1. SCOPE OF DRUG ANALYSIS

A drug may be defined as a substance meant for diagnosis, cure, mitigation and prevention, treatment of diseases in human beings or animals, for altering in structure or function of the body of human beings or animals [1]. Pharmaceutical chemistry [2-6] is a science that makes use of general laws of chemistry to study drugs i.e. their preparation, chemical nature, composition, structure, influence on an organism, the methods of quality control and the conditions of their storage etc. The family of drugs may be broadly classified as:

1. Pharmacodynamic agents

2. Chemotherapeutic agents

Pharmacodynamic agents refer to a group of drugs, which stimulate or depress various functions of body so as to provide some relief to the body in case of body abnormalities, without curing the disease. They are mainly used in case of non-infectious diseases; so as to correct the abnormal body functions. Non-selective central nervous system modifiers (depressants or stimulants), adrenergic stimulants and blocking agents, cholinergic and cholinergic blocking agents, cardiovascular agents, diuretics, antihistaminic agents and anticoagulating agents are some examples of this group. These agents have no action on infective organisms, which cause various diseases.

Chemotherapeutic agents are agents, which are selectively more toxic to the invading organisms without harmful effect to the host. Some of the examples of this group are organometallic agents, antimalarials, antibacterials, antiprotozoals,
antifungal agents, anthelmentics, antiseptics, antitubercular agents, antineoplastics, etc.

Every country has legislation [7] on bulk drugs and their pharmaceutical formulations that sets standards and obligatory quality indices for them. These regulations are presented in separate articles relating to individual drugs and are published in the form of book called “Pharmacopoeia” such as I.P. [8], U.S.P. [9], B.P. [10] and Martindale: The Extra Pharmacopoeia [11].

Pharmaceutical analysis [12] deals not only with medicaments (drugs and their formulations) but also with their precursors i.e. with the raw material on which degree of purity and the quality of medicament depends. The quality of a drug is determined after establishing its authenticity by testing its purity and the quality of pure substance in the drug and its formulations.

Quality [13] is important in every product or service but it is vital in medicine as it involves life. Unlike ordinary consumer goods there can be no “second quality” in drugs. Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production.

As a matter of fact, it is built in from the time of inception of the thought to make a product, to the time; it is finally made and sent out with an okay quality report. In popular practice, the quality of medicines or pharmaceutical products is assured through quality control. It is therefore, essential that quality assurance department must adopt “good laboratory practice” to ensure reliability and accuracy
of results given out by them. The assurance of the quality and reliability of pharmaceuticals, together with their careful control are our moral obligations towards the sick human beings. Consequently the manufacture and control of drugs are very responsible task and they need substantial knowledge of the science.

The decision to release or reject a product is based upon one or two types of control actions or combination of both. If the product is single entity of high purity, the analytical data is the basis for the decision but most of time; the formulation is a physical mixture of several potent drugs. With the growth of pharmaceutical industry during the last several years, there has been a rapid progress in the field of pharmaceutical analysis involving complex instrumentation, providing simple analytical procedures for complex formulations is a matter of foremost importance.

Some of medicinal products are still being assayed by the time-tested procedures of gravimetric and titrimetric techniques, though use of electronic balances and recording titrators have improved these classical procedures considerably. A wide diversity in type of analytical technique has been characteristic of assay method for pharmaceuticals. Simple distillation is useful for determining alcohol contents of the galenicals, or other substances being volatile in current of steam such as menthol, thymol, and even certain alkaloids such as ephedrine. Moisture contents have been determined by drying in a desiccator or in a heated oven. Use of moisture balance in which sample pan is directly heated by infrared lamp without removing the sample from the balance has been an innovation, though the most specific and convenient procedure being Karl-Fisher titration. Separation
techniques, particularly chromatographic methods, are valuable in analysis of pharmaceuticals. Modern spectrophotometer which incorporates features such as microprocessor control, diode array detector has become essential tools for analysis. Assay methods based on absorption in the ultraviolet and visible region of electromagnetic spectrum are used extensively. Some colorless substances required to be analysed are converted to a derivative having color, the intensity of color measured at suitable wavelength and compared with that of known amount of reference substance of known purity. The fluorimeter measures fluorescence that may be present in the sample such as riboflavin or may be developed into the sample such as thiamine hydrochloride. Solvents used for dilution for UV-visible spectrophotometric assay require special purification different from the requirement for other uses. It is preferable that blanks are run on the solvent and reagents used to obtain a correction for their inherent absorbances.

