1. INTRODUCTION

1.1 Introduction to medicinal plants

Medicinal plants have been identified and used throughout human history. Medicinal plants were existing even before human being, made their appearance on the earth\(^1\). Man’s existence on this earth has been made possible only because of the vital role played by plant kingdom in sustaining life\(^2\). Medicinal plants have been regarded as sacred and used by early civilizations to treat sickness and to embellish man’s well being\(^3\). The relationship between man and plants have been close throughout the development of human culture, with the increase in the understanding of human diseases there have been continued interest in the drugs from the plant kingdom\(^4\). Plants have ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions, and to defend against attack from predators such as insects, fungi and herbivorous mammals\(^5\). The history of medicine and surgery dates back in the remote past. In India the earliest records referring to curative properties of certain herbs are contained in Rigveda (3500 – 1800 BC), then came to important works of Charaka and Susruta\(^6\). A new herbal 1551 by William Turner was the earliest English book which gave a truly scientific account of plants\(^7\). It has been estimated that from 25000 to 75000 species of higher plant exists on the earth. A reasonable estimated of about 10% has been used in Traditional System of Medicine (TSM). However perhaps only about 1% of these (250-750 species) are acknowledge through scientific studies to have therapeutic value when used in extract form by human. Natural products have been derived from higher plants, microbes or animals and those can be of terrestrial, marine or aquatic origin. The medicinal preparations based on these raw materials were in the form of crude drug such as dried herbs, or an extract there of and are invariably derived from a mixture of several materials. With the advent of European scientific methods, many of these reputed medicinal plants came under chemical investigation leading to the isolations of active principles. Beginning with 1800 AD there was continuous activity in this area and many of the well known medicinal plants were chemically analyzed and their active principles characterized. Soon after their isolation and characterization these compounds, either in pure state or in the form of well characterizes extracts, became part of pharmacopeias of several countries. This is where herbal medicine and modern medicine have a common link\(^8\). In modern medicine also, plants occupy a very
significant place as raw materials for some important drugs, although synthetic drugs brought about a revolution in controlling different diseases. But these synthetic drugs are out of reach to millions of people those who live in remote places depends on traditional healers, whom they know and trust, judicious use of medicinal herbs can ever cure deadly disease. The Indian system of Medicine is a TSM which encompasses 3 systems namely Ayurveda, Siddha and Unani, practiced by Vaidyas, Siddhars and Hakims respectively. The medicines that come under Ayurveda, Siddha and Unani system of treatment are called as Indian System of Medicines. The drug and Cosmetic Act defines the ISM as “Ayurvedic, Siddha and Unani drug includes all medicines, intended for internal or external use in diagnosis, treatment, mitigation or prevention of disease or disorder in human beings or animals”.

1.1.1 A brief look into the history of herbal medicine.

The Egyptian pharmacopoeia always had a supply of medications of plant and animal, as well as mineral origins. There were 25 types of medicinal plants, as basic nutritional and medicinal plants. The knowledge of Indian physician is documented by the so called Bower manuscript found in 1889 in the ruins of Mingsi in central Asia. In ancient Chinese pharmacology and herbal medicine were most extensive field of medicine, they contained 8160 prescription for the use of various drugs, with instructions on how to use, how to collect and prepare various drugs from the medicinal plants. The main problem in undertaking an extensive herbal drug research programme is the identification and standardization of each and every herbal drug plant and its product says Dr. Shantan Mehrotra Head, Pharmacognosy Division, NBRI. Femen is a non-hormonal herbal formula, associated with dismenorrhoea and pre-menstrual syndromes. Rider is an herbal aphrodisiac which boost body energy level and also relaxes mind. Government of India has also an important role in this. It should set up a special group to explore ways and means of encouraging R & D by Indian Pharma Companies. Hence the research in herbal drugs is the only answer to survival in the stiff competitive world.

1.2 Herbal Medicine

1.2.1 What is herbal medicine?

Herb has various meanings, but in simple terms, it refers to “crude drugs of vegetable origin utilized for the treatment of diseases states, often of a chronic nature, or to
attain or maintain a condition of improved health”. Herbal preparations called ‘Phytopharmaceuticals’, ‘Phytomedicinal’ or ‘Phytomedicine’, are preparations made from different parts of herbs or plants. They come into different formulations and dosage forms including tablets, capsules and elixir, powder, extract, tincture, cream and parental preparations. A single isolated or active principle derived from plants such as digoxin or reserpine tablets is not considered herbal medicine\textsuperscript{15}.

1.2.2 Why herbal medicines?

Herbal medicines are in great demand in the developed as well as developing countries for primary healthcare because of their wide biological activities, higher safety margins and lesser costs. They also offer therapeutics for age-related disorders like memory loss, osteoporosis, immune disorders, etc for which no modern medicine is available. Public, academic and government interest in herbal medicines is growing exponentially due to the increased incidence of the adverse drug reactions and economic burden of the modern system of medicine\textsuperscript{8}.

