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AN ANALYTICAL METHOD DEVELOPMENT OF HPLC

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ABSTRACT

Many different strategies of high performance liquid chromatographic method development are being used today. This overview describes a strategy for the systematic development of High performance liquid chromatographic (HPLC) methods. It is an analytical tool which is able to separate, detect and quantify the drug, its various impurities and drug related degrdants that can form on synthesis or storage. HPLC involves the understanding of chemistry of drug substance and facilitates the development of the analytical method.

Reversed phase chromatography has found both analytical and The preparative applications in the area of biochemical separation and purification. Molecules that possess some degree of hydrophobic character, such as proteins, peptides and nucleic acids, can be separated by reversed phase chromatography with excellent recovery and resolution. This review covers the importance of RP-HPLC in analytical method development and their strategies along with brief knowledge of critical chromatographic parameters need to be optimized for an efficient method development.

INTRODUCTION

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. The technique, chromatography was originally developed by the Russian botanist M.S Tswett in 19031 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.

HPLC as compared with the classical LC technique is characterized 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. **(1)**

Principle

High pressure liquid chromatography is the full form for HPLC and as given in the name, there is use of high pressure in the *principle* of its operation. Also due to its efficiency in analysis of compounds it is regarded as High performance liquid chromatography. Some have even gone to the extent of calling it as High patience liquid chromatography based on the long human time requirement and patience needed in its operation. HPLC is one of the modern chromatography systems which are widely used in the fields of clinical research, biochemical research, industrial quality control etc. Applications of HPLC include detection, analysis, determination, quantification, derivation of molecules from mixtures of biological, plant and medical importance. High performance liquid chromatography is basically a highly improved form of column chromatography. Instead of allowing the solvent to drip through a column under just the force of gravity, it is externally forced through the column under high pressures of up to 400 atm. This makes the chromatographic process a lot faster. It also allows the use of very small particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing through it. Thus, it allows a much better separation of the components of the mixture. High performance liquid chromatography is now one of the most powerful tools in analytical chemistry as it has the ability to identify, separate and quantitate the compounds that are present in any sample that can be dissolved in any liquid. Today, trace concentrations of compounds as low as *parts per trillion* [ppt] may easily be identified. HPLC can be,and has been, applied to just about any sample, such as food, pharmaceuticals, forensic samples, nutraceuticals, cosmetics, industrial chemicals and environmental matrices (2,3)Two variants are in use in HPLC based on the relative polarity of the solvent and the stationary phase.

Theory of Reversed Phase Chromatography

Reversed phase chromatography has found both analytical and preparative applications in the area 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. (4) The separation mechanism in reversed phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilised hydrophobic ligand, i.e. the stationary phase. The actual nature of the hydrophobic binding interaction itself is a matter of heated debate(5) but the conventional wisdom assumes the binding interaction to be the result of a favourable entropy effect. The initial mobile phase binding conditions used in reversed phase chromatography are primarily aqueous which indicates a high degree of organised water structure surrounding both the solute molecule and the immobilised ligand. As solute binds to the immobilised hydrophobic ligand, the hydrophobic area exposed to the solvent is minimised. Therefore the degree of organised water structure is diminished with a corresponding favourable increase in system entropy. In this way, it is advantageous from an energy point of view for the hydrophobic moieties, i.e. solute and ligand, to associate (6)

Water adjacent to hydrophobic regions is postulated to be more highly ordered than the bulk water. Part of this ‘structured’ water is displaced when the hydrophobic regions interact leading to an increase in the overall entropy of the system. Separations in reversed phase chromatography depend on the reversible adsorption/desorption of solute molecules with varying degrees of hydrophobicity to a hydrophobic stationary phase. The samples are subjected to flow by a mobile liquid phase through the stable stationary phase. The sample compounds are separated into individual components based on their relative affinity towards the two phases during their travel**.**

Types of Chromatography

Based on the type of stationary material used for the separation, it is of two types:

1) Normal phase: The stationary material in normal phase is polar in nature and therefore, the compounds with higher polarity elute out last while non polars come out first.

2) Reverse phase: The stationary material in reverse phase is non-polar in nature and therefore, the compounds with lower polarity elute out last and vice-versa. Mostly in HPLC analysis, the type that is used nowadays is reverse phase as many of the biological, phyto-chemical compounds and drugs that are being analysed by using HPLC are polar in nature **(7,8,9,10)**