The methods of estimation of drugs are divided into physical, chemical, physico-chemical and biological ones. Physico-chemical and physical methods are used the most. Physical methods of analysis involve the study of the physical properties of the substance. They include determination of solubility, transparency or degree of turbidity, color, density, specific gravity (for liquids), moisture content, melting, freezing and boiling points. Physico-chemical methods [14, 15] are used to study the physical phenomena that occur as a result of chemical reactions. Among the physico-chemical methods, the most important are optical (refractometry, polarimetry, emission and fluorescence methods of analysis, photometry including
photocolorimetry and spectrophotometry covering UV, visible and IR regions, nephelometry or turbidimetry) and chromatographic (column, paper, thin-layer, gas liquid [16], HPLC [17]) methods. Methods such as Nuclear Magnetic Resonance (NMR) and Para Magnetic Resonance (PMR) are becoming more and more popular. The combination of mass spectroscopy with gas chromatography is one of the most powerful tools available. The chemical methods include the gravimetric and volumetric procedures, which are based on complex formation, acid base, precipitation and redox reactions. Titrations in non-aqueous media and complexometric have also been used in pharmaceutical analysis.

The complete analysis of a substance consists of 5 main steps.

1. Sample preparation / Sampling

2. Dissolution of the sample, conversion of the analyte into a form suitable for measurement.

3. Measurement

4. Calculation and interpretation of the measurement

In the development of organic therapeutic agents, pharmaceutical scientists have explored numerous approaches to find and develop organic compounds that are now available in pharmaceutical formulations suitable for the treatment of diseases and often for the maintenance of human health. It is well to remember that the chemical, physical, conformational and biochemical properties of organic compounds are functions of their structures. Some times the activity of the drug is dependent chiefly on its physical and chemical properties, whereas in other instances
the arrangement, the position and size of the groups in a given molecule are important and lead to a high degree of specificity. The word ‘quality’ with reference to formulation is comprehensive and refers to characteristics like the potency, uniformity, purity, pharmacological actions, stability, etc. It is not only the moral responsibility of manufacturers to produce effective, safe and non-toxic forms but also their legal responsibility.

The following sampling techniques are generally adopted for preparing sample solutions of different pharmaceutical formulations.

**Sampling techniques:**

Due to the great variability of formulation, which is to be assayed, skilful sampling in drug analysis is very essential. The extent of variation depends upon the product and the manner of its selection. Usually the following methods are adopted for sampling of pharmaceutical formulations.

**Liquids**

These are mixed thoroughly several times by inverting the solution. If any sediment remains behind, it should disperse in the liquid before it consider as a sample for analysis.

**Powders**

They are thoroughly mixed before a portion of the sample is taken for analysis.

**Tablet**

Tablets are thoroughly mixed before a portion of the sample is taken for analysis.
Capsules

About ten capsules are weighed accurately. They are opened with a razor blade and the contents are emptied into a small beaker and mixed thoroughly. In the case of dry filled capsules, the adhering powder to the shells is cleaned with absorbent cotton.

One of the major decisions to be made by an analyst is the choice of the most effective procedure for a given analysis. For this, he must be familiar with the practical details, the theoretical principles and also that he must be conversant with the conditions under which each method is reliable, aware of possible interferences which may arise and capable of minimizing or circumventing such problems. He must also be concerned with question regarding accuracy and precision. In addition he must not overlook factors such as time and costing.

Important factors, which must be taken into account when selecting an appropriate method of analysis, are

a. Nature of the information sought.

b. Size of sample available and the proportion of the constituent sought.

c. The purpose for which the analytical data are required.

Different types of chemical analysis:

1. Proximate analysis - The amount of each element in a sample is determined with no concern as to the actual compounds present.

2. Partial Analysis - It deals with the determination of selected constituents in the sample.
3. Trace constituent analysis – It concerns with the determination of specified compounds present in minute quantity.

4. Complete analysis – In this, a proportion of each component of the sample is determined.

Factors affecting the choice of analytical methods

a. The type of analysis required.

b. Problem arising from the nature of the material.

c. Possible interference from components of the material other than those of interest.

d. The concentration range, which needs to be investigated.

e. The accuracy required.

f. The facilities available.

g. The time required for complete analysis.

h. Similar type of analysis performed.

2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The term ‘Chromatography’ covers those processes aimed at the separation of the various species of a mixture on the basis of their distribution characteristics between a stationary and a mobile phase.