1.2.3 Popularity of herbal medicine\textsuperscript{16}

The traditional medicine is largely gaining popularity over allopathic medicine because of the following reasons favorable to it,

- Rising cost of medicinal care
- As these are from natural origin, so free from side effects
- Goes to root cause and removes it, so that the disease does not occur again
- Freedom from approaching various specialty
- Cure for many obstinate diseases
- Easy availability of drugs from natural sources.

Steps necessary for promoting herbal medicine\textsuperscript{17}

Phytochemistry or natural product chemistry research is the backbone of herbal industry and directly/indirectly responsible for both failure and success of herbal drugs. For promoting the use of herbals in modern medicine, phytochemistry should be envisaged for,

- Isolation, purification and characterization of new phytoconstituents.
Use of newly isolated phytoconstituent as ‘lead’ compound for the synthetic design of analogues with either improved therapeutic activity or reduced toxicity.

Conservation of lead phytoconstituents into medicinally important drugs.

1.3 Herbal drug standardization

A system to ensure that every packet of medicine that is being sold has the correct substances in the correct amount and will induce its therapeutic effect is known as standardization. In recent years, there has been great demand for plant derived products in developed countries. These products are increasingly being sought out as medicinal products, nutraceuticals and cosmetics. There are around 6000 herbal manufacturers in India. More than 4000 units are producing Ayurveda medicines. Due to lack of infrastructures, skilled manpower, reliable methods and stringent regulatory laws most of these manufacturers produce their product on very tentative basis. In order to have a good co-ordination between the quality of raw materials, in process materials and the final products, it has become essential to develop reliable, specific and sensitive quality control methods using a combination of classical and modern instrumental method of analysis. Standardization is an essential measurement for ensuring the quality of the herbal drugs. ‘Standardization’ expression is used to describe all measures, which are taken during the manufacturing process and quality control leading to reproducible quality. It also means adjusting the herbal drug preparation to a defined content of a constituent or a group of substances with known therapeutic activity respectively by adding excipients or by mixing herbal drugs or herbal drug preparations. ‘Evaluation’ of a drug means confirmation of its identity, determination of its quality, purity and detection of its nature of adulteration. Standardization of herbal drugs is not an easy task as numerous factors influence the bio-efficacy and reproducible therapeutic effect. In order to obtain quality oriented herbal products, care should be taken right from the proper identification of plants, season and area of collection, their extraction, purification process and rationalizing the combination in case of Polyherbal drugs.

1.3.1 Standardization by marker compound

The best tool developed for standardization is by chromatography. It describes botanical identity and chemical sanctity of herb. One of such technique is marker compound testing and finger print analysis. Secondary metabolites present in herb are
considered as marker compounds. Different chromatographic methods are used to analyze the marker compounds in herbs with the help of modern sophisticated tools like HPTLC, HPLC etc\textsuperscript{20}.

1.3.2 Current status of standardization

WHO has emphasized on the need to ensure the quality control of herbs and herbal formulations by using modern techniques. Several pharmacopeias like British Herbal Pharmacopeia, Japanese Pharmacopeia, United States Pharmacopeia, British Herbal Compendium, and German Commission – E etc. lay down monographs for herbs to maintain their quality. Ayurvedic Pharmacopeia of India recommends basic quality parameters for 80 common Ayurvedic herbal drugs. BHP contains 233 monographs and quality control tests, Chinese Herbal Pharmacopeia contain 1751 monographs of substances and articles, BHC contains 84 monographs of medicinal plants. German Commission E has 330 monographs for drug used in German folk medicine.

1.3.3 WHO guidelines for quality standardized herbal formulations\textsuperscript{21,22}

The standardization of crude drug materials includes the following steps.

1.3.3.1 Authentication: Parameters like stage of collection, parts of the plant collected, regional status, botanical identity; phytomorphology, microscopical and histological analysis, taxonomical identity, etc, should be considered for the authentication.

1.3.3.2 Foreign Matter: Herbs collected should be free from insect parts or animal excreta and other contaminants like dust, soil, stones and extraneous matter etc. Foreign matter sometimes also consists of parts of the organ of the plant other than require for drug by definition or beyond limits.

1.3.3.3 Organoleptic evaluation: Sensory characters taste, appearance, odour of the drug; textures etc. are the various organoleptic parameters which should be evaluated first for identification and purity before any further tests are undertaken.

1.3.3.4 Presence of tissues of diagnostic importance: Microscopical identity of medicinal plant material is indispensable for the identification of broken or powdered material. An examination by microscopy alone cannot always provide complete identification, though when used in association with other analytical method.
1.3.3.5 **Ash values**: This method is designed to measure the total amount of material remaining after ignition. It is called total ash. The ash remaining after ignition of plant material is also determined by acid insoluble ash and water soluble ash.

1.3.3.6 **Extractive values**: This method determines the amount of active constituents extracted with different solvents like water, alcohol, ether, chloroform etc. from a given amount of medicinal plant material.

