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HPLC METHOD DEVELOPMENT AND VALIDATION: A REVIEW

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ABSTRACT

Many different strategies of HPLC method development are used today. This review describes a strategy for the systemic development of HPLC method. Validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. Chromatographic method plays significant role in the pharmaceutical industry from the drug discovery, development formulations and quality control. This review describes general approach towards validation process and validation parameters to be considered during validation of a HPLC method. A new simple, selective, accurate, rapid and reversed phase HPLC technique was established as per ICH guidelines.

Introduction:

High performance liquid chromatography (HPLC) which is also know as high pressure liquid chromatography. It is a popular analytical technique used for the separation, identification and quantification of each constituent of mixture. The analytical technique of HPLC is used pharmaceutical industry. HPLC is used at all the different stages in the creation of a new drug, and also is used routinely during drug manufacture. The information obtained may be qualitative indicating what compounds are present in sample or quantitative, providing the actual amount of compounds in the sample. Solid mixtures are also analyzed by first converting them to a liquid or gaseous state, using suitable sample preparation techniques.

Chromatography equipment look rather intimidating to any one who has not handled them before, but on a closer look and as you get familiar with the equipment you realize that behind the network of wires, complex plumbing and circuitry is simple machine with only a few major parts. Different combination of these parts namely pumps, detectors and injectors yield an infinite numbers of configurations based on the application. The liquid phase is pumped at constant rate to the column packed with the stationary phase(a solid or liquid supported on a solid). The also involve a mobile phase (liquid or a gas). A mobilephase flow through the stationary phase. HPLC is basically highly improved form of column liquid chromatography adsorption/ retention of substance on stationary phase. Separationof adsorb substance using mobile phase.

What Is Chromatography?

A Russian botanist Mikhail Tswett (1872- 1919) is credited with the first use of chromatography in 1906 when he separated plant pigment such as chlorophylls and xanthophyll. Chromatography is a technique used for separation of the components of mixture by distribution of two phase. Chromatography is the most widely used laboratory technique for separation, identification and quantification of components of liquid and gaseous mixture.

Table:1 Types of chromatography

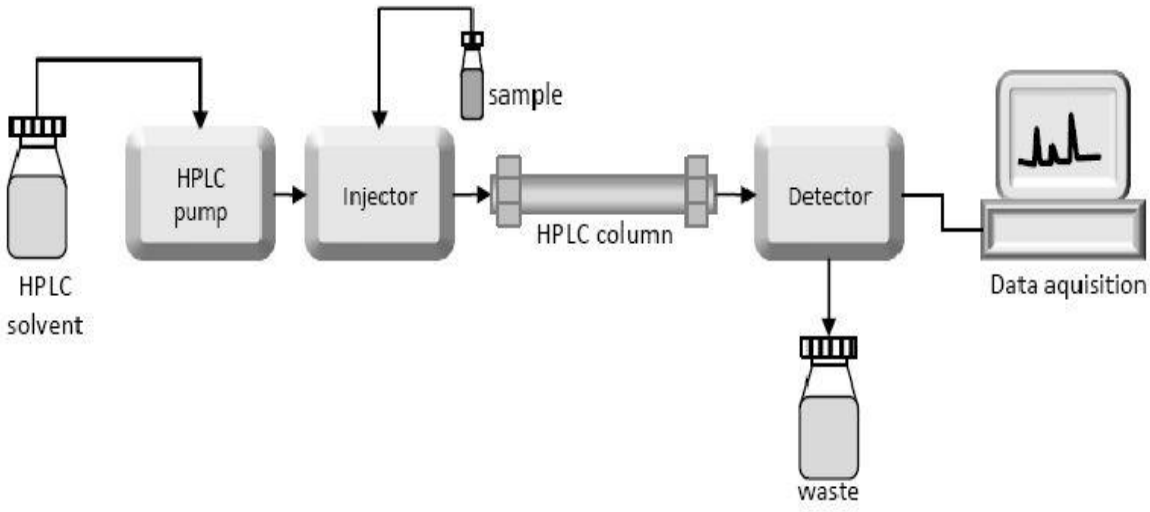
Type of chromatography	Mobile Phase	Stationary Phase
Gas chromatography	Gas	Solid/liquid
Liquid chromatography	Liquid	Solid/liquid
Supercritical- fluid chromatography	Supercritical fluid	Solid/liquid

