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HPLC Instrumentation and Validation

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ABSTRACT
High pressure liquid chromatography–frequently called high performance liquid chromatography (HPLC or, LC) is the premier analytical technique in pharmaceutical analysis and is predominantly used in the pharmaceutical industry.Only compounds dissolved in solvents can be analyzed with HPLC. HPLC separates compounds dissolved in a liquid sample and allows qualitative and quantitative analysis of what components and how much of each component are contained in the sample.For food scientists, high-performance liquid chromatography (HPLC) is a powerful tool for product composition testing and assuring product quality.

Keywords: High performance liquid chromatography, instrumentation, Validation, mobile phase.

I. INTRODUCTION

1.1 Chromatography

Chromatography is an analytical technique commonly used for separating a mixture of chemical substances into its individual components, so that the individual components can be thoroughly analyzed.

1.2 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography is a powerful tool in analysis. This page looks at how it is carried out and shows how it uses the same principles as in thin layer chromatography and column chromatography. High Performance Liquid Chromatography is basically a highly improved form of Column Chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster. It also allows you to use a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture. The other major improvement over column chromatography concerns the detection methods which can be used. These methods are highly automated and extremely sensitive.

1.3 Normal phase HPLC

This is essentially just the same as you will already have read about in Thin Layer Chromatography or Column Chromatography. Although it is described as "normal", it isn't the most commonly used form of HPLC. The column is filled with tiny silica particles, and the solvent is non-polar - hexane, for example. A typical column has an internal diameter of 4.6 mm (and may be less than that), and a length of 150 to 250 mm. Polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar compounds will. The non-polar ones will therefore pass more quickly through the column.

1.4 Reversed phase HPLC

In this case, the column size is the same, but the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent is used - for example, a mixture of water and an alcohol such as methanol. In this case, there will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column. There won't be as much attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution. Polar molecules in the mixture will therefore spend most of their time moving with the solvent. Non-polar compounds in

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the mixture will tend to form attractions with the hydrocarbon groups because of van der Waals dispersion forces. They will also be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or methanol molecules, for example. They therefore spend less time in solution in the solvent and this will slow them down on their way through the column. That means that now it is the polar molecules that will travel through the column more quickly. Reversed phase HPLC is the most commonly used form of HPLC.

1.5 Injection of the sample

Injection of the sample is entirely automated, and you wouldn't be expected to know how this is done at this introductory level. Because of the pressures involved, it is not the same as in gas chromatography.

