of HPLC is the most popular chromatography technique used for purification and all types of biological molecules.
- the system is widely used in clinical and pharmaceutical work as it possible to apply biological fluids such as serum and urine directly to the column.
- RP-HPLC has biggest impact on the separation of oligo peptides and proteins.
- Wide range of applications in organic chemistry.
- Chromatography separation of anions can be carried out by using ion exchange ion pair chromatography and ion exclusion chromatography.
- Chromatography separation of cation superficially sulphonated inert polymer resins have been used.
- Most widely used in Agri chemicals i.e. analysis of pesticides in cleaning water.
- mainly applied in food analysis
- Widely applied in forensic science for the separation of morphine and metabolites extracted from blood plasma.
- Modern applications are mainly in pharmaceutical field.

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