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Analytical Method by High Performance Liquid Chromatography

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ABSTRACT

Pharmaceutical analysis, a branch of pharmacy, plays a very significant role in quality control of pharmaceuticals through a rigid check on raw materials used in manufacturing of formulation and on finished products. Analytical chemistry has since long, occupied an important place in the development of science and technology. It is primarily concerned about determining the qualitative and quantitative composition of material under study. In this article we have focused on the different analytical method and brief description of chromatographic technique, especially High performance Liquid Chromatography. The qualitative analysis gives us the information about the nature of sample by knowing about the presence or absence of certain components. The quantitative analysis deals about the content present in the sample. The development in analytical sciences has been more significant and prominent in recent years than the past.

Keywords: Pharmaceutical analysis, High performance Liquid Chromatography, Instrumentation, Quality assurance.

INTRODUCTION:

Quality assurance is a wide ranging concept covering all matters that individually or collectively influence that quality of the product. It plays a central role in determining the safety and efficiency of medicines. Highly specific and sensitive analytical techniques hold the key role to the design, development, standard and quality control of medicinal product. [1]

Quality of the drug product is very vital, as it involves life. Proper manufacture and quality control of pharmaceuticals is the vital segment of strong primary healthcare program worldwide. Quality is the total sum of all factors which contribute directly or indirectly to the safety; efficacy and acceptability of the product. [2]

This has really broaden our vistas and helped to develop new methods of analysis. In pharmacy analytical chemistry is responsible for developing sensitive, reliable and more accurate methods for the estimation of drug in pharmaceutical dosage form. [3]

1.0 Analytical chemistry:

Analytical chemistry is an important part of pharmaceutical analysis. Analytical Chemistry may be defined as the science and art of determining the components of materials in terms of the elements or compound contained. Analytical Chemistry seeks ever improved means of measuring the chemical composition of natural and artificial materials. The techniques of this science are used to identify the substances which may be present in a material and to determine the exact amounts of the identified substances.

Analytical chemistry is important in nearly all aspects of chemistry, for example, agricultural, clinical, environmental, forensic, manufacturing metallurgical and pharmaceutical chemistry. [2] Analytical techniques play an important role in assuring and maintaining the quality of substance and are critical components of Q.A. /Q.C. The reliability, utility, accuracy, interception and specificity of the measurement are the responsibility of an analytical chemist. In general terms pharmaceutical analysis comprises of those procedures which are necessary to determine the identity, strength, quality and purity of drugs and chemicals. [3]

The discipline of analytical chemistry consists:

i) Qualitative Analysis: Qualitative analysis deals with the identification of elements, ions or compounds present in the sample.

ii) Quantitative Analysis: Quantitative analysis deals with the determination of how much amount of one or more constituents are present in the sample. [4]

Importance of Analytical Chemistry:

- Development of theory of analytical method in every possible way.
- Improvement and scientific substantiation of the exiting analytical methods.
- Scientific elaboration of new analytical methods, which meet the requirement of advancing science and modern production.
- Analysis of natural substances, environment and also industrial material.

Selection of Analytical Method: Method should be,

- As simple as possible,
- Most specific,
- Most productive, economical and convenient,
- As accurate and precise as required,
- Should be fully optimized before transfer for validation of its characteristics such as accuracy, precision, sensitivity etc. [5]

Classification of Analytical Methods:

Analytical methods Classified into two categories; Classical methods and Instrumental methods as,

❖ **Classical Methods:** For qualitative analysis the separated compounds are treated with reagents that could be recognized by either colour, by their boiling or melting points, their solubility.

1. **Volumetric Methods:** In volumetric, also called titrimetric, procedures, the volume or mass of a standard reagent required to react completely with the analyte is measured.

2. **Gravimetric Methods:** In gravimetric measurements, the mass of the analyte or some compound produced from the analyte is determined. The extent of their general application is, however, decreasing with the passage of time and with the advent of instrumental methods to supplant them.