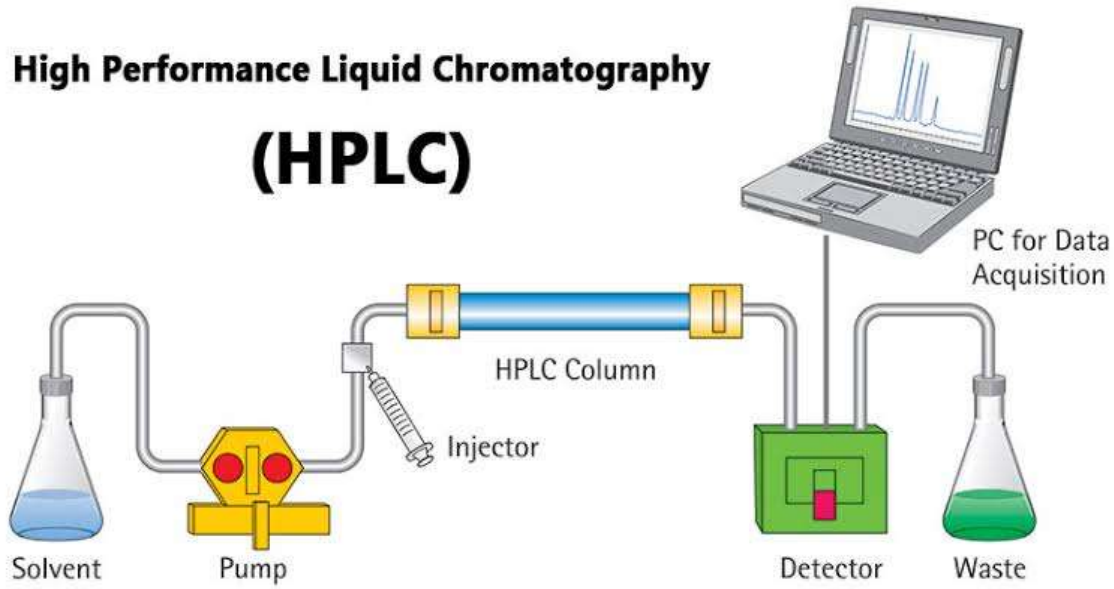
1.6 Retention time

The time taken for a particular compound to travel through the column to the detector is known as its retention time. This time is measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound. Different compounds have different retention times. For a particular compound, the retention time will vary depending on:

i) The pressure used (because that affects the flow rate of the solvent)
ii) The nature of the stationary phase (not only what material it is made of, but also particle size)
iii) The exact composition of the solvent
iv) The temperature of the column that means that conditions have to be carefully controlled if you are using retention times as a way of identifying compounds.

INSTRUMENTATION

1. Solvent Delivery System
2. Pump
3. Solvent Degassing System
4. Sample Injector
5. Guard Column
6. Analytical Column
7. Detector
8. Recorders and Integrators



1 Solvent Delivery System

The mobile phase is pumped under pressure at a high pressure at about 1000 to 3000 psi; from one or several reservoirs and flows through the column at a constant rate. With micro particulate packing, there is a high pressure drop across a chromatography column. 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 separations eluting power increases with increasing polarity of the solvent but for reversed phase separations, eluting power decreases with increasing solvent polarity. Some other properties of the solvents, which need to be considered for a successful separation, are boiling point, viscosity,

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detector compatibility, flammability and toxicity. Optimum separating conditions can be achieved by making use of mixture of two solvents.

2 Pump

The most important component of HPLC in solvent delivery system is the pump, because its performance directly effects the retention time, reproducibility and detector sensitivity. The particle size of the stationary phase is 5-10 µm. So resistance to flow is observed. This is the reason that high pressure is required and this is provided by using pumps. The different types of pumps include: Constant pressure pump which uses a constant pressure to the mobile phase; the flow rate through the column is determined by the flow resistance of the column and any other restrictions between the pump and the detector outlet. Constant flow pump generates a given flow of liquid, so that the pressure developed depends on the flow resistance.

2.1 Constant pressure Pumps

The simplest type of constant pressure pump is the pressurized coil pump or gas displacement pump. consisting of some form of pneumatic device for the direct pressurization of the mobile phase with an inert gas, give a reliable pulse-free flow and have the advantage of low cost and simplicity. They are however not as accurate as constant volume pumps but can be used where flow accuracy and reproducibility are less critical. But this is now only of historical interest.

2.2 Syringe Pump

The syringe type pump consists of a syringe the plunger of which is driven by a stepping motor through a gear box. The rate of delivery from the syringe is controlled by varying the voltage on the motor. The main advantage of this type of pump is that it is capable of providing a pulse-free flow at high pressure and the flow rate is independent of the operating pressure, if the compressibility of the liquid is ignored. Its main disadvantage is that it has a finite solvent capacity. Dual syringe systems found in some instruments are of course expensive, but with suitable gradient formers they also provide the gradient elution capacity. Due to its high cost and lack of flexibility this type of pump is little used in today's commercial instruments.

2.3 ReciprocatingPumps

Reciprocating Pumps fall into two types: diaphragm and piston pumps. Among the several solvent delivery systems (direct gas pressure, pneumatic intensifier, reciprocating etc.) reciprocating pump with twin or triple pistons is widely used, as this system gives less baseline noise, good flow rate reproducibility etc

3 Solvent Degassing System

The constituents of the mobile phase should be degassed and filtered before use because several gases are soluble in organic solvents. When solvents are pumped at high pressure, gas bubbles are formed which will interfere with the separation process. Numerous methods are employed to remove the dissolved gases in the mobile phase. They include heating and stirring, vacuum degassing with an aspirator, filtration through 0.45 filters, vacuum degassing with an air-soluble membrane, helium purging, ultra-sonication or purging or combination of these methods. HPLC systems are also provided an online degassing system, which continuously removes the dissolved gases from the mobile phase.