1.3.3.7 **Moisture content and volatile matter**: An excess of water in medicinal plant material will encourage microbial growth there for limits for water content should be set for every given plants. Determination of moisture content or loss on drying helps in the estimation of the amount of volatile matter (i.e. water dries off from the drug). This procedure is more appropriate for substances appearing to contain water as the only volatile constituent.

1.3.3.8 **Chromatographic and Spectroscopic evaluation**: TLC, HPTLC, HPLC methods will provide qualitative and semi quantitative information about the main active constituents present in the crude drug as chemical markers in the TLC fingerprint evaluation of herbals (FEH). The quality of the drug can also be assessed on the basis of the chromatographic fingerprint.

1.3.3.9 **Heavy metals**: Contamination of medicinal plant materials with arsenic and heavy metals can be attributed to many causes including environmental pollution and traces of pesticides.

1.3.3.10 **Pesticide residue**: Pesticides like DDT, BHC, toxaphene and aldrin, mixed with the herbs during the time of cultivation cause serious side effects in human beings. The WHO and Food and Agricultural Organization (FAO) have set limits of pesticides.

1.3.3.11 **Microbial contamination and aflatoxins**: Usually medicinal plants containing bacteria and molds are coming from soil and atmosphere. Analysis of the limits of *E. coli* and molds clearly throws light towards the harvesting and production practices. The substance known as aflatoxins will produce serious side effects if consumed along with the crude drugs. Aflatoxins should not be present or should be completely removed.

1.3.3.12 **Radioactive contamination**: Microbial growth in herbs is usually avoided by irradiation. This process may sterilize the plant material but the radioactivity
hazard should be taken into account. The radioactivity of the plant samples should be checked accordingly to the guidelines of International Atomic Energy (IAE) in Vienna and that of WHO.

1.3.4 Quality control parameters for the herbal formulations

1.3.4.1 Physical parameters: It include color, appearance, odour, clarity, viscosity, moisture content, pH, disintegration time, friability, hardness, flow-ability, flocculation, sedimentation, settling rate and ash values.

1.3.4.2 Chemical parameters: It includes limit tests for heavy metal, extractive values, chemical assays for active constituents etc.

1.3.4.3 Chromatographic analysis of herbals: It can be done using TLC, HPLC, HPTLC, GC, UV, GC-MS and Fluorimetry etc.

1.3.4.4 Microbiological parameters: It includes total viable content, total mold count, total enterobacteria and their count.

1.4 High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) is an outcome of the various theories and instrumentation that were originally advocated for Liquid Chromatography (LC) and Gas Chromatography (GC). By the late 1960’s, analysts used the excellent experience for achieving the goal of superb and quick separations of complex mixtures, with the aid of electronic integrators to get an exact access of areas under elution bands, and above all the ‘computer-printouts’ of the complete analysis. In many favorable instances the smallest possible quantities ranging from nanogram to picogram could be detected with utmost ease and convenience.

1.4.1 Theory

The particle size of the stationary phase material plays vital and crucial role in HPLC. In fact, high-efficiency-stationary-phase materials have been researched and developed exclusively for HPLC having progressively smaller particle size termed as micro particulate column packings. These silica particles are mostly uniform, porous, with spherical or irregular shape, and having diameter ranging from 3.5 to 10μm.

1.4.1.1 Bonded-Phase supports: The bonded-phase supports usually overcome for troubleshooting the problems which is mostly encountered with adsorbed-liquid phases. Here the molecules, comprising the stationary phase, i.e., the surfaces of the
silica particles, are covalently bonded to a silica-based support particle. However, the most popular bonded-phase, siloxanes, is formed by heating the silica particles-in dilute acid for a day or two so as to generate the reactive silonal group. These bonded phases are found to be fairly stable between the pH range 2 to 9 and up to temperatures of about 80°C. The nature of the R group of the silane solely determines the surface polarity of the bonded phase. A fairly common bonded phase is made with a linear C\textsubscript{18} hydrocarbon, also known as ODS (octadecyl silane) bonded phases, wherein the groups appear to be protruding out from the silica particle surface just as the bristles on a toothbrush. It takes care of almost 75% of the samples in HPLC. When such micro particulate bonded phases are packed compactly into a column by means of a suitable device, the small size of these particles offers a significant resistance to solvent flow; therefore, the mobile phase has to be pumped through the column under a high positive pressure. For an analytical HPLC, the mobile-phase is pumped through the column at a flow rate of 1-5cm\textsuperscript{3}/min. At this juncture two varying situations arise; Isocratic elution - i.e., when the composition of the mobile-phase is constant, and Gradient elution-i.e., when the composition of the mobile phase can be made to change in a predetermined fashion during the course of separation.

1.4.1.2 In-line detector: The detector is an electrical signal whose variation is displayed on a potentiometer recorder or a computing integrator or a video-screen. Modern HPLC units are provided with detectors having selective devices thereby categorically restricting the response to all the solutes present in a mixture.

