ORIGINAL RESEARCH PAPER
Volume - 10 | Issue - 06 | June - 2021 | PRINT ISSN No. 2277 - 8179 | DOI : 10.36106/ijsr
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

A REVIEW ON METHOD DEVELOPMENT AND VALIDATION BY USING HPLC

Pharmacy

Dipali S. Doifode* Vidyabharati College of Pharmacy, C.K. Naidu Road, Camp, Amaravati, Maharashtra. *Corresponding Author

Shailesh G. Jawarkar Vidyabharati College of Pharmacy, C.K. Naidu Road, Camp, Amaravati, Maharashtra.

Monika P. Jadhao Vidyabharati College of Pharmacy, C.K. Naidu Road, Camp, Amaravati, Maharashtra.

Manali M. Bode Vidyabharati College of Pharmacy, C.K. Naidu Road, Camp, Amaravati, Maharashtra.

ABSTRACT

Analytical method development and validation are the continuous and inter-dependent task associated with the research and development, quality control and quality assurance departments. High performance liquid chromatography (HPLC) has been widely used for years as an analytical method and is a key tool for the separation and analysis of pharmaceutical drugs, for drug monitoring and for quality assurance and life science research. Most of the drugs in multi component dosage forms can be analysed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC methods development and validation play important roles in new discovery, development, manufacture of pharmaceutical drugs and various other studies related to humans and animals. This review gives information regarding principle, types, instrumentation and along with various application of the method and Validation of HPLC method as per ICH Guidelines covers all the performance characteristics of validation, like Accuracy, precision, specificity, linearity, range and limit of detection, limit of quantification, robustness.

KEYWORDS

HPLC, Method development, Validation.

INTRODUCTION

Analytical methods including chromatographic methods are commonly used for the quantitative and qualitative analysis of raw materials, drug substances, drug products, and compounds in biological samples in pharmaceutical industry. The components monitored include chiral or achiral drugs, process impurities, residual solvents, excipients such as preservatives, degradation products, extractable and leachable from container and closure or manufacturing process, pesticide in drug product from plant origin, and metabolites.

Chromatography may be regarded as an analytical technique employed for the purification and separation of organic and inorganic substances. It is also very useful for the fractionation of complex mixtures, separation of closely related compound like isomers. Chromatography relatively new technique which was 1st developed by M. Tswett, a botanist in 1906 in Warsaw. [1]

Basically, this technique is based on differences in the rate at which component of a mixture move through a porous medium (Stationary phase) under the influence of some solvent or gas (mobile phase) at a high pressure. The instrumental method like High Performance Liquid Chromatography (HPLC) was derive from the classical column chromatography and, is widely used tool of analytical chemistry now a days. High Performance Liquid Chromatography is more versatile than gas chromatography since (a) it is not limited to volatile and thermally stable samples, and (b) the choice of mobile and stationary phases is wider. [2]

Chromatography

HPLC

High Performance Liquid Chromatography (HPLC) was derived from the classical column chromatography and, is one of the most important tools of analytical chemistry today. The principle is that a solution of the sample is injected into a column of a porous material (stationary phase) and a liquid (mobile phase) is pumped at high pressure through the column. The separation of sample is based on the differences in the rates of migration through the column arising from different partition of the sample between the stationary and mobile phase. Depending upon the partition behaviour of different components, elution at different time takes place. [3]

HPLC is the method of choice for checking peak purity of new chemical entities, monitoring reaction changes is in synthetic procedures or scale up, evaluating new formulations and carrying out quality control / assurance of the final drug products. The Goal of HPLC method is to try & separate, quantify the main drug, any reaction impurities, all available synthetic intermediates and any degradants. High Performance Liquid Chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid. HPLC is the most accurate analytical methods widely used for the quantitative as well as qualitative analysis of drug product and used for determining drug product stability. HPLC principle is the solution of sample is injected into a column of porous material (stationary phase) and liquid phase (mobile phase) is pumped at higher pressure through the column. The principle of separation followed is the adsorption of solute on stationary phase based on its affinity towards stationary phase. [4]

The technique of HPLC has following features.