Chromatographic methods can be classified most practically according to the stationary and mobile phases, as shown in the Table.1.1.
Table.1.1: Classification of Chromatographic methods

<table>
<thead>
<tr>
<th>Stationary phase</th>
<th>Mobile phase</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid</td>
<td>Liquid</td>
<td>Adsorption column, Thin-layer, Ion exchange chromatography, etc.</td>
</tr>
<tr>
<td></td>
<td>Gas</td>
<td>Gas -Solid chromatography</td>
</tr>
<tr>
<td>Liquid</td>
<td>Liquid</td>
<td>Partition column, Thin-layer, HPLC, Paper chromatography, etc.</td>
</tr>
<tr>
<td></td>
<td>Gas</td>
<td>Gas – Liquid Chromatography.</td>
</tr>
</tbody>
</table>

The importance of Chromatography is increasing rapidly in pharmaceutical analysis. The exact differentiation, selective identification and quantitative determination of structurally closely related compounds are possible with chromatography. Another important field of application of chromatographic methods is the purity testing of final products and intermediates (detection of decomposition products and by-products). As a consequence of the above points, chromatographic methods are occupying an ever-expanding position in the latest editions of the pharmacopoeias and other testing standards.

The modern form of column chromatography has been called high performance, high pressure, high-resolution and high-speed liquid chromatography.
HPLC is a special branch of column chromatography in which the mobile phase is forced through the column at high speed. As a result, the analysis time is reduced by 1-2 orders of magnitude relative to classical column chromatography and the use of much smaller particles of the adsorbent or support make it possible to increase the column efficiency substantially.

The essential equipment consists of an eluent reservoir, a high-pressure pump, an injector for introducing the sample, a column containing the stationary phase, a detector and recorder. The development of highly efficient micro particulate bonded phases has increased the versatility of the technique and has greatly improved the analysis of multi component mixtures.

The systems used are often described as belonging to one of four mechanistic types such as adsorption, partition, ion exchange and size-exclusion. Adsorption chromatography arises from interaction between solutes on the surface of the solid stationary phase. Partition chromatography involves a liquid stationary phase, which is immiscible with the eluent and coated on an inert support. Adsorption and partition systems can be normal phase (stationary phase more polar than eluent) or reversed phase (stationary phase less polar than eluent). Ion-exchange chromatography involves a solid stationary phase with anionic or cationic groups on the surface to which solute molecules of opposite charge are attracted. Size-exclusion chromatography involves a solid stationary phase with controlled pore size. Solutes
are separated according to their molecular size, the large molecules unable to enter the pores eluting first.

![Typical High performance liquid chromatography system](image)

**Figure 1.1:** Typical High performance liquid chromatography system

**Solvent delivery system**

The mobile phase is pumped under pressure 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. Optimum separating conditions can be achieved by making use of mixture of two solvents. Some other
properties of the solvents, which need to be considered for a successful separation, are boiling point, viscosity, detector compatibility, flammability and toxicity.

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. 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.

**Solvent degassing system**

The constituents of the mobile phase should be degassed and filtered before use. Several methods are employed to remove the dissolved gases in the mobile phase. They include heating and stirring, vacuum degassing with an aspirator, filtration through 0.45μ filter, vacuum degassing with an air-soluble membrane, helium purging, ultra sonication or purging or combination of these methods. HPLC systems are also provided with an online degassing system, which continuously removes the dissolved gases from the mobile phase.

**Gradient elution devices**

HPLC columns may be run isocratically, i.e., with constant eluent or they may be run in the gradient elution mode in which the mobile phase composition varies during run. Gradient elution is a means of overcoming the problem of dealing with a complex mixture of solutes.
**Sample introduction systems**

Two means for analyte introduction on the column are injection into a flowing stream and a stop flow injection. These techniques can be used with a syringe or an injection valve. Automatic injector is a microprocessor-controlled version of the manual universal injector. Usually, up to 100 samples can be loaded into the auto injector tray. The system parameters such as flow rates, gradient run time, volume to be injected, etc. are chosen, stored in memory and sequentially executed on consecutive injections.

**Liquid chromatographic detectors**

The function of the detector in HPLC is to monitor the mobile phase as it emerges from the column. Generally, there are two types of HPLC detectors, bulk property detectors and solute property detectors.

**Bulk property detectors**

These detectors are based on differential measurement of a property, which is common to both the sample and the mobile phase. Examples of such detectors are refractive index, conductivity dielectric constant detectors, etc.

**Solute property detectors**

Solute property detectors respond to a physical property of the solute, which is not exhibited by the pure mobile phase. These detectors measure a property, which is specific to the sample, either with or without the removal of the mobile phase prior to
the detection. Solute property detectors which do not require the removal of the mobile phase before detection include spectrophotometric (UV or UV-Visible) detector, fluorescence detectors, polarographic, electro-chemical and radio activity detectors, whilst the moving wire flame ionization detector and electron capture detector both require removal of the mobile phase before detection. UV-Vis and fluorescent detectors are suitable for gradient elution, because many solvents used in HPLC do not absorb to any significant extent.

**Column and Column-packing materials**

The heart of the system is the column. In order to achieve high efficiency of separation, the column material (micro-particles, 5-10 \( \mu \text{m} \) size) packed in such a way that highest numbers of theoretical plates are possible.