1.4.1.3 Post-Column derivatization: There are certain stubborn and fairly difficult components that are not easily detectable in HPLC. Therefore, such component(s) have to be appropriately converted into their corresponding detectable form once they emerge from the column 26, 27.

1.4.2 Instrumentation\textsuperscript{24,25}

Following diagram showing modern HPLC essentially comprises of the following main components.
1.4.2.1 Solvent reservoir and degassing system

Mobile-phase consisting of a mixture of organic solvents or an aqueous-organic mixture or a buffer solution may be employed depending upon the chromatographic method to be used. Special grades of solvents are commercially available for HPLC that have been adequately refined to eliminate completely the UV – absorbing impurities and any particular matter. Many liquids dissolve appreciable amounts of atmospheric gases e.g., air or suspended air-bubbles that may be a major cause of practical problems in HPLC, specifically affecting the operation of pump and detector. All such problems may be avoided by degassing the mobile phase by subjecting the mobile – phase under vacuum, distillation, spurring with a fine spray of an inert gas of low solubility such as Argon or Helium or by heating and ultrasonic stirring.

1.4.2.2 Pressure, flow and temperature

HPLC columns are packed usually up to 700 times atmospheric pressure and therefore the operating inlet-column pressure in HPLC may be to a maximum of 200 times atmospheric pressure.
The flow can be measured periodically at the column outlet by collecting the liquid for a known period, and thereafter, either measuring the volume or weighing it physically.

In reality, the maintenance of strict ‘temperature control’ plays a vital role in measuring the retention-data correctly and precisely. It makes use of the refractrometer detectors specifically. In HPLC, difficult separations may be achieved by increasing the temperature carefully, but this must be done initially on a hit and trial basis.

1.4.2.3 Pumps and sample injection system

The two major functions of the pump in a modern HPLC are; to pass the mobile-phase through the column at a high pressure and at a constant controlled flow rate. The pump is a very delicate and sensitive part of HPLC unit; therefore, all buffer solutions should be removed carefully after use either by pumping water or an appropriate solvent for several minutes.

In Reciprocating pump the piston is moved in and out of a solvent chamber by an eccentric cam or gear. The forward-stroke closes the inlet-check value while the outlet valve opens and the respective mobile phase is duly pumped into the column. Consequently, the return-stroke-closes the outlet valve and it refills the chamber.

There are three different modes of sample injection system used in HPLC,

1.4.2.3.1 Septum injectors: They usually permit the introduction of the sample by a high pressure syringe through a self-sealing elastometer septum.

1.4.2.3.2 Stop-flow septum less injection: Here, most of the problems associated with septum-injectors have been duly eliminated. The flow of the mobile-phase through the column is stopped for a while, and when the column reaches an ambient pressure the top of the column is opened and the sample introduced at the top of the packing.

1.4.2.3.3 Micro volume sampling valves: Highly sophisticated modern HPLC frequently make use of micro volume sampling valves for injection which not only give fairly good precision, but also are adaptable for automatic injection. These valves enable samples to be introduced reproducibly into pressurized columns without causing the least interruption of the mobile-phase flow.
1.4.2.4 Columns

Following are the various dimensions, fittings and types of packings for HPLC columns:

1.4.2.4.1 Dimensions

Material: Stainless-steel (highly polished surface)

External Diameter: 6.35mm,

Internal Diameter: 4-5mm (usual: 4.6 mm), and

Length: 10-3cm (usual: 25cm).

1.4.2.4.2 Fittings: Each end of the column is adequately fitted with a stainless-steel gauze or frit with a mesh of 2μm or less so as to retain the packing material (usually having a particle diameter 10, 5 and 3μm).

1.4.2.4.3 Types of Packing: Modern HPLC makes use of packing which essentially consist of small and rigid particles with a very narrow particle size distribution. Types of packing are invariably used in HPLC column, namely: styrene-divinylbenzene copolymers, porous-layer beads and porous-silica particles

1.4.2.5 Detectors: Types of detectors used in HPLC are as discussed below.

I. UV-detectors: It is based on the principle of absorption of UV visible light from the effluent emerging out of the column and passed though a photocell placed in the radiation beam.

II. Diode array detector (Or Multichannel detector): It is also a UV detector wherein a polychromatic light is made to pass through the flow cell. A strategically placed grating diffracts the out coming radiation and subsequently meets an array of photodiodes whereby each photodiode receives a different narrow wavelength band. Here, a microprocessor scans the array of diodes several times in one second and the resulting spectrum is visualized on the screen of a VDU or subsequently stored in the instrument for a printout as and when required.

III. Fluorescence detector: A plethora of compounds (solute) present in the mobile-phase on being passed as column effluent through a cell irradiated with Xenon or Deuterium source first absorb UV radiation and subsequently emit
radiation of a longer wavelength in two different manners, namely; instantly-termed as ‘Fluorescence’, and after a time-gap-known as ‘Phosphorescence’.