❖ **Instrumental Methods:**

These methods are based upon the measurement of some physical properties as conductivity, electrode potential, light absorption or emission, mass-to-charge ratio and fluorescence of substance. There are many techniques available for the analysis of analytes, which can be broadly classified as,

a) Spectroscopic Techniques:

- Ultraviolet and visible spectrophotometer
- Fluorescence and phosphorescence spectrophotometer
- Atomic spectrophotometers (emission & absorption)
- Infra-red spectrophotometer
- Raman spectroscopy
- X-ray spectroscopy
- Radiochemical techniques including activation analysis
- NMR spectroscopy ESR spectroscopy

b) Electrochemical Techniques

- Potentiometry
- Voltametry
- Stripping techniques
- Amperometric techniques
- Coulometry
- Electrogravimetry
- Conductance techniques

c) Chromatographic Techniques

- Gas chromatography (GC)
- High performance liquid chromatography (HPLC)
- High-performance thin layer chromatography (HPTLC)
- Supercritical fluid chromatography (SFC)
- Ultra pressure liquid chromatography (UPLC)

d) Miscellaneous Techniques

- Thermal analysis
- Mass spectrometric
- Kinetic techniques

e) Hyphenated Techniques

- GC-MS
- ICP-MS
- GC-IR
- MS-MS
- CE-MS
- LC-NMR
- LC-MS
- LC-MS-NMR

Amongst all the techniques mentioned above UV-Visible spectrophotometers and High Performance Liquid Chromatography (HPLC) are the most widely used techniques for quantitative analysis of pharmaceutical substances, and are briefly discussed further.

2.0 Chromatographic techniques: [7-8]

Chromatography is separation of a mixture into individual components using a stationary phase and a mobile phase. The stationary phase may be solid or a liquid supported on a solid or gel or may be packed in column. The mobile phase may be gaseous or liquid. Chromatographic separation relies on relative movement of two phases. In chromatography one phase is fixed (stationary phase) and other is mobile (mobile phase) the mobile phase passes over the stationary phase. The separation of component is a result of the differential affinity of the components for the mobile phase and a stationary phase.

In the beginning of 19th century, a Russian scientist Tswett while working on plant extracts encountered colour bands that moved down the column. He coined the name chromatography for the technique. As Tswett had coined the name, which is till date popular and prevalent, he is considered as the father of chromatography. Chromatography as a word stems from the Greek origin Chroma, which means colour, and graphien which means to write. Indeed the greatest advantage of the chromatography method over other analytical procedures is the ability of separating specific analytes, a feature that appeals to all branches of science, and gives that ability to discover and analyse unknown elements and chemical compounds. Chromatography includes group of different methods that allow the separation of complex chemical mixtures. All chromatography techniques consist of two phases such as mobile phase and immiscible stationary phase.

Classification:

1) **Based on the nature of stationary and mobile phase:**

- Gas –solid chromatograph
- Gas- liquid chromatography
- Solid- liquid chromatography e.g. TLC, column chromatography, HPLC
- liquid-liquid chromatography e.g. Paper partition chromatography, column partition chromatography

2) **Based on the principle of separation:**

- Adsorption chromatography
- Partition chromatography

3) **Based on the modes of chromatography:**

- Normal phase chromatography
- Reversed phase chromatography

4) **Other types of chromatography:**

- Ion – exchange chromatography
- Exclusion chromatography

Adsorption chromatography, the analytes interact with solid stationary surface and are displaced with the eluent for active sites on surface. Partition chromatography, result from a thermodynamics distribution between two liquid or liquid like phases on the basis of relative polarities of stationary and mobile phases. Partition chromatography can be divided in to normal phase and reverse phase chromatography. In normal phase chromatography the stationary bed is strongly polar in nature (e.g., silica gel) and the mobile phase is non-polar (such as n-hexane or tetrahydrofuran).

Polar sample are thus retained on the polar surface of the column packing longest than less polar materials while in reversed –phase chromatography the stationary phase is non-polar in nature, while the mobile phase is polar liquid , such as mixtures of water and methanol or acetonitrile. Here the more non-polar the material is, the longer it will be retained.