Stationary Phase: The substance on which adsorption of the analyte (the substance to be separated during chromatography) take place. It can be a solid, a gel or a solid liquid combination.

Mobile Phase: Solvent which carries the analyte (a liquid or a gas)

Principle:

The separation principle of HPLC is distribution of sample or analyte between a mobile phase and stationary phase. The compounds bind at specific regions of stationary phase based on physical and chemical properties. The different constituents of a sample are eluted at different times. The analyte recognizes by the detectors unit (e.g. UV detector). A signals are recorded into computer software and then show in a chromatogram. The principle of separation in normal phase mode and reverse phase mode is adsorption. HPLC work on the principle that some molecules take longer than other to pass through a chromatography column.



Different type of HPLC act on the basis of two laws

1) Adsorption law
2) Partition law

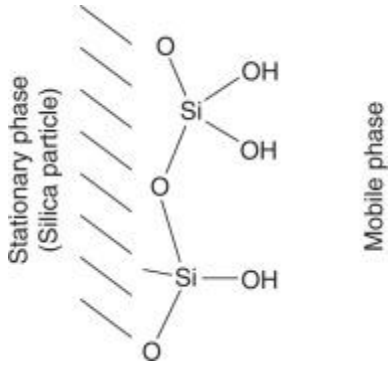
"When a solid surface is exposed to a gas or a liquid, molecules from the gas or the solution phase accumulated or concentrated at the surface of the solid." this phenomenon is called as **adsorption**.

"The ratio of the partition or distribution of a compound between two immiscible phases remain constant." The constant is called as "**partition constant or distribution constant."**

Types of HPLC:

1. Normal phase HPLC:

The NP- HPLC uses polar stationary phase and non-polar mobile phase. In NP- HPLC the stationary phase is more polar than the mobile phase. The most common used stationary phase is silica gel $[(SiO_2)_x . (H_2O)_y]$.



2. Reverse phase HPLC:

Reverse phase HPLC is more commonly used compared to NP- HPLC. This technique can be used to separate, identify and are quantitates components in mixture of soluble organic components based on there hydrophobicity. The stationary phase is non-polar,like C18 bonded silica. The mobile phase is polar usually being water and polar organic solvent.

3. Size-exclusion HPLC:

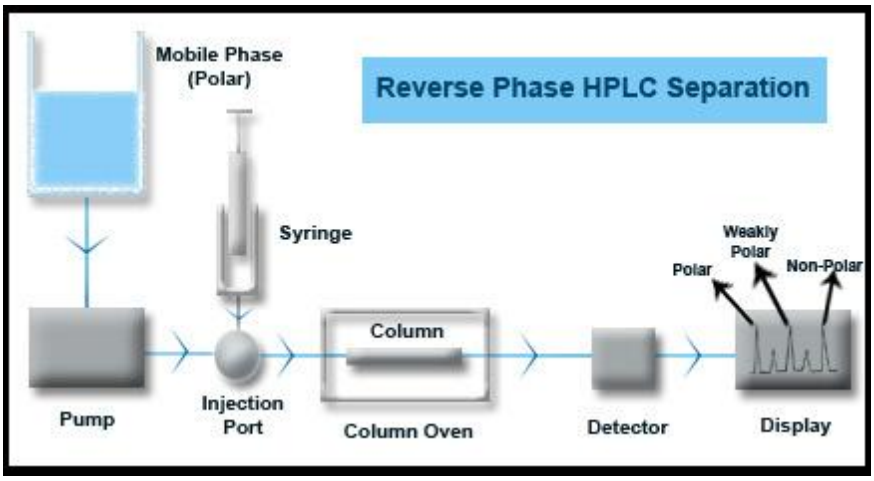
The column is filled with material having precisely controlled pore sizes, and the particle are separated according to its their molecular size.