IV. **Refractive index detectors**: It is also known as ‘RI-Detector’ and ‘Refract meter’. Light from the source is focused into the cell, which consists of sample and reference sample; and the two chambers are separated by a diagonal sheet of glass. After passing through the cell, the light is diverted by a beam-splitter to two photocells. A change in the observed refractive index (RI) of the sample stream causes a difference in their relative output, which is adequately amplified and recorded duly.

V. **Multipurpose detectors**: A multipurpose detector essentially comprises of three detectors combined and housed together in a single unit.
   a. **Fluorescence function**: It can monitor emission above 280nm, based on excitation at 254nm,
   b. **UV-Function**: It is fixed wavelength 254nm detector, and
   c. **Conductance-function**: The metal inlet and outlet tubes serve as electrodes to measure the conductance of the ions.

VI. **Electrochemical detectors**: In actual practice, however, it is rather difficult to utilize the functions of electrochemical reduction as a means of detection of HPLC by virtue of the fact that the serious interference (i.e., large background current) generated by reduction of oxygen in the mobile phase. As complete removal of oxygen is almost difficult, therefore, electrochemical detection is normally based upon the oxidation of the solute.

1.4.2.6 **Strip chart recorder**: The signal emerging from the detector of a HPLC is recorded continuously as function of time most commonly with the help of a potentiometric recorder

1.4.2.7 **Data handling device and microprocessor control**: Modern HPLC is adequately provided with complete data handling devices. Thousands of samples routinely analyzed in Quality Assurance laboratories in Pharmaceutical Industries / Bulk Drug Industries etc. are duly processed and the data stored in the computerized data-handling devices.
1.4.3 Classification of HPLC\textsuperscript{26,27}

1.4.3.1 Classification according to stationary phase

I. **Normal phase mode:** In the normal phase mode, the stationary phase is polar and the mobile phase is non-polar. Non-polar compounds travel faster and are eluted first. This is because of the lower affinity between the non-polar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore, take more times to elute. Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute.

II. **Reversed phase mode:** Reversed phase mode is the most popular for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. The stationary phase is non-polar hydrophobic packing with octyl or octadecyl functional group bonded to silica gel and the mobile phase is polar solvent. An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pair and complexation) to control retention and selectivity. The polar compound gets eluted first in this mode and non-polar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are octadecylsilane (ODS) or C\textsubscript{18}, C\textsubscript{8}, C\textsubscript{4}, etc., (in the order of increasing polarity of the stationary phase).

1.4.3.2 Classification according to principle\textsuperscript{27,28}

I. **Ion exchange chromatography:** Here, the stationary phase contains ionic groups like NR\textsubscript{3}\textsuperscript{+} or SO\textsubscript{3}\textsuperscript{−}, which interact with the ionic groups of the sample molecules. This is suitable for the separation of charged molecules only. Changing the pH and salt concentration can modulate the retention.

II. **Ion pair chromatography:** It may be used for the separation of ionic compounds and this method can also substitute for ion exchange chromatography. Strong acidic and basic compounds may be separated by reversed phase mode by forming ion pairs (columbic association species...
formed between two ions of opposite electric charge) with suitable counter ions. This technique is referred to as reversed phase ion pair chromatography or soap chromatography.

III. **Affinity chromatography**: It uses highly specific biochemical interactions for separation. The stationary phase contains specific groups of molecules which can adsorb the sample, if certain stearic and charge related conditions are satisfied. This technique can be used to isolate proteins, enzymes as well as antibodies from complex mixtures.

IV. **Size exclusion chromatography**: It separates molecules according to their molecular mass. Largest molecules are eluted first and the smallest molecules last. This method is generally used when a mixture contains compounds with a molecular mass difference of at least 10%. This mode can be further subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solvents).

The efficiency of HPLC column is check using following parameters.

I. **Capacity Factor (k’)**: It measures the degree of retention of an analyte relative to an unretained peak and express by following equation.

\[
K' = \frac{(t_R - t_0)}{t_0}
\]

Where,

- \(t_R\), the retention time for the sample peak and
- \(t_0\), the retention time for an unretained peak.

The peak should be well-resolved from other peaks and the void volume, generally \(k' > 2.0\)

II. **Backpressure**: It is the pressure required to pump the mobile phase through the column and calculated by the following equation

\[
\Delta P \propto FL\eta / dp^2 dc^2
\]

Where,

- \(\eta\), is the viscosity of mobile phase,
- \(F\), is flow rate
- \(L\), column length
- \(d_c\), diameter and
- \(d_p\), particle size
III. Resolution ($R_s$): It is the ability of a column to separate chromatographic peaks. It can be improved by increasing column length, decreasing particle size, increasing temperature, changing the eluent or stationary phase. It can also be expressed in terms of the separation of the apex of two peaks divided by the tangential width average of the peaks.

\[
R_s = \frac{\Delta t_R}{0.5(W_1 + W_2)};
\]

Where,
\[
W_1 = \text{Peak width of first peak}; \quad W_2 = \text{Peak width of second peak}
\]
\[
t_1 = \text{Retention time of first peak}; \quad t_2 = \text{Retention time of second peak}
\]
\[
\Delta t_R = t_2 - t_1
\]

![Figure 1.2 Resolution](image)

For reliable quantitation, well-separated peaks are essential for quantitation: $R_s > 2$; between the peak of interest and the closest potential interfering peak (impurity, excipient, degradation product, internal standard, etc.) are desirable.