3.0 Spectrophotometry: [9-11]

Spectrophotometric techniques are the most important instrumental techniques available to the pharmaceutical analyst for estimation of complex mixture of drugs. The basis of all instrumental techniques is that they measure the interaction of electromagnetic radiation with matter in quantized, i.e. specific energy levels. In spectrophotometry absorption of the electromagnetic radiation of definite and narrow wavelength range by molecules, ions and atoms of chemical substance is measured. There are various spectrophotometric techniques available as follows,

- UV-Visible absorption spectrophotometry
- Atomic emission and atomic absorption spectrophotometry
- Spectrofluorimetry
- Infrared spectrophotometry
- Nuclear magnetic resonance spectroscopy
- Mass spectrometry
- Raman spectrometry

The study of spectroscopy can be carried out under following two heads,

a) Atomic spectroscopy: This spectroscopy is concerned with the interaction of electromagnetic radiation with atoms which are commonly in their lowest energy state.

b) Molecular spectroscopy: This spectroscopy deals with the interaction of electromagnetic radiation with molecules. This results in transition between rotational and vibration energy levels in addition to electronic transition.

The fundamental law that governs the quantitative spectrophotometric analysis is the Beer- Lambert's law which states that,'When a beam of monochromatic light is passed through a transparent cell containing a solution of an absorbing substance, reduction of intensity of the light may occurs; the rate of reduction in intensity with the thickness of the medium is proportional to the intensity of the light and the concentration of the absorbing substances'. Mathematically Beer- Lamberts law is expressed as:

$$A = a.b.c$$

Where,

A = absorbance or optical density

a = absorptivity or extinction coefficient

b = path length in cm

c = concentration of solute in solution

4.0 High Performance Liquid Chromatography: [12-17]

HPLC is an analytical process utilizing special instruments designed to separate, quantify and analyse components of chemical mixture. Samples of interest are introduced to a solvent flow path; carried through a column packed with specialized materials for component separation; and component data is obtained through the combination of a detection mechanism coupled with a data recording system. A typical HPLC separation is based on the selective distribution of analytes between a liquid mobile phase and an immiscible stationary phase. The sample is first introduced by means of an injection port into the mobile phase stream that is delivered by a high- pressure pump. Next, the components of this sample mixture are separated on the column, a process monitored with a flow-through detector as the isolated components emerge from the column.

The analysis in HPLC is either in qualitative or quantitative determination of different components present in the sample. The qualitative analysis determines the sample quality and quantitative analysis involves comparison of standard and samples (their area or height). It is based on two requirements they are reproducible chromatogram and linear response of the detector for analytes of interest. In most of the cases HPLC method development is carried out with ultraviolet (UV) detection using either a variable-wavelength (spectrophotometric) or a diode-array detector (DAD). For many samples, good analytical results will be obtained only by careful selection of the wavelength used for detection. DAD permits the acquisition of UV spectra for all sample components during method development. Various methods are used for quantitative analysis in HPLC.

4.1 Types of High Performance Liquid Chromatography:

a) Normal phase HPLC: Normal phase HPLC (NP-HPLC) was the first kind of HPLC chemistry used, and separates analytes based on polarity. This method uses a polar stationary phase and a non-polar mobile phase, and is used when the analyte of interest is fairly polar in nature. Use of more polar solvents in the mobile phase will decrease the retention time of the analytes while more hydrophobic solvents tend to increase retention times.

b) Reversed phase HPLC: RP-HPLC is the choice for the majority of samples. It consists of a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been treated with alkyl dimethyl silylchloride (RMe2SiCl), where R is a straight chain alkyl group such as octadecyl ($C_{18}H_{37}$) or octyl (C_8H_{17}). The retention time is therefore longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily.

c) Isocratic and Gradient HPLC: Two basic elution modes are used in HPLC. The first is called isocratic elution. In this mode, the mobile phase, either a pure solvent or a mixture, remains the same throughout the run. A typical system is outlined in Figure 1.1.