4. Ion-Exchange HPLC:

The separates molecules based on differences between the overall charge of the proteins. The stationary phase has an ionically charge surface of opposite charge to the sample ions.

The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

Instrumentation of HPLC:



1. Solvent reservoir:

i. The reagent bottle that holds our HPLC solvent can used as reservoir.

ii. The reservoir and its attachment to the pump should be made of material that will not contaminate the mobile phase.

e.g. Teflon, glass, or stainless steel.

2. Pump:

i. HPLC pumps from the basic parts of HPLC instrumentation. Unlike other chromatography techniques, HPLC needs to generate pressure by pump. HPLC pump generate pressure on the solvent so as to pass through the dense column.

ii. Suitability to the wide range of solvents used.

iii. Create uniform pressure without fluctuations.

iv. Deliver constant flow rate i.e. the volume of solvent pump per minute.

v. Easy to use and operable for long duration.

Three types of HPLC pumps:

a) Screw-driven syringe pump

b) Reciprocating pumps and

c) Pneumatic pumps

3. Sample injector:

i. The injection of a sample at atmospheric pressure into the system, at high pressure, represents a critical step in the chromatographic process.

ii. The injector can be a single injection or an automated injection system.

iii. The range of 0.1-100mL of volume with high reproducibility and under high pressure (up to 4000 psi)

4. Columns:

3) Columns are usually made of polished stainless steel, are between 50 and 300mm long and have an internal diameter of between 2 and 5 mm.

4) They are commonly filled with a stationary phase with a particle size of 3-10 mm.

5) Columns with internal diameter of less than 2 mm are often referred to as microbore columns.

6) HPLC columns can be used to separate many types of analytes.

7) HPLC Columns commonly used for separation of carbohydrates, amino acid and proteins.

8) Reversed phase HPLC columns have nonpolar packing.

5. Detector:

i. The HPLC detector, located at the end of the column detects the analytes as they elute from the chromatographic column.

ii. The commonly used detectors are UV-spectroscopy, fluorescence, mass- spectrometric and electrochemical detectors.

6. Data Collection Devices:

i. Signals from the detector may be collected on chart recorders or electronic integrators that vary complexity and in their ability to process, store and reprocess chromatographic data.

ii. 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 Development:**

Analytical method development and validation plays important roles in the discovery development and manufacture of pharmaceuticals. These methods used to ensure the identity, purity, potency, & performance of drug products. Analytical method development is considered as a critical process in pharmaceuticals. Availability of the different types of column, operating, parameters, mobile phase composition, diluent and pH values make it critical to develop an analytical method. The wide variety of equipment column, eluent, operational parameter involved makes HPLC method development seem complex.

Steps involve in method development are:

1. Understand the physiochemical properties of drug molecule.
2. Set up HPLC conditions.
3. Preparation of sample solution for method development.
4. Method optimization.
5. Validation of method.

Understand the physiochemical properties of drug molecule

i. Understand the physiochemical properties of drug molecule to study the physical properties like solubility, polarity, pK_a and pH of the drug molecule.

ii. Polarity is a physical property of a compound.

iii. In non-polar covalent bond, the electron are shared equally between two atom.

iv. A polar covalent bond is one in which one atom has a greater attraction for the electron than the other atom.

v. pH and pK_a plays an important role in HPLC method development.

vi. The pH value is defined as the negative of the logarithm to the bas 10 of the concentration of the hydrogen ion. $pH= -\log_{10}[H_3O^+]$

vii. The acidity or basicity of a substance is defined most typicallyby the pH value.

viii. The acidity of an aqueous solution is determine by the concentration of $[H_3O^+]$ ions.

ix. The concentration of hydrogen ion indicated as $[H^+]$.

x. The pH of a solution can be changed simply by adding acid or base to the solution.