IV. Tailing factor ($T$): It is the measure of the symmetry of a peak, given by the following equation,

\[
T = \frac{W_{0.05}}{2f}
\]

Where,
\[
W_{0.05} \text{ is the peak width at 5\% height and}
\]
\[
f \text{ is the distance from peak front to apex point at 5\% height.}
\]
Ideally, peaks should be totally symmetrical. The accuracy of quantitation decreases with increase in peak tailing because of the difficulties encountered by the integrator in determining where/when the peak ends and hence the calculation of the area under the peak. Integrator variables are preset by the analyst for optimum calculation of the area for the peak of interest.

Recommendations: T of $\leq 2$

V. **Theoretical plate number / Efficiency (N):** It is a measure of column efficiency, that is, how many peaks can be located per unit run-time of the chromatogram. It depends on elution time but in general should be $> 2000$.

1.4.4 **Strategy for method development in HPLC**

For selection of suitable chromatography method for organic compounds, first Reversed Phase should be tried, if not successful than Normal Phase. Following parameters are helpful for HPLC method development using Reverse Phase. Detector can be chosen on the basis of analyte structure and its solubility. If the analytes are UV active, use UV/RID/LSD. While selecting wavelength using PDA detector, avoid matrix interference based on wavelength maxima and UV cut off of mobile phase. If the analytes are not UV active then use derivatization method. Column selection starts with C$_{18}$ inertsil and short column (100mm or 150mm). Always use buffer or modifier if the analytes are ionisable, because ionisable compounds will show two peaks as it exists in two forms. So if the mobile phase is buffered into basic or acidic then analyte will go to single form. The mobile phase composition can be started with the use of water and methanol or acetonitrile according to solubility in 50: 50 ratios. Along with the selection of mobile phase other parameters like wavelength, ambient temperature and flow rate are also considered for the strategy of method development. For preparation of sample use concentration between 100-500 ppm as per peak response. Keep the peak response below 1000mV = 1V or 1mAU=1AU to keep the UV detector linearity.

1.4.5 **Steps for method development**

Documentation is the first step of the development process. All data relating to the study must be recorded in laboratory notebook or an electronic database.

1.4.5.1 **Analyte standard characterization:** Information about physical and chemical properties, toxicity, purity, hygroscopic nature, solubility, stability and
structure of the analyte is collected. The standard analyte of 100% purity is obtained and properly stored in refrigerator/desiccators/freezer. In case of multiple analyte in the sample matrix, the number of components is noted, data is assembled and the availability of standards for each one is determined. Consider only those methods (MS, GC, HPLC etc.,) that are compatible with sample stability.

1.4.5.2 Method requirements: The goals or requirements of the analytical method are considered and the analytical figures of merit like detection limits, selectivity, linearity, range, accuracy and precision should be defined.

1.4.5.3 Literature search and prior methodology: The literature related to the physical and chemical properties, solubility and relevant analytical methods of analyte are surveyed from books, periodicals, chemical manufacturers and regulatory agency compendia such as USP / NF, AOAC and ASTM publications, Chemical Abstracts Service (CAS) and automated computerized literature searches.

1.4.5.4 Choosing a method: Using the information in the literatures and prints, methodology is modified wherever necessary. Sometimes it is necessary to acquire additional instrumentation to reproduce, modify, improve or validate existing methods for in-house analytes and samples.

If there are no prior methods for the analyte in the literature, from analogy, the compounds that are similar in structure and chemical properties are investigated and are worked out. There is usually one compound for which analytical method already exist that is similar to the analyte of interest.

1.4.5.5 Instrumental setup and initial studies: Installation, operational and performance qualification of instrumentation are setup using laboratory standard operating procedures (SOP’s). Always new consumables e.g. solvents, filters and gases are used. Method development is never started, on a HPLC column that has been used earlier. The analyte standard in a suitable injection solution, in known concentrations and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix. If the sample is extremely close to the standard (e.g., bulk drug), then it is possible to start work with the actual sample. Analysis is done using analytical conditions described in the existing literature.
1.4.5.6 **Optimization**: During optimization one parameter is changed at a time, and set of conditions are isolated, rather than using a trial and error approach. Work has been done from an organized methodical plan, and every step is documented in a lab notebook.

1.4.5.7 **Documentation of analytical figures of merit**: The originally determined analytical figures of merit limit of quantitation (LOQ), Limit of detection (LOD), linearity, time per analysis, cost, sample preparation etc., are documented.

1.4.5.8 **Evaluation of method development with actual samples**: The sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all other matrix components.

1.4.5.9 **Percent recovery**: Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery (average +/- standard deviation) from sample to sample and whether recovery has been optimized has been shown. It is not necessary to obtain 100% recovery as long as the results are reproducible and known with a high degree of certainty.