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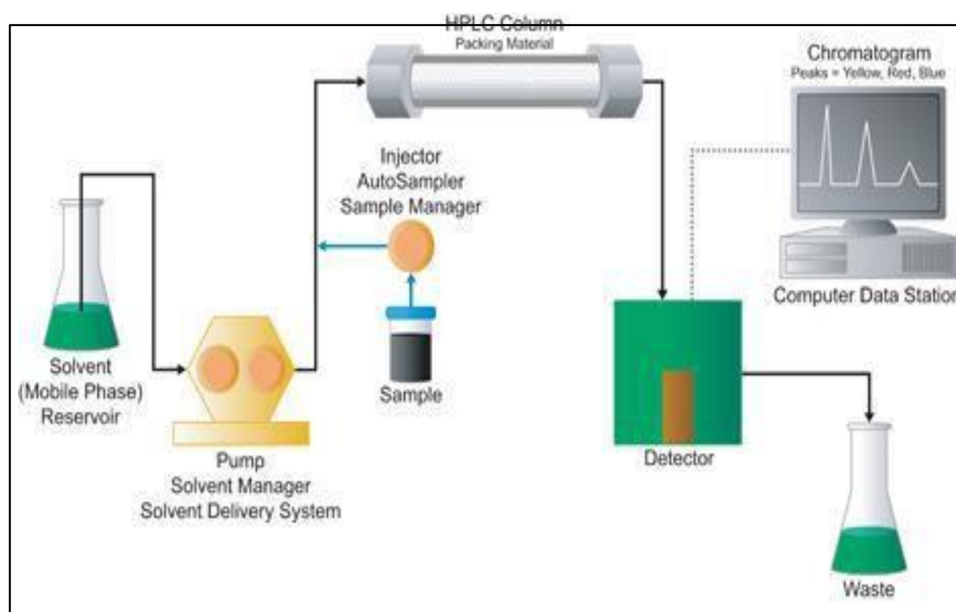

Figure 1.1: Isocratic LC System

The second type is called gradient elution, wherein, as its name implies, the mobile phase composition changes during the separation. This mode is useful for samples that contain compounds that span a wide range of chromatographic polarity. As the separation proceeds, the elution strength of the mobile phase is increased to elute the more strongly retained sample components.

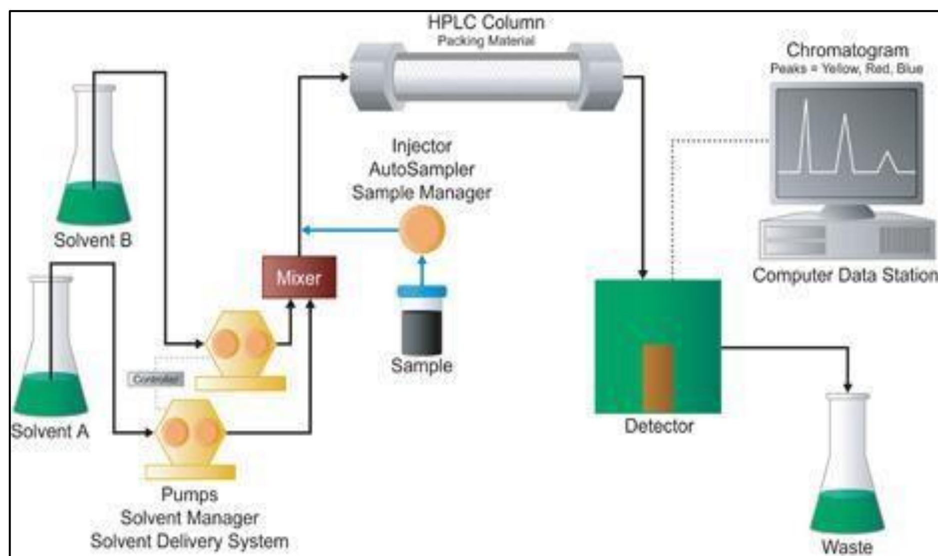

Figure 1.2: Gradient LC System

In the simplest case, shown in Figure 1.2, there are two bottles of solvents and two pumps. The speed of each pump is managed by the gradient controller to deliver more or less of each solvent over the course of the separation. The two streams are combined in the mixer to create the actual mobile phase composition that is delivered to the column over time. At the beginning, the mobile phase contains a higher proportion of the weaker solvent [Solvent A]. Over time, the proportion of the stronger solvent [Solvent B] is increased, according to a predetermined timetable. Note that in Figure1.2, the mixer is downstream of the pumps; thus the gradient is created under high pressure.