- 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.
- Rapid analysis.

Separation principle [5]

In HPLC, individual components are separated using a column, based on the difference in the degree of interaction between the sample components and the column. Components with a low degree of interaction with the column are eluted first. These interactions include adsorption, hydrophilic interactions, hydrophobic interactions, electro affinity, penetration and exclusion.

Types of HPLC [6]

Types of HPLC generally depend on phase system used in the process. Following types of HPLC generally used in analysis

I. Based on mode of separation

Normal phase chromatography:

Also known Normal phase HPLC (NP-HPLC), this method separates analytes based on polarity. NP-HPLC uses a polar stationary phase and a non-polar mobile phase. The polar analyte interacted with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase increases the elution time.

Reversed phase chromatography:

Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. RPC operates on the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar analyte, and the non-polar stationary phase. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand in the aqueous eluent.

II. Based on principle of separation

1. Absorption Chromatography
2. Ion-exchange chromatography
3. Ion-pair chromatography

It is a form of chromatography in which ions in solution can be "paired" or neutralized and separated as an ion pair on a reversed-phase column. Ion-pairing agents are usually ionic compounds that contain a hydrocarbon chain that imparts a certain hydrophobicity so that the ion pair can be retained on a reversed-phase column.

4.gel permeation chromatography

This type of chromatography lacks an attractive interaction between the stationary phase and solute.

The liquid or gaseous phase passes through a porous gel which separates the molecules according to its size

4. Affinity Chromatography

III. Based on elution technique

1. Isocratic elution: A separation that employs a single solvent or solvent mixture of constant composition.

2. Gradient elution: Here two or more solvent systems that differ significantly in polarity are employed. After elution is begun; the ratio of the solvents is varied in a programmed way, sometimes continuously and sometimes in a series of steps. Separation efficiency is greatly enhanced by gradient elution.

IV. Based on scale of operation

1. Analytical HPLC

No recovery of individual components of substance

2.Preparative HPLC

Individual components of substance can be recovered

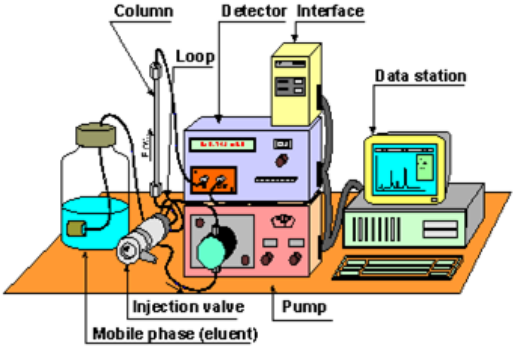

Fig- schematic diagram for HPLC

Instrumentation

HPLC Pumps [7]

pumps are classified according to their flow rate:

- Nano LC pumps: 1 µL/min or less
- Micro LC pumps: several tens of µL/min
- Semi-micro LC pumps: several hundreds of µL/min
- Analytical pumps: several mL/min
- Preparative pumps: several tens of mL/min or more

The pump flow rate for normal analysis is several mL/min.

Types of Detectors

There is a variety of detectors that can be used depending on the target sample. To detect only a few specific components, a highly sensitive fluorescence detector or a UV/Vis detector can be used. To detect a wide range of components, then a PDA detector, an evaporative light-scattering detector, or a differential refractive-index detector is more appropriate.

Table 1.- Types of Detectors

Detector type	Measurement principle
UV/Vis detector	Absorbance
PDA detector	Absorbance
Differential refractive index detector	Refractive index
Fluorescence detector	Fluorescence
Electrochemical detector	Oxidation / reduction
Electrical conductivity detector	Conductivity
Mass spectrometry detector	MS
Optical rotation detector	Optical rotation
Circular dichroism detector	Circular dichroism
Evaporative light scattering detector	Light scattering

Column Types and separation Modes [8]

There are various types of columns and separation modes that can be used, and the optimum choice depends on the nature of the sample and the analysis that is required. When an organic solvent is used as the mobile phase, a normal-phase column (mainly silica gel) can separate and analyse samples composed of fat-soluble components based on adsorption. When a water/methanol solvent is used as the mobile phase, separation can be achieved based on hydrophobic interactions in reverse-phase mode. GPC columns separate sample components based on their molecular size using pores. Ion exchange columns separate ion components based on electrical affinity.