4 SampleInjector

Two means for analyte introduction on the column are injection in to a flowing stream and a stop flow injection. Several devices are available either for manual or auto injection. a. Septum injectors- for injecting the sample through a rubber septum. b. Stop flow- in which the flow of the mobile phase is stopped for a while and the sample is injected through a valve device. c. Rheodyne injector (loop valve type) - it is the most popular type. This has a fixed volume loop like 20-50 µl or more. The injector has two modes, i.e., load position when the sample is loaded in the loop and the inject mode, when the sample is injected. The injector can be a solitary infusion or a computerized infusion framework. An injector for a HPLC framework should give infusion of the fluid specimen inside the scope of 0.1 mL to 100 mL of volume with high reproducibility and under high pressure (up to 4000 psi). Automatic injector is a microprocessor-controlled version of the manual universal injector. Usually, up to 100 samples is capable of being loaded on to the auto injector tray. The system parameters such as flow rates, volume to be injected, gradient, run time, etc. are selected, stored in memory and sequentially executed on consecutive injections**.**

5 GuardColumn

Guard column has very small quantity of adsorbent and improves the life of the analytical

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column. It also acts as a prefilter to remove particulate matter, if any, and other material. Guard column has the same material as that of analytical column. It does not contribute to any separation but is necessarily used before the analytical column to protect & increase lifetime of column. Operator usually slurry or dry packs short guard column regularly with same or similar packing used in analytical column (old column material) are used for guard columns. However, the particle size of the packing material is bigger in size than analytical column in order to avoid the pressure drop in the HPLC system.

6 AnalyticalColumn

Columns are typically made of polished stainless steel, glass, and polyethylene or poly ether ether ketone (latest). Column length is around 50 mm to 300 mm and has an internal diameter across of somewhere around 2mm to5 mm. They are generally loaded with a stationary phase with a particle size of 3 μm to 10 μm. 1gm of stationary phase provides surface area ranging from 100860 sq.m. with an average of 400sq.m. Columns with inner diameters of <2 mm are regularly normally suggested to as microbore segments. Rather the temperature of mobile phase and the column should be kept consistent during investigation. The functional group present in stationary phase depends on the type of chromatographic separation. In normal phase mode it contains the silanol groups (hydroxyl group). In the reverse phase mode C_{18} (Octa Decyl Silane), C_8, C_4, CN, NH_2columns are used.

7 Detector

Several ways of detecting are used when a substance has passed through the column. A detector used depends upon the property of the compound to be separated.

7.1 UVDetector

This is the most commonly used type of detector as it can be rather sensitive, has a wide linear range, is relatively unaffected by temperature fluctuations and is also suitable for gradient elution. It records compounds that absorb ultraviolet or visible light. Many organic compounds absorb UV light of various wavelengths. The amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time. Absorption takes place at a wavelength above 200nm, provided that the molecule has at least:

(a) A double bond adjacent to an atom with a lone electron pair
(b) Bromine, iodine or sulfur
(c) A carbonyl group or a nitro group
(d) Two conjugated double bonds
(e) An aromatic ring

7.2 Refractive Index (RI) Detectors

Refractive index (RI) detectors are nonselective and often used to supplement UV models. These detectors sense the difference in refractive index between the column eluent and a reference stream of pure mobile phase. They are the closest ones in HPLC to a universal detector, as any solute can be detected as long as there is a difference in RI between the solute and the mobile phase.

7.3 Fluorescence Detectors

Compounds that have fluorescence or of with fluorescing derivatives can be obtained are picked up with high sensitivity and specificity by this detector. The sensitivity may be up to 1000 times greater than with UV detection. Light of a suitable wavelength is passed through the cell and the higher wavelength radiation emitted is detected in a right-angled direction.