4.2 Instrumentation and theory of operation of HPLC:

The technique of HPLC was developed in the late 1960"s. This technique based on same modes of separation as column chromatography, ion exchange chromatography, and gel permeation but differs from column chromatography in that the mobile phase is pumped through column with high pressure.

a) Solvent delivery system: A mobile phase is pumped under pressure from one or several reservoir and flows through the column at a constant rate. For normal phase separation eluting power increases with increasing polarity of the solvent but for reversed phase separation, eluting power decrease with increasing polarity. A degasser is needed to remove dissolved air and other gases from the solvent. Special grades of solvents are available for HPLC and these have been purified carefully in order to remove absorbing impurities and particulate matter to prevent these particles from damaging the pumping or injection system or clogging the column.

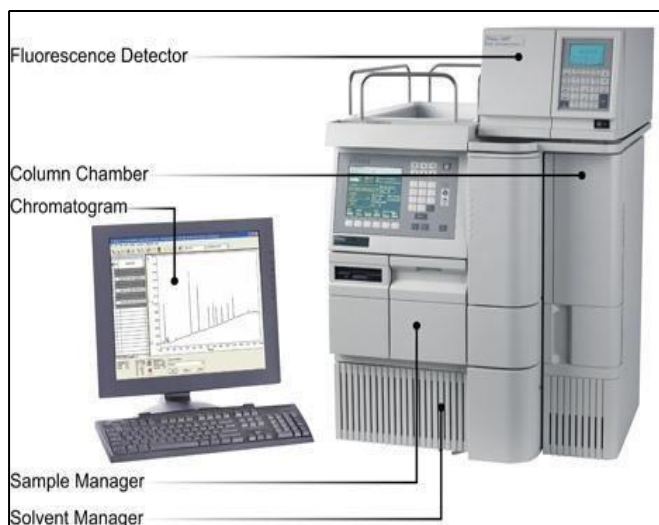

Figure 1.3: Typical HPLC Waters System

b) Pumps:

1. Pumps are required to deliver a constant flow of mobile phase at pressure ranging from 1 to 550 bar (14.6 to 8000psi).

2. Mechanical pump give a pulsating supply of mobile phase.

3. Flow rate range: 1 to 10ml/min.

The pump is one of the most important components of HPLC, since its performance directly affects retention time, reproducibility and detector sensitivity. Three main types of pumps are used in HPLC to propel the liquid mobile phase through the system.

i. Displacement pump: It produces a flow that tends to independent of viscosity and back pressure and also output is pulse free. But it possesses limited capacity (250 ml).

ii. Reciprocating pump: It has small internal volume (35 to 400 µl), their high output pressure (up to 10,000 psi) and their constant flow rates. But it produces a pulsed flow.

iii. Pneumatic or constant pressure pump: They are pulse free; suffer from limited capacity as well as a dependence of flow rate on solvent viscosity and column back pressure. They are limited to pressure less than 2000 psi.

c) Injection systems:

Insertion of the sample onto the pressurized column must be as a narrow plug so that the peak broadening attributable to this step is negligible. The injection system itself should have no dead (void) volume. There are three important ways of introducing the sample into injection port.

i. **Loop injection:** In which, a fixed amount of volume is introduced by making use of fixed volume loop injector.

ii. **Valve injection:** In which, a variable volume is introduced by making use of an injection valve.

iii. **On column injection:** In which, a variable volume is introduced by means of a syringe through a septum.

a) Column:

HPLC column are made up of high quality stainless steel, polished internally to a mirror finish. Standard column are 4-5mm in diameter and 10-30 cm in length.