Table 2.-column Types And Separation

Mode	Stationary phase	Mobile phase	Interaction	Features
Normal phase	Silica gel	Organic solvent	Adsorption	Separation of fat-soluble components
Reversed phase	Silica C_{18} (ODS)	Water / MeOH	Hydrophobic	The most commonly used method
GPC (non-aqueous)	Polymer	Organic solvent	Gel permeation	Molecular weight distribution measurement
GFC (aqueous)	Hydrophilic polymer	Buffer	Gel permeation	Biopolymer separation
Ion exchange	Ion exchanger	Buffer	Electric affinity	Separation of ionic components

Analytical method development [9]

Analytical method could be spectral, chromatographic, electrochemical, hyphenated or miscellaneous. Analytical method development is the process of selecting an accurate assay procedure to determine the composition of a formulation. It is the process of proving that an analytical method is acceptable for use in laboratory to measure the concentration of subsequent samples Analytical methods should be used within GMP and GLP environments and must be developed using the protocols and acceptance criteria set out in the ICH guidelines Q2(R1).

c) When there are no analytical methods for the formulation of the drug due to the interference caused by the formulation excipients.

d) Analytical methods for the quantitation of the analyte in biological fluids are found to be unavailable.

e) The existing analytical procedures may need costly reagents and solvents. It may also involve burdensome extraction and separation procedures.

Validation of method

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use. The methods validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to support analytical procedures21. All analytical methods that are intended to be used for analyzing any clinical samples will need to be validated. The validation of analytical methods is done as per ICH guidelines. [10]

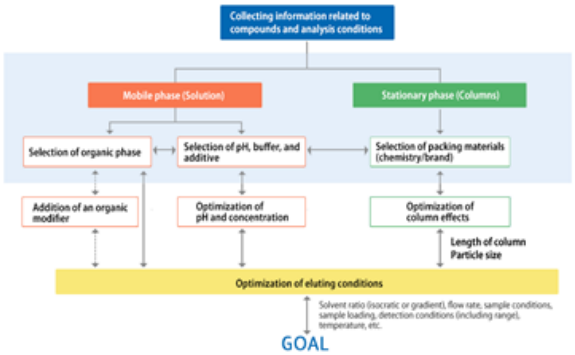

Fig- steps involved in method development

Components of method validation [11]

The following are typical analytical performance characteristics which may be tested during methods validation:

- Accuracy
- Precision
- Repeatability
- Intermediate precision
- Linearity
- Detection limit
- Quantitation limit
- Specificity
- Range
- Robustness
- System suitability determination
- Forced degradation studies
- Solution stability studies

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.

Precision

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. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision .

Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology)

Detection limit

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.

Quantitation limit

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 is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

Linearity

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.

Range

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.

Robustness

The robustness of an analytical procedure 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.

Forced Degradation Studies [12]

Forced degradation or stress studies are undertaken to deliberately degrade the sample. These studies are used to evaluate an analytical method's ability to measure an active ingredient and its degradation products, without interference, by generating potential degradation products. During validation of the method, drug substance are exposed to acid, base, heat, light and oxidizing agent to produce approximately 10% to 30% degradation of active substance. The studies can also provide information about the degradation pathways and degradation products that could form during storage. These studies may also help in the formulation development, manufacturing, and packaging to improve a drug product. Reasons for carrying out forced degradation studies include: development and validation of stability- indicating methodology, determination of degradation pathways of drug substances and drug products, discernment of degradation products in formulations that are related to drug substances versus those that are related to non–drug substances (e.g., excipients).

Solution Stability Studies

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.