Silica (\( \text{SiO}_2 \cdot X \text{H}_2\text{O} \)) is the most widely used substance for the manufacture of packing materials. It consists of a network of siloxane linkages (Si-O-Si) in a rigid three dimensional structure containing inter connecting pores. Thus wide ranges of commercial products are available with surface areas ranging from 100 to 800 \( \text{m}^2/\text{gm} \) and particle sizes from 3 to 50 \( \mu \text{m} \).

The silanol groups on the surface of silica give it a polar character, which is exploited in adsorption chromatography using non-polar organic eluents. Silica can be drastically altered by reaction with organochloro silanes or organo alkoxy silanes giving Si-O-Si-R linkages with the surface. The attachment of hydrocarbon chain to silica produces a non-polar surface suitable for reversed phase chromatography where
mixtures of water and organic solvents are used as eluents. The most popular material is octadecyl-silica (ODS-Silica), which contains C\textsubscript{18} chains, but materials with C\textsubscript{2}, C\textsubscript{6}, C\textsubscript{8} and C\textsubscript{22} chains are also available. During manufacture, such materials may be reacted with a small mono functional silane (e.g. trimethyl chloro silane) to reduce further the number of silanol groups remaining on the surface (end-capping). There is a vast range of materials which have intermediate surface polarities arising from the bonding to silica of other organic compounds which contain groups such as phenyl, nitro, amino and hydroxyl. Strong ion exchangers are also available in which sulphonic acid groups or quaternary ammonium groups are bonded to silica. The useful pH range for columns is 2 to 8, since siloxane linkages are cleaved below pH-2 while at pH values above 8, silica may dissolve.

In HPLC, generally two types of columns are used, normal phase columns and reversed phase columns. Using normal phase chromatography, particularly of non-polar and moderately polar drugs can make excellent separation. It was originally believed that separation of compounds in mixture takes place slowly by differential adsorption on a stationary silica phase. However, it now seems that partition plays an important role, with the compounds interacting with the polar silanol groups on the silica or with bound water molecules.

While normal phase seems the passage of a relatively non-polar mobile phase over a polar stationary phase, reversed phase chromatography is carried out using a polar mobile phase such as methanol, acetonitrile, water, buffers etc., over a non-polar
stationary phase. Ranges of stationary phases ($C_{18}$, C$_8$, -NH$_2$, -CN, -phenyl, etc.) are available and very selective separations can be achieved. The pH of the mobile phase can be adjusted to suppress the ionization of the drug and thereby increase the retention on the column. For highly ionized drugs, ion-pair chromatography is used.

**Derivatization**

In HPLC derivatization is used to enhance the sensitivity and selectivity of detection when available detectors are not satisfactory for the underivatized compounds. Both ultra violet absorbing and fluorescence derivatives have been widely used. Ultra violet derivatization reagents include N-succinimidyl p-nitrophenyl acetate, phenyl hydrazine and 3, 5-dinitro benzyl chlorides, while fluorescent derivatives can be formed with reagents such as dansyl chloride, 4-bromo methyl-7-methoxy-coumarin and fluorescamine. Derivative formation can be carried out before the sample is injected on to the column or by online chemical reactions between the column outlet and the detector.

**Gradient elution**

Gradient elution or solvent programming is the change of solvent composition during a separation in which the solvent strength increases from the beginning to the end of the separation. It is well suited to the analysis of samples of unknown complexity since good resolution is automatically provided for a wide range of sample polarities. There are two types of gradient systems: Low-pressure gradient mixtures and high-pressure gradient mixtures. In the former the solvents are mixed at
atmospheric pressure and then pumped to the column, where as in the later, solvents are pumped in to a mixing chamber at high pressure before going in to the column.

3. METHOD DEVELOPMENT

The development of any new or improved method for the analysis of an analyte usually depends on tailoring the existing analytical approaches and instrumentation. Method development [18, 19] usually involves selecting the method requirements and on the type of instrumentation. In the development stage of an HPLC method, decision regarding the choice of column, mobile phase, detector and method of quantitation must be addressed.

Once the instrumentation has been selected, it is important to determine the chromatographic parameters for the analyte of interest. It is necessary to consider the properties of the analyte(s) that may be useful to select the nature of the column to be used, establish the approximate composition and pH of the mobile phase for separation of the components, wavelength to be employed or mass/charge ratio to be scanned at for detection of the component, the concentration range to be followed and choice of a suitable internal standard for quantification purpose etc. Such information may be already available in the literature for the analyte or related compounds.