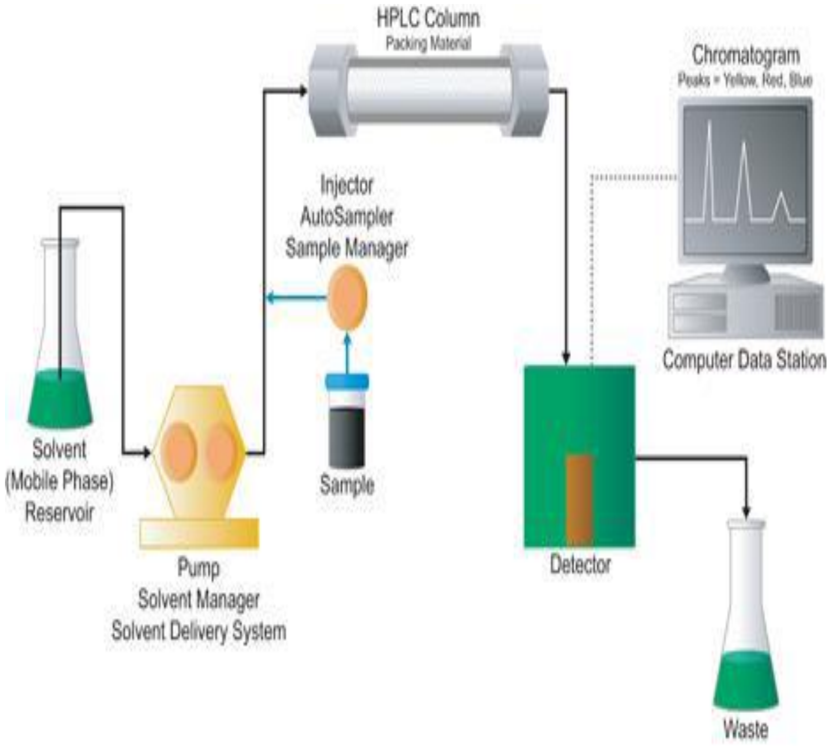

Fig no 1 Basic instrumentation of HPLC

METHOD VALIDATION

Validation is defined by the International Organization for Standardization (ISO) as “verification, where the specified requirements are adequate for an intended use where the term verification is defined as “provision of objective evidence that a given item fulfills specified requirements.(11) The applicability and scope of an analytical method should be defined before starting the validation process It includes defining the analytes, concentration range description of equipment and procedures, validation level and criteria required. The validated range is defined by IUPAC as “the interval of analyte concentration within which the method can be regarded as validated” (12 ,13)This range does not have to be the highest and lowest possible levels of the analyte that can be determined by the method .Instead, it is defined on the basis of the intended purpose a of the method(14,15) .The method can be validated for use as a screening or quantitative method. It can also be validated before use on single equipment, different equipments in the laboratory, different laboratories or even for international use at different climatic and environmental conditions .The criteria of each type of validation will of course be different with the validation level required.The various validation parameters include linearity, accuracy, precision, ruggedness, robustness, LOD, LOQ and selectivity or specificity. (16)

Linearity

Linearity is the ability of analytical procedure to obtain a response that is directly proportional to the concentration (amount) of analyte in the sample. If the method is linear, the test results are directly or by well-defined mathematical transformation proportional to concentration of analyte in samples within a given range. Linearity is usually expressed as the confidence limit around the slope of the regression line.(17,18)

Accuracy

Accuracy is defined by ISO as “closeness of agreement between a measured quantity value and a

true quantity value of a measure”. It is a qualitative characteristic that cannot be expressed as a numerical value. It has an inverse relation to both random and systematic errors, where higher accuracy means lowererrors (19,20,21)

Accuracy is evaluated by analyzing test drug at different concentration levels. Typically, known amount of related substances and the drug substance in placebo are spiked to prepare an accuracy sample of known concentration of related substance. Samples are prepared in triplicate. ICH recommends accuracy evaluation using a minimum of nine determinations over a minimum of three concentration levels covering the range specified.(22,23)

It is determined by comparing the found concentration with the added concentration. The methods of determining accuracy include analysis of analysis of an analyte of known purity (i.e., reference material), comparisons of results of the proposed analytical procedure with those of a second well-characterized procedure and standard addition method. The accuracy may also be inferred once precision, linearity and specificity have been established (24,25,26)

Precision

It expresses 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(29 30) Repeatability is also referred to as intra-assay precision. It is a measure of precision of analysis in one laboratory by one operator using one piece of equipment over a relatively short time-span. It is degree of agreement of results when experimental conditions are maintained as constant as possible, and expressed as RSD of replicate. ICH recommends a minimum of nine determinations covering the specified range for the procedure (e.g., three concentrations/three replicates as in the accuracy experiment), or a minimum of six determinations at 100% of the test concentration for evaluation of repeatability which should be reported as standard deviation, relative standard deviation (coefficient of variation) or confidence interval. ICH defines intermediate precision as long-term variability of the measurement process and is determined by comparing the results of a method run within a single laboratory over a number of weeks. It is also called as inter day precision (27,28) . It reflects discrepancies in results obtained by different operators, from different instruments, with standards and reagents from different suppliers, with columns from different batches or a combination of these but in the same laboratory (29). The objective of intermediate precision validation is to verify that the method will provide same results in the same laboratory once the development phase is over. Reproducibility expresses precision of analysis of the same sample by different analysts in different laboratories using operational and environmental conditions that may differ but are still within the specified parameters of the method. The objective is to verify that the method will provide the same results despite differences in room temperature and humidity, variedly experienced operators, different characteristics of equipments (e.g., delay volume of an HPLC system), variations in material and instrument conditions (e.g. in HPLC, mobile phase composition, pH, flow rate of mobile phase), equipments and consumables of different ages, columns from different suppliers or different batches and solvents, reagents and other material with different quality.

Selectivity and Specificity

Selectivity and specificity are sometimes used interchangeably to describe the same concept in method validation. Selectivity of an analytical method is defined by the ISO as “property of a measuring system, used with a specified measurement procedure, whereby it provides measured quantity values for one or more measure and such that the values of each measure and are independent of other measure ands or other quantities in the phenomenon, body, or substance being investigated” Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. The specificity of a test method is determined by comparing test results from an analysis of samples containing impurities, degradation products, or placebo ingredients with those obtained from an analysis of samples without impurities, degradation products, or placebo ingredients. Specificity can best be demonstrated by resolution between the analyte peak and the other closely eluting peak(s).

CONCLUSION

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. The method development and validation are continuous and interrelated processes that are conducted throughout the drug development process. The analytical validation verifies that a given method measures a parameter as intended and establishes the performance limits of the measurement. Reproducible quality HPLC results can only be obtained if proper attention has been paid to the method development, validation and system's suitability to carry out the analysis. (30, 31)

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