APPLICATIONS-

HPLC industry Applications

There is a wide variety of applications throughout the process of creating a new drug from drug discovery to the manufacture of formulated products that will be administered to patients. This Process to create a new drug can be divided into 3 main stages

1. Drug discovery
2. Drug development
3. Drug manufacturing.

Pharmaceutical applications:

1. Tablet dissolution study of the pharmaceutical dosage form.
2. To control drug stability, Shelf-life determination.
3. Identification of active ingredients.
4. Pharmaceutical quality control.
5. Tablet dissolution of pharmaceutical dosage forms [13]

Environmental applications

1. Detection of phenol compounds in drinking water.
2. Identification of diphenhydramine in sedimented samples.
3. Bio-monitoring of pollutant.
4. Rapid separation and identification of carbonyl compounds by HPLC.
5. LC/MS/MS solution for pharmaceuticals and personal care products in water, sediment, soil and biosolids by HPLC/MS/MS.
6. Determination of 3-mercaptopropionic acid by HPLC

Forensics applications:

1. Quantification of the drug biological samples.
2. Identification of anabolic steroids in serum, urine, sweat & hair.
3. Forensic analysis of textile dyes.
4. Determination of cocaine and other drugs of abuse in blood, urine,

etc.
5. Determination of benzodiazepines in oral fluid using LC/MS/MS.

Clinical applications:

Catecholamines such as epinephrine and dopamine are highly important for many biological functions. Analyzing their precursors and metabolites can provide diagnosis of diseases such as Parkinson's disease, heart disease, and muscular dystrophy.

2. Quantification of ions in human urine analysis of antibiotics in blood plasma.
3. Estimation of bilirubin & biliviridin in blood plasma in case of hepatic disorders.
4. Detection of endogenous neuropeptides in extracellular fluids of the brain.

Pharmaceutical impurity profiling analysis

1. Structure elucidation of impurities with LC/MS.
2. Rapid condition scouting for method development.
3. Using a fast LC method for higher sample throughput. [14]

Recent applications[15]

Analytic method development9-17 and validation are key elements of any pharmaceutical development program. HPLC analysis method is developed to identify, quantity or purifying compounds of interest. HPLC helps a lot in stability studies of drug formulations. HPLC helps a lot in stability studies of atropine, antibiotics, & biotechnologybased drugs like insulin, streptokinase, etc

1. It is used in inorganic chemistry for separating anions & cations.
2. It is used in forensic science for the separation of phenyl alkylamines (morphine and its metabolites) from blood plasma, and for the detection of poisons or intoxicants such as alcohol, carbon monoxide, cholinesterase inhibitors, heavy metals, hypnotics, etc.
3. It is used in environmental studies for analyzing the pesticide content in drinking water
4. It is utilized in food analysis for separating water soluble and fat-soluble vitamins from variety of food products, fortified food and animal feed.
5. It is also used for determining antioxidants and preservatives present in the food.
6. It is used in the cosmetic industry for the assay and quality control of various cosmetics like lipsticks, creams, ointments, etc.
7. It is used for separating various components of plant products with bear structural resemblance Eg: Analysis of cinchona, digitalis, ergot extracts and licorice.
8. It is used in the agrichemical industry for the separation of herbicides
9. It is used in the separation and analysis of amino acids, carbohydrates, proteins, lipids and steroidal hormones.
10. It is used for separating coal and oil products from their crude sources.

CONCLUSION

In recent years development of the analytical methods for identification, purity evaluation and quantification of drugs has received a great deal of attention in the field of pharmaceutical analysis. This review describes the general technique of HPLC method development and validation of optimized method. The knowledge of the pKa, pH and solubility of the primary compound is of utmost importance prior to the HPLC method development. Final optimization can be performed by changing the temperature, gradient slope, and flow rate as well as the type and concentration of mobile-phase modifiers Optimized method is validated with various parameters (e.g. specificity, precision, accuracy, detection limit, linearity, etc.) as per ICH guidelines. The role of HPLC in the pharmaceutical industry is very vital particularly in preformulation, process development, during formulation development and drug discovery and to verify drug purity.

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