Set up HPLC conditions

i. A buffer is a partially neutralized acid which resist changes pH.

ii. Buffering capacity increase as the molar concentration (molarity) of the buffer salt/acid solution increase.

iii. The closer of buffered pH is to the pK_a ,the greater the buffering capacity.

iv. Consideration of the affect of pH on analyte retention, type of buffer to use, and its concentration, solubility in the organic modifier and its affect on detection are important in reversed phase chromatography (RPC) method development of ionic analytes.

Preparation of sample solution for method development

The drug substance being analyzed should be stable in solution (diluent). The sample solution should be filtered; the use of a 0.22 or 0.45µm pore-size filter is generally recommended for removal of particulates. Filtration is prevention maintenance tool for HPLC analyses.

Method optimization

The experimental condition optimize to get desired separate. The stability indicating planned/systemic examination on parameters including pH (if ionic), mobile phase components and ratio gradient, flow rate, temperature, sample amount, Injection volume anddiluent solvent types.

- **Validation of method:**

In the pharmaceutical industry, validation is essential part of quality control and quality assurance. Various regulatory authorities give particular emphasis on the validation of all the process used in the industry.

1. Validation process:

i. Develop a validation protocol or operating procedure for the validation
ii. Define the application, purpose and scope of the method
iii. Define performance parameters and acceptance criteria
iv. Define validation experiments
v. Verify relevant performance characteristic of equipment
vi. Quality material e.g. standard and reagents
vii. Perform pre-validation experiments
viii. Adjust method parameters and acceptance criteria if necessary
ix. Perform full validation experiments
x. Develop sop_s for executing method in routine
xi. Define criteria foe revalidation
xii. Define type and frequency of system suitability tests for routine
xiii. Document validation experiments and result of validation

Validation protocol:

The validation protocol is an ideal tool for training all the employees working for validation. The validation protocol should be include:

1) Introduction
2) Organizational structure
3) Process and product description
4) Specific process consideration
5) Key acceptance criteria
6) Documentation format
7) Required sop_s
8) Planning and scheduling
9) Change control

For each individual validation project plan should be developed. The plan should be including a time table with specific task, derivable and owners.

Revalidation:

The operating parameter need to be a specified with ranges clearly defined. In case of method for quantitation of impurities, if a new impurity is found that makes the method deficient in its specificity. So any such change needs revalidation.