The validity of analytical method can be verified only by laboratory studies. Therefore documentation of the successful completion of such studies is a basic requirement for determining whether a method is suitable for its intended applications.

1.4.5.10 **Statistical analysis and representative calculations**: The consistency and suitability of the developed method are substantiated through the statistical analysis like standard deviation, relative standard deviation and theoretical plates per meter.

**For Accuracy**: Standard deviation \( \sigma = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}} \)

Where;
- \( x \) is sample,
- \( x_i \) is mean value of samples,
- \( n \) is number of samples

**Relative Standard Deviation** = \( \frac{\sigma}{x_i} \times 100 \)

**Molar extinction coefficient (mol\(^{-1}\) cm\(^{-1}\))** = \( \frac{A \times L}{c} \)
Where;

\[ A \text{ is Absorbance of drug, } \quad C \text{ is concentration of drug, } \]
\[ L \text{ is Path length} \]

Sandell’s sensitivity \((\mu g/cm^2/0.001 \text{ absorbance units}) = \frac{C}{A} \times 0.001\)

Where;

\[ C \text{ is concentration of drug,} \]
\[ A \text{ is Absorbance of drug} \]

1.4.5.11 Eluent Choice: Acetonitrile is the preferred organic solvent because of low viscosity and high UV transparency. Aqueous eluents are selected on the basis of nature of compounds; water for neutral compounds, 10mM \(H_3PO_4\) pH 2.3 for weak to medium acids (partly ion suppression), and 5mM phosphate buffer pH 7.5 for weak to medium bases or acids in ionization form. Unknown samples are analyzed first with water, then with an acid and a neutral buffer, acid and basic compounds can be recognized by change of retention time.

1.4.6 Detection: Universal detection is possible at 210nm. Before analyzing an unknown sample, the performance of the column and the instrument is tested with a column test mixture.

Developed method can be further optimized by, shorter analysis time, change to an isocratic method, shorter column if enough resolution was obtained, better resolution, longer column, use of sorbent with smaller particles (4µm, 3µm), better selectivity, other stationary phase, e.g. Phenyl, CN, Polymer columns, pH control with ion forming compounds, use of methanol or THF instead of acetonitrile, better sensitivity and detection at the absorption maximum of the substance. Gradient elution, small particles and micro bore columns are the factors lead to narrower and higher peaks.

1.4.7 Selection of Mobile Phase\(^{28,29}\)

In reverse phase HPLC, the stationary phase is an adsorbent packed into a column and the mobile phase is composed of water or organic solvents plus other additives. The mobile phase is continuously pumped at a fixed flow rate through the system after mixing (if required) by the pump. Dipole moment (polarity), acidity (proton donor), basicity (proton acceptor) are the fundamental properties of an organic solvent which affects chromatographic selectivity.

The solvents commonly used in reverse phase HPLC whose covalent properties differ
significantly are methanol, acetonitrile and tetrahydrofuran.

![Figure 1.3 Relevant Solvent properties](image)

The diagram shows that tetrahydrofuran (THF) presents the greatest dipole interaction followed by acetonitrile and then methanol. THF is often considered the strongest of the organic modifiers and results in the largest changes in retention per percentage change in modifier concentration. Methanol is the most basic of the three solvents while acetonitrile is the most acidic. When changing from one solvent to another it is more convenient to use the information from a nomogram that compares the relative elution strengths of each solvent. In this way an equivalent mobile phase composition can be chosen that has same eluting power as the original i.e. overall separation will occur in the same time frame but with differing selectivity. In such cases the phases being compared are said to be isoeluotropic. This means that the elution time of the last compound will be approximately the same, whilst the selectivity between peaks within the chromatogram may be significantly different. In this way the separation conditions may be optimized, by altering the mobile phase composition, in order to achieve the desired resolution between all analytes.

1.4.7.1 Solvent miscibility

For analytes employing a variety of modes of chromatography (Normal phase, Reverse phase, Ion-pair, Ion exchange chromatography etc.), there are various factors that need to be considered when choosing a solvent for use in a particular analysis. The most important consideration from a practical perspective is that of solvent
miscibility. That is does the solvent mix well without precipitation or the formation of immiscible emulsions. If immiscible solvents are used, mixing can become non-uniform and the formation of an accurate and reproducible mobile phase composition is impossible. An unstable baseline may result due to cavitations in the mixer or pump chamber or flow cell. Ultimately, de-wetting can occur in which traces of an immiscible solvent coat the internal components of the HPLC instrument leading to problems with mobile phase formation and solubility issues with buffers and sample components.