This is followed by optimization and preliminary evaluation of the method. Optimization criteria must be determined with cognizance of the goals common to any new method. Initial analytical parameters of merit like sensitivity (measured as response per amount injected), limit of detection, limit of quantitation and linearity of
calibration plots are to be determined. As a precautionary measure, it is important that method development be performed using only the analytical standards that are highly pure and have been well identified and characterized and whose purity is known.

During the optimization stage, the initial sets of conditions that have evolved from the first stages of development are improved or optimized in terms of resolution, peak shape, plate counts, peak asymmetry, capacity, elution time, detection limits, limit of quantitation and overall ability to quantify the specific analyte of interest. Results obtained during optimization must be evaluated against the goals of the analysis set forth by the analytical figures of merit. This evaluation reveals if additional improvement and optimization are needed to meet the initial method requirements.

Optimization of the method should yield maximum sensitivity, good peak symmetry, minimum detection and quantitation levels, a wide linearity range, and a high degree of accuracy and precision. Other potential optimization goals include baseline resolution of the analyte of interest from other sample components, unique peak identification, and on-line demonstration of purity and interfacing of computerized data for routine sample analyses. Absolute quantitation should use simplified method that requires minimal sample handling and analysis time.

Optimization of the method may follow either manual or computer driven approaches. The manual approach involves varying one experimental condition at a time, while holding all others constant and evaluating the changes in response. The variables might include flow rate, mobile or stationary phase composition,
temperature, detection wavelength and pH. This univariate approach of system optimization is usually time consuming and expensive. However, it may provide a much better understanding of the principle involved and of the interaction of the variables. In computer driven automated method development, efficiency is optimized while experimental input is minimized. This approach can be applied to many types of methods. It significantly reduces the time of analysis, energy, and cost of analysis.

**Systematic approach for chromatographic separation of pharmaceutical compounds**

The first step in the method development is to characterize the drug whether it is regular or special. The regular compounds are those that are neutral or ionic. The inorganic ions, bio-molecules, carbohydrates, isomers, enantiomers and synthetic polymers, etc are called special compounds. The selection of initial conditions for regular compounds depends on the sample type.

**The column and Flow rate**

To avoid problems from irreproducible sample retention during method development, it is important that columns be stable and reproducible. A C$_8$ or C$_{18}$ column made from specially purified less acidic silica and designed specifically for the separation of basic compounds is generally suitable for all samples and is strongly recommended. If temperatures >50$^\circ$ c are used at low pH, sterically protected bonded phase column packing are preferred. The column should provide reasonable resolution in initial experiments, short run times, and an acceptable pressure drop for different mobile phases. A 5$\mu$, 150 x 4.6 mm column with a flow rate of 2 mL/min is
good for different mobile phases as initial choice. These conditions provide reasonable plate number (N=8000), a run time of <15 min for a capacity factor k<20 and a maximum pressure drop <2500 psi for any mobile phase made from mixtures of water, acetonitrile and/or methanol.

**The mobile phase**

The preferred organic solvent for the mobile phase mixture is acetonitrile (ACN) because of its favorable UV transmittance and low viscosity. However, Methanol (MeOH) is a reasonable alternative. Amine modifiers like tetra hydro furan (THF) are less desirable because they may require longer column equilibration times, which can be a problem in method development and routine use of the method. They may occasionally introduce additional problems like erratic base line and poor peak shape. However, some samples may require the use of amine modifiers when poor peak shapes or low plate number are encountered.

The pH of the mobile phase should be selected with two important considerations. A low pH that protonates column silanols and reduces their chromatographic activity is generally preferred. A low pH (<3) is usually quite different from the pKa values of common acidic and basic functional groups. Therefore, at low pH the retention of these compounds will be more rugged. For columns that are stable at low pH, a pH of 2 to 2.5 is recommended. For less stable columns, a pH of 3.0 is a better choice.
**Separation temperature**

Mostly the temperature controllers operate best above ambient (>30°C). Higher temperature operation also gives lower operating pressures and higher plate numbers, because of decrease in mobile phase viscosity. A temperature of 35-40°C is usually a good starting point. However, ambient temperature is required if the method will be used in laboratories that lack column thermostating.

**Sample size**

Initially, a 25-50 μL injection (25-50μg) can be used for maximum detection sensitivity. Smaller injection volumes are required for column diameters of below 4.5mm and / or particles smaller than 5μm. The samples should be dissolved initially in water (1 mg/mL) or dilute solution of acetonitrile in water. For the final method development stage, the best sample solvent is the mobile phase. The samples which can not be dissolved in water or the mobile phase should be dissolved initially in either acetonitrile or methanol and then diluted with water or mobile phase before injection.