Different types of column that are used include:

i. **Guard columns:** They are placed anterior to the separating column. They are for protective purpose. They are dependable columns designed to filter or remove particles that clog the separation of the column. These are used in the following cases Compounds and ions that could ultimately cause “baseline drift”, decreased resolution, decreased sensitivity and create false peaks.

ii. **Preparative columns:** These columns are utilized when the objective is to prepare bulk of sample for laboratory applications. Accessories important to mention are the backpressure regulator and the fraction collector. Back- pressure regulator is designed to apply constant pressure to the detector outlet, which prevents the formation of air bubbles within the system. This, in turn, improves chromatographic baseline stability. It is usually designed to operate regardless of flow rate, mobile phase, or viscosity.

iii. **Capillary columns:** These are also known as micro columns, capillary columns have a diameter much less than a millimetre and there are three types: Open-tubular, Partially packed and Tightly packed.

Micro bore and small-bore columns are also used for analytical and small volume assays. A typical diameter for a small bore column is 1-2 mm. However besides the advantage of smaller sample and mobile phase volume, there is a noted increase in mass sensitivity without significant loss in resolution.

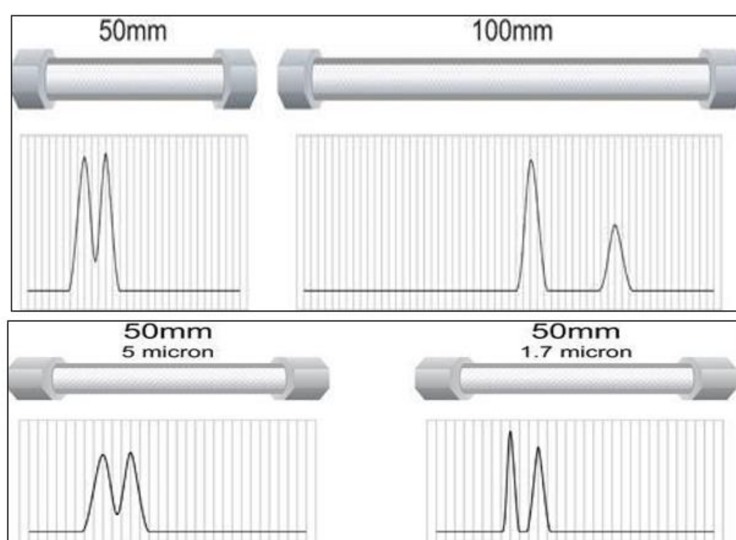

Figure 1.4: Types of Column

b) Detectors:

The detection of the separated compounds in the elute from the column is based upon the bulk property of the elute or the solute property of the individual components. Generally, a detector is selected. That will respond to a particular property of the substance being separated.

i. Bulk property detectors: It compares overall changes in a physical property of the mobile phase with and without an eluting solute. e.g. refractive index, dielectric constant or density.

ii. Solute property detectors: It responds to a physical property of the solute which is not exhibited by the pure mobile phase. e.g. UV absorbance, fluorescence or diffusion current. Such detectors are about 1000 times more sensitive giving a detectable signal for a few nanograms of sample.

c) Recorder:

Recorders are used to record the responses obtained from detector after Amplification.

4.3 Applications of HPLC:

1. Analytical HPLC:

Here the focus is to obtain information about the sample compound, which includes relative comparison, quantification and resolution of a compound.

2. Preparative HPLC:

It refers to the process of isolation and purification of compound. Importance is the degree of solute purity and the throughput, which is the amount of compound, produced per unit time.

3. Identification:

For this purpose a clean peak of known sample assay has to be observed from the chromatogram. Selection of column mobile phase and flow rate matter to certain level in this process by comparing with reference compound does identification and it can be assured by combing two or more detection method.

4. Chemical Separation:

This can be accomplished using HPLC by utilizing the fact that, certain compounds have different migration rates given at a particular column and mobile phase. The extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase.

5. Quantification:

It is the analyte confirmation by using the known reference standards. Quantification of known and unknown areas with respect to the principle peak by various methods like,

- Area normalization method
- Internal standard method
- External standard method

HPLC is used to estimate the concentration of API as well as dosage formulation.

4.4 Advantages of HPLC:

1. Capable of separating complex mixtures at low operating temperature.

2. Identification of an unknown solution.

3. Quantification of a compound in a known solution.

4. Capable of separating materials according to size and /or chemical properties.

5. Can be used to separate delicate or heat labile compound.

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