7.4 Electrochemical (Amperometric) Detectors

Electrochemistry provides a useful means of detecting traces of readily oxidizable or reducible organic compounds with great selectivity. The detection limit can be extraordinarily low and the detectors are both simple and inexpensive. The potential between the working and reference electrodes may be selected. The working electrode is made up of glassy carbon, carbon paste or amalgamated gold. Frequently a silver/silver chloride electrode is used as the reference. The diffusion current recorded is proportional to the concentration of the compound eluted. This is applicable only when compounds have functional group which can be either oxidized or reduced.

7.5 Conductivity Detectors

This is the classical ion chromatography detector and measures the eluate conductivity, which is proportional to ionic sample concentration (provided that the cell is suitably constructed). Its sensitivity decreases as the specific conductivity of the mobile phase increases. The active cell volume of 2ml is very small. Good conductivity detectors have automatic temperature compensation

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(conductivity is highly temperature-dependent) and electronic background conductivity suppression.

7.6 Light Scattering Detectors

The evaporative light-scattering detector (ELSD) is an instrument for the nonselective detection of nonvolatile analytes. The column eluate is nebulized in a stream of inert gas. The liquid droplets are then evaporated, thus producing solid particles which are passed through a laser, LED, or polychromatic light beam. The resulting scattered light is registered by a photodiode or photomultiplier.

7.7 Photo Diode Array Detectors (PDA) Detectors

A Photodiode Array Detector is a lined array of discrete photodiodes on an integrated circuit (IC) chip for spectroscopy. It is placed at the image plane of a spectrometer to allow a range of wave length to be sensed concomitantly. PDA can be programmed for any wavelength range and all the compounds that absorb at this range can be identified in a single range. It can also analyze peak purity by matching spectra within a peak. The resulting spectra are 3-D plot of Response Vs Time Vs Wavelength.

Other Detectors

7.8 Photoconductivity Detectors

These are sensitive, selective detectors for organic halogen and nitrogen compounds. The eluate is split up as it leaves the column. One half passes through the reference cell of a conductivity detector and the other half is irradiated with 214 or 254nm UV light whereupon suitable sample molecules become dissociated into ionic fragments. The ensuing high level of conductivity is recorded in the measuring cell.

7.9 Infrared Detectors

Every organic molecule absorbs infrared light at one wavelength or another. When an IR detector is used, the mobile phase chosen must not be self-absorbent at the required wavelength. Hexane, dichloromethane and acetonitrile are suitable mobile phases for ester detection whereas ethyl acetate is not. The sensitivity is no greater than that of refractive index detectors.

7.10 Radioactivity Detectors

These are used especially for detecting the b-emitters H_3, C_{14}, P_{32}, S_{35} and I_{131}. The scintillator required for this relatively weak radiation is either added as a liquid between the column and the detector or is contained as a solid in the cell.

8 Recorders and Integrator

Recorders: the signals from the detector after amplification (if necessary) are recorded as a series of peaks, each one representing a compound in the mixture. Baseline and the peaks are recorded with respect to time.

Retention time for all the peaks can be found from the recordings. The area under the peak is proportional to the amount of substance passed through detector, and this area can be calculated automatically by the computer linked to the display.

Integrator: improved version of recorders by which signals from the detector are gathered on graph recorders or electronic integrators that fluctuate in many-sided quality and in their capacity to process, store and reprocess chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret.

METHOD VALIDATION

Validation of an analytical method is the process by which it is established by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application. Validation is required for any new or amended method to ensure that it is capable of giving reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories. The type of validation program required depends entirely on the particular method and its proposed applications.

1 Scope of Process Validation

Validation is one of the wide and most difficult areas because it engaged in all levels of product manufacturing. The scope of validation in different fields is follows:

1.1 Analytical
1.2 Instrument Calibration
1.3 Process Utility services
1.4 Raw materials
1.5 Packaging materials
1.6 Equipment
1.7 Facilities
1.8 Manufacturing operations
1.9 Product Design
1.10 Cleaning
1.11 Operators

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2 Importance of Validation

2.1 Assurance of quality
2.2 Time bound
2.3 Process optimization
2.4 Reduction in rejections
2.5 Increased output
2.6 Avoidance of capital expenditures
2.7 Fewer complaints about process related failures
2.8 Reduced testing in process and in finished goods
2.9 More rapid and reliable start-up of new equipments
2.10 Easier scale-up for development work.