**Equilibration of the column with the mobile phase**

The analytical column is completely equilibrated with the mobile phase before injecting the sample for analysis and retention data are collected for interpretation. This is done for ensuring accurate retention data. Equilibration is required whenever the column, mobile phase or temperature is changed during method development; usually by flow rate of at least 10 column volumes of the new mobile phase before the
first injection. Some mobile phases may require a much larger column equilibration time (e.g. mobile phases that contain Tetrahydrofuran, amine modifiers such as triethylamine and tetra butyl amine and any ion pair reagent).

Column equilibration and reproducible data can be confirmed by first washing the column with at least 10 column volumes of the new mobile phase before injecting the sample and then a second washing with at least five column volumes of the new mobile phase and reinjection of the sample. If the column is equilibrated, the retention times should not change by more than 0.02 min between the two runs.

**Column performance**

The following values are used to assess overall system performance

1. Relative Retention
2. Theoretical Plates
3. Capacity Factor
4. Resolution
5. Peak Tailing Factor
6. Plates per metre

The chromatographic peak shape and plate number are calculated to assess the column performance. The asymmetry factor $A_s$ should fall between 0.9 – 10.5 and number of theoretical plates should be $>4000$ for a 15 cm; 5μm column at a flow rate of 2 mL / min. The number of theoretical plates for well packed HPLC columns under optimized test conditions is given in the Table. 1.2.
Table 1.2: Number of theoretical plates for well packed HPLC columns

<table>
<thead>
<tr>
<th>Particle diameter (μm)</th>
<th>Column length (cm)</th>
<th>Plate number (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>15</td>
<td>6000-7000</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>8000-10000</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>7000-9000</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>10000-12000</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>17000-20000</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>6000-7000</td>
</tr>
<tr>
<td>3</td>
<td>7.5</td>
<td>9000-11000</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>12000-14000</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>17000-20000</td>
</tr>
</tbody>
</table>

Evaluating peak shape and plate number

The requirements for a given separation usually determine the type and configuration of the column to be used. There are different suppliers for a given type of column. These columns vary generally in performance. Therefore certain information concerning column specifications and performance is needed for use in method development and their routine performance.

The column plate number (n) is an important characteristic of a column and ‘n’ signifies the ability of the column to produce sharp, narrow peaks for achieving good resolution of band pairs with small α values. The Table 1.2 shows the typical plate numbers (small, neutral sample molecules) for well packed HPLC columns of various
lengths and practical sizes. A 15 or 25 cm column of 5 μ particles are preferred as a starting point for method development. This configuration provides a large enough N value for most separations and such columns are quite reliable. A column which gives large N value can easily recognize closely overlapping peaks. Short columns of 3 μ particles are useful for carrying out very fast separation (< 5 min). But these columns are less used because they are more susceptible to sampling problems, more operators dependent and more affected by band broadening.

**Peak asymmetry and peak tailing**

Columns and experimental conditions that provide symmetrical peaks always preferred. Peaks with poor symmetry can result in inaccurate plate number and resolution measurement, imprecise quantitation, degraded resolution, poor retention reproducibility.

Peak shape is measured in terms of peak asymmetry factor (A_s) and peak tailing factor (PTF). Peak asymmetry factor (A_s) is measured at 10% of full peak height. Good columns produce with A_s values of 0.95 to 1.1. For accurate measurement of symmetry, bands should be measured with a magnified time scale because asymmetrical bands also often appear symmetrical when observed in a compressed chromatogram.

**Retention**

The time between the sample injection point and the analyte reaching the column is called the retention, \( t_R \).
Capacity factor $k'$

It measures how many times the analyte is retained to an unretained component.

$$k' = \left( \frac{t_2}{t_a} \right) - 1$$

A $k'$ value zero means that the compound is not retained and elutes with the solvent front. A $k'$ value of 1 means that the component is slightly retained by the column while $k'$ value of 20 means that component is highly retained and spends much time in interacting with the stationary phase.

Selectivity, $\alpha$

Separation between two components is only possible if they have different migration rates through column. Selectivity or separation factor is a measure of differential retention of two analytes. It is defined as the ratio of the capacity factors ($k'$) of two peaks.

$$\alpha = \left( \frac{t_2-t_a}{t_1-t_a} \right)$$

Column efficiency ($n$) or number of theoretical plates

The term plate number ‘$n$’ is a quantitative measure of the efficiency of the column and is related to the ratio of the retention time and the standard deviation of the peak width ‘$\sigma$’. Since it is difficult to measure ‘$\sigma$’ or Width at base of the peak), a relationship using width at half height or $W_{1/2}$ is often used to calculate ‘$n$’ as described in the USP.

$$n = 16 \left( \frac{t}{W} \right)^2$$

Height Equivalent of a Theoretical Plate (HETP) or Plate Height (H)

$$\text{HETP} = \frac{L}{n}$$
Resolution, $R$

It is the degree of separation of two adjacent peaks and is defined as the difference in retention times of the two peaks divided by the average peak width. As the peak width of adjacent peaks tends to be similar, the average peak width can be equal to the width of one of the two peaks.

$$R = \frac{2(t_2 - t_1)}{(W_2 + W_1)}$$

Tailing factor, $T$

It is a measure of peak asymmetry. It is given by the equation

$$T = \frac{W_{0.05}}{2f}$$

Tailing factor for most peaks should fall between 0.9 and 1.4 with a value of 1.0 indicating a perfectly symmetrical peak.