1.4.7.2 UV cut off of solvents\textsuperscript{30,31}

UV detectors are commonly used in HPLC analysis. The UV absorbance of an analyte containing a chromophore is plotted against time. The higher the UV absorbance the higher the concentration of species present at that time. When using UV detection it is necessary to select a solvent that has no significant UV absorption at the wavelength at which measurements are to be taken. Using a solvent with high UV cut-off at the selected wavelength can result in increased noise level and a loss in sensitivity, therefore UV cut-off must be considered. A table of UV cut-off points for typical HPLC solvents is shown below. Where possible a solvent with a UV cut-off of 20nm below the wavelength selected for the analysis should be used.

**Table 1.1 UV cut-off points for some common HPLC solvents**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>UV cut off (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>190</td>
</tr>
<tr>
<td>Water</td>
<td>190</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>195</td>
</tr>
<tr>
<td>Hexane</td>
<td>200</td>
</tr>
<tr>
<td>Methanol</td>
<td>210</td>
</tr>
<tr>
<td>Ethanol</td>
<td>210</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>220</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>220</td>
</tr>
<tr>
<td>Chloroform</td>
<td>240</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>265</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>210, 220</td>
</tr>
<tr>
<td>Toluene</td>
<td>285</td>
</tr>
</tbody>
</table>
1.4.7.3 Purity of solvents\textsuperscript{30, 31}

The purity of the solvent is important as it can affect the sensitivity of the analysis. An impure solvent can cause an increased level of baseline noise, as well as ghost peaks. It is recommended that HPLC grade or better solvents are used. Gradient grade solvents are guaranteed not produce ghost peaks when using UV detection.

1.5 Validation Parameters\textsuperscript{30}

Analytical monitoring of a pharmaceutical product or of specific ingredients within the product is necessary to ensure its safety and efficacy throughout all phases of its shelf life. Such monitoring is in accordance with the specifications elaborated during product development. Analytical validation is the corner stone of process validation without a proven measurement system it is impossible to confirm whether the manufacturing process has done what it purports to do. All new methods developed are validated.

Validation is an act of proving that any procedure, process, equipment, material, activity or system performs as expected under given set of conditions and also give the required accuracy, precision, sensitivity, ruggedness, etc. When extended to an analytical procedure, depending upon the application, it means that a method works reproducibly, when carried out by same or different persons, in same or different laboratories, using different reagents, different equipments, etc.

The ICH documents give guidance on the necessity for revalidation in changes in the analytical procedures, changes in the synthesis of the drug substances and changes in the composition of the drug product.

The various validation parameters are;

1.5.1 Accuracy: It is defined as the closeness of the test results obtained by the method to the true value. It is expressed as percent recovery by the assay of a known amount of analyte added. It is determined by applying the method of samples or mixtures of excipients to which known amount of analyte is added above and below the normal levels expected in the samples. It is then calculated from the test results as the percentage of the analyte recovered by the assay.

1.5.2 Precision: It is a measure of degree of reproducibility of the analytical method. It provides an indication of random error. It is expressed as standard deviation (SD) or relative standard deviation (RSD).
1.5.2.1 **Interday precision**: Variation of results at different day is called interday variation.

1.5.2.2 **Intraday precision**: Variation of results within the same day is called intraday variation.

1.5.3 **Linearity and range**: Linearity is the ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional to the concentration of an analyte in sample within a given range. Range of analytical method is the interval between the upper and lower limit of analyte. The results are expressed in terms of correlation coefficient of the linear regression analysis.

1.5.4 **Limit of detection (LOD)**: It is the lowest level of analyte that can be detected, but cannot be determined in a quantitative fashion using a specific method under the required condition. The determination of limit of detection of instrumental procedure is carried out by determining signal to noise ratio by comparing test results from the samples. The signal to noise ratio is determined by dividing the base peak by standard deviation of all data points below a threshold. LOD is calculated by taking the concentration of the peak of interest divided by three times the signal to noise ratio.

1.5.5 **Limit of quantitation (LOQ)**: It is a parameter of quantitative assays for low levels of compounds in sample matrices such as impurities in bulk drugs and degradation products in finished pharmaceuticals. It is the lowest concentration of analyte in a sample that may be determined with acceptable accuracy and precision when required procedure is applied. Numbers of blank samples are analyzed; standard deviation is calculated and multiplied by factor 10.

1.5.6 **Selectivity and Specificity**: If an analytical procedure is able to separate and resolve the various components of a mixture and detect the analyte quantitatively then the method is called selective. If the method determines or measures quantitatively the component of interest in the sample matrix without separation then the method is called specific.

1.5.7 **Robustness**: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. It provides an indication of the reliability of procedure during normal usage. For the determination of robustness, a number of method parameters, such as
pH, flow rate, column temperature, injection volume, detection wavelength or mobile phase composition, are varied within a realistic range, and the quantitative influence of the variables is determined. If the influence of the parameter is within a previously specified tolerance, the parameter is said to be within the method’s robustness range.

1.5.8 Ruggedness: Ruggedness is a measure of the reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst. It is the degree of reproducibility of results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental conditions, operators and materials. It is determined by the analysis of aliquots from homogeneous lots in different laboratories.
Reference

27. www.researchgate.net/how_can_we_develop_HPLC_method_for_unknow_same.