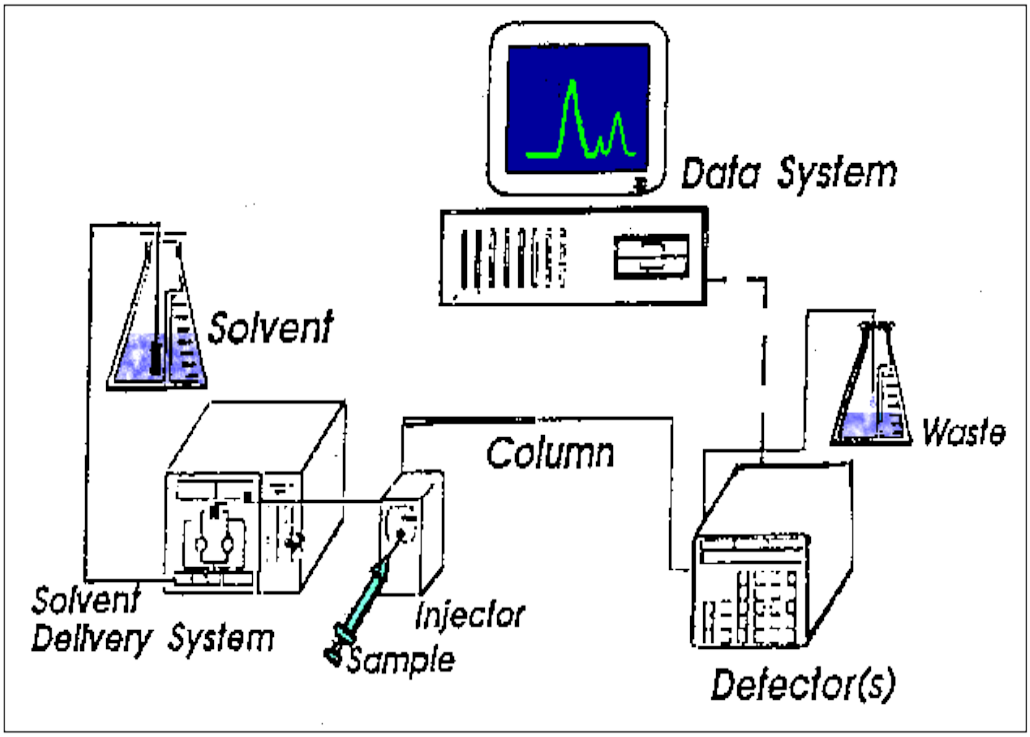
Validation parameters:

The analytical method which need to be validated are classified as per ICH are classified as following:

1) Identification tests.
2) Quantitative test for impurities.
3) Assay of drug substance and drug products.

How to operate HPLC (High perform liquid chromatography):

1. Turn on the switches of the pump and detector.
2. Turn on the computer first and then printer.



3. The word “Hewlett Packard System Hardware test” will be show on monitor and that means the computer is checking the system for examples; hard disk. If the computer does not find any problems, the monitor will show the word ‘pass”.
4. Next, type “C:/>win” to get into the HPLC system.
5. The word “HP Chemistation” will appears on the monitor and then double click at “instrument online HPLC system”.

6. The monitor will show the word “HPLC system”which gives you “peak” (when the sample is injected), the flow rate of mobile phase or solvent, wave length and pressure.

7. For the pump, click the button “pump on” and then “enter”.

8. For the detector, click the button “lamp on”and then “enter”. After that, adjust the magnitude of wave length as you prefer.

9. Adjust the condition of the pumpby keying in the values to the computer at the icon “the instrument” and “set up pump”.

9.1 Adjust the flow rate of mobile phase starting from 0.1ml/min

9.2 Select the proper kind of sample to be the mobile phase; for examples

A. Acetonitrile

B. Water

C. Acetic acid

D. Methanol

The mobile phase can be mixed together, for example B 10%,D 90%.

10. Gradually increase the flow rate of the mobile phase by the step of 0.1ml/min. As the time passes, the pressure increase. When the word “Ready” appears on the monitor, the pressure is constant. Then increase the flow rate of mobile phase one more step. Repeat the same way until reaching the preferred flow rate. Wait until the base line steady then prepare to inject the sample.

11. Clean the syringe with the sample before injecting the sample into HPLC. Then inject and load the sample into HPLC, the component of the sample will be detected by the detector and the time is counted.

12. peak of each component will be show on the monitor at the different retention time. When there are no more peak detected, press F8 button to stop detecting the sample.

13.computer will calculate and integrate the area of each peak. Then the result will be presented in the relation between area and the retention time. Peak and other results can be viewed by clicking at the following icons; “View”, “Data analysis”, “load signal” and “Integration result”.

Application of HPLC:

- Clinical diagnosis of diseases, disorder.
- In scientific research for discovery.
- In pharmaceutical labs for analysis.
- In the food industry quality control.
- For standards control by government.
- For separation of similar molecules.

Conclusion:

HPLC is probably the most universal type of analytical procedure; its application areas include, quality control, forensic analysis, environmental monitoring and clinical testing.

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