Formulae for calculating the different system performance parameters are given in Table 1.3.

**Table: 1.3.** Formulae for different system performance parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative retention (selectivity)</td>
<td>$\alpha = \frac{(t_2-t_a)}{(t_1-t_a)}$</td>
</tr>
<tr>
<td>Capacity factor</td>
<td>$k' = \frac{t_2}{t_a} - 1$</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>$T = \frac{W_{0.05}}{2f}$</td>
</tr>
<tr>
<td>Resolution</td>
<td>$R = \frac{2(t_2 - t_1)}{(W_2 + W_1)}$</td>
</tr>
<tr>
<td>Theoretical plates</td>
<td>$n = 16 \left(\frac{t}{W}\right)^2$</td>
</tr>
<tr>
<td>HETP</td>
<td>$L/n$</td>
</tr>
</tbody>
</table>
Where, $\alpha =$ relative retention

$t_2 =$ retention time of the second peak measured from the point of injection

$t_1 =$ retention time of the first peak measured from the point of injection

$t_a =$ retention time of the inert peak not retained by the column measured from the point of injection

$n =$ theoretical plates

$t =$ retention time of the component

$W =$ Width of the base of the component peak using tangent method.

$k' =$ Capacity factor.

$R =$ Resolution between a peak of interest (peak 2) and the peak preceding it (peak 1)

$W_2 =$ Width of the base of component peak 2.

$W_1 =$ Width of the base of component peak 1.

$T =$ Peak asymmetry, or tailing factor.

$W_{0.05} =$ Distance from the leading edge to the tailing edge of the peak, measured at a point 0.5 % of the peak height from the baseline.

$f =$ Distance from the peak maximum to the leading edge of the peak.

$N =$ Plates per meter.

$L =$ Column length, in meters.


4. METHOD VALIDATION

Validation is the process of ensuring that a test procedure performs within acceptable standards of reliability, accuracy and precision for its intended purpose. It is the act of conforming that a method does what it is intended to do.
It is difficult to completely separate method development and optimization from validation; these areas often overlap. In the validation stage, an attempt should be made to demonstrate that the method works with samples of the given analyte, at the expected concentration in the matrix, with a high degree of accuracy and precision. Complete method validation can occur only after the method is developed and optimized. In validation studies, suitability of the final method for the given analyte and a selection of sample matrix is demonstrated, using specified instrumentation, samples, and data handling: ultimately, the method can be transferred from one laboratory to another that is suitably equipped and staffed. A method that provides all or most of the original method requirements is deemed optimized and becomes ready for validation.

There is no single validation approach that must always be employed for a new method; the analyst’s primary concern should be to select an approach that will prove to be a true validation. Acceptance of any new method by others in the field will depend on the specific validation approaches used. It is the responsibility of the individual analyst to select the correct validation method(s). Validation approaches include the Zero-, Single-, and Double-blind spiking methods; inter-laboratory collaborative studies; and comparison with a currently accepted (compendium) method.

**The Zero-blind method**

The Zero-blind approach involves a single analyst using the method with samples at known levels of analyte to demonstrate recovery, accuracy, and precision.
The method is subject to analyst bias, and though the method is, in general, fast, simple and useful, it leads to subjective results and doubt on the part of the unbiased reviewer or end user. However, as a first approximation and a demonstration of validation potential requiring minimal time, manpower, samples, and cost, a Zero-blind study is a good place to start the overall validation process. Clearly, if this approach fails to validate a method, then there is no reason to proceed with further validation of the method.

**The Single-blind method**

The Single-blind approach involves one analyst preparing samples at varying levels unknown to a second analyst, who also analyses the samples. The results are then compiled and compared by the first analyst. Although this approach is unbiased at the start, it loses its blindness at the most crucial stage when both sets of data are compared. While perhaps more valuable and believable than the Zero-blind approach, the Single-blind approach still invites bias on the part of the first analyst to bring two sets of data into better agreement. This approach is appropriate at the very start of the method validation, after the Single-blind approach has proven successful, but before one decides to involve additional analysts or management.