3 Basic concept of Process Validation

3.1 Calibration, verification and maintenance of process equipment.
3.2 Prequalification or revalidation.
3.3 Establishing specifications and performance characteristics.
3.4 Selection of methods, process and equipment to ensure the product meets specifications.
3.5 Qualification or validation of process and equipment.
3.6 Testing the final product, using validated analytical methods, in order to meet specifications.

4. Significance of Process Validation

Process Validation is defined as establishing documented evidence which provides a high degree of assurance that a specific system, related equipment and process consistently meet the approved specifications and produce products meeting predetermined quality attributes. Process validation is a basic factor for drug product safety and quality and thus a fundamental component of the quality assurance system used by pharmaceutical manufacturers. The basic principle of Quality Assurance is that a drug should be produced that is fit for its intended use. Effective Process Validation contributes significantly to assure the drug quality; this principle incorporates the understanding that the following conditions exist:

4.1 Quality, safety, and efficacy are designed or built into the product.
4.2 Quality cannot be adequately assured merely by in process and finished-product inspection or testing.
4.3 Each step of a manufacturing process is controlled to assure that the finished product meets all design characteristics and quality attributes including specifications.

5 Types of process validation

5.1 Prospective validation
5.2 Concurrent validation
5.3 Retrospective validation
5.4 Revalidation

5.1 Prospective validation

Prospective validation is defined as the establishment of documented evidence that a system does what it purports to do based on a pre planned protocol. The objective of prospective validation is to prove or demonstrate that the process will work in accordance with a validation master plan or protocol prepared for pilot product trails. It is not limited to, List of analytical methods, as appropriate.

1. Proposed in-process controls with acceptance criteria
2. Additional testing to be carried out, with acceptance criteria and analytical validation, as appropriate.
3. Sampling plan.
4. Methods for recording and evaluating results.
5. Functions and responsibilities.

5.2 Retrospective validation

Validation of such processes should be based on historical data. The steps involved require the preparation of a specific protocol and the reporting of the results of the data review, leading to a conclusion and a recommendation. The basis for retrospective validation is Valid in-process specifications for such characteristics shall be consistent with drug product final specifications and shall be derived from previous acceptable process average and process variability estimates where possible and determined by the application of suitable statistical procedures where appropriate. Some of the essential elements are;

1. Batch size/strength/manufacturer/year/period.
2. Master manufacturing/packaging documents.
3. Current specifications for active materials/finished products.
4. List of process deviations, corrective actions and changes to manufacturing

5.3 Concurrent Validation : In-process monitoring of critical processing steps and end-product testing of current production can provide documented evidence to show that the manufacturing process is in a state of control. It is similar to the prospective, except the operating firm will sell the product during the qualification runs, to the public as its market price.

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5.4 Revalidation

It is the repetition of a validation process or a part of it. Some of the changes in revalidation are

Changes in the source of active raw material manufacturer.

Changes in packaging material

1. Changes in the process and in the plant/facility.

II. CONCLUSION

The literature review of HPLC was done vigilantly and it was found to be one of the most widely used system among the chromatographic techniques. The choice of detection approach is critical to guarantee that all the components are detected accurately. Chromatography is a separation technique used to separate the individual compound from a mixture using a stationary and mobile phase. The discovery of chromatography is a millstone event in biomedical research. Chromatographic separation is based on the principles of adsorption, partition, ion exchange, molecular exclusion, affinity and Chirality. HPLC is a highly assertive analytical technique which uses sophisticated technologies that have been extensively practiced from decades. Modernizations such as ultrahigh-pressure liquid chromatography, nano liquid chromatography, liquid chromatography-mass spectrometry, chiral phase separations, core-shell columns, and novel stationary phases have helped HPLC to acquire higher performance levels; in diverse factors, yielding faster speed, higher resolution, greater sensitivity, and increased precision. The practice of HPLC is restricted to analyzers, but is now widely performed by students, chemists, biologists, production workers, and other novices in academia, research, and quality control laboratories.

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