**The Double-blind Method**

The Double-blind approach involves three analysts. The first analyst prepares samples at known levels, the second does the actual analysis, and the third analyst (or administrator) compares both sets of data received separately from the first two analysts. Neither the first nor the second analyst has access to the set of data
generated by the other. This double-blind approach is the most objective approach, assuming no bias on the part of the third analyst.

**The Analysis of standard Reference Materials**

The analysis of a standard reference material (SRM) or an authenticated sample is a generally accepted method of validation. The USP, NIST, and other, private organizations specialize in preparing, guaranteeing, and marketing standard reference materials of various analyte species in different sample matrices. It may be necessary, however, to contract the preparation of a unique sample in a particular matrix in order to utilize this procedure for method validation. When using SRMs, the analyst must demonstrate that the method provides accurate and precise measurements of the analyte in a particular sample matrix. Analyst bias can also be an issue, especially when the analyst knows the amounts and levels of the SRM.

**The Inter-laboratory collaborative study**

The inter-laboratory collaborative study is perhaps the most widely accepted procedure to validate any new analytical method, but it suffers from serious practical drawbacks. The collaborative approach is costly and time consuming; it can take years from start to finish. During that time, the analysts may have to expend considerable effort coordinating the process, shipping samples and receiving results, statistically analyzing and interpreting the results, and then finally interpreting and verifying the data. Although the approach is operator dependent (generating laboratory-to-laboratory variability), when all laboratories involved come up with over-lapping quantitative values in comparison with known levels present, the method
is generally accepted as full validation. This approach is rarely employed when a method is being described for the first time in the literature

**Comparison with a currently accepted method**

Comparison with a currently accepted analytical method is yet another validation approach. This is usually done by a single analyst, but it can be done by two analysts using a split sample. This approach uses results from the currently accepted method as verification of the new method’s results. Agreement between results initially suggests validation. Disagreement is a serious cause for concern of future acceptability of the new method. However, disagreement could also suggest that the currently accepted method is invalid, creating additional problems. If the analyst can prove that the currently accepted method is indeed invalid, the analyst must then initiate an alternative approach to validate the new method.

The question will eventually arise as to how many samples should be analysed in any validation approach. Ideally, the method should be validated for the analyte using several samples, different sample types, with several of each type determined separately for statistical and validation purposes. A Single, Zero-blind or a Single-blind study is obviously less meaningful and less acceptable than an inter-laboratory collaborative, true Double-blind study of several sample matrices at widely different concentration levels. Initial validation approaches are generally less rigorous and demanding than one’s performed standard reference material (SRM) development.

Assay procedures are intended to measure the analyte present in a given sample. In the context of this document, the assay represents a quantitative
measurement of the major component(s) in the drug substance. For the drug product
similar validation characteristics also apply when assaying for the active or other
selective component(s). The same validation characteristics may also apply to assays
associated with other analytical procedures (e.g. Dissolution).

5. VALIDATION OF ASSAY METHODS

Reliable analytical methods are required to comply with national and
international regulations in all the areas of analysis. It is accordingly internationally
recognized that a laboratory must take appropriate measures to ensure that it is
capable of providing and does provide data of the required quality. Method validation
is therefore an essential component of the measures that a laboratory should
implement to allow it to produce reliable analytical data [20]. A number of protocols
and guidelines [21-30] on method validation and uncertainty have been prepared,
most notably in Association of Official Analytical Chemists (AOAC) international,
International Conference on Harmonization (ICH), Pharmacopoeias and Eurachem
documents.

Method validation makes use of a set of tests based on the analytical method
to establish and document the performance characteristics of the method and to
demonstrate whether the method is fit for particular analytical purpose. Typical
performance characteristics of LC methods are specificity, selectivity, precision,
linearity, robustness, recovery, range, limit of quantification, limit of detection, and
ruggedness. Validation should refer to an “analytical system” (system precision)
rather than an “analytical method” (method precision), the analytical system
comprising a defined method protocol, a defined concentration range for the analyte, and a specified type of test material. The method validation protocol will be prepared by referring the analytical system as a whole and analytical procedure.

The guidelines [31-35] recommended that the validation parameters must be carried for compendial methods are specificity of the procedure, stability of the sample solution and intermediate precision. The non-compendial methods should be validated for accuracy, precision (repeatability and intermediate precision), specificity, detection limit, quantification limit, linearity, range and robustness based on the type of the test procedure.

**Specificity:** Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

- For chromatographic procedures, representative chromatograms should be used to demonstrate specificity and individual components should be appropriately labeled.
- For SIAMs, this should be including samples stored under relevant stress conditions: light, heat, humidity, acid/base hydrolysis and oxidation.
- An investigation of specificity should be conducted during the validation of the methods for determination of impurities and the assay. For the assay, the two (reference and sample) results should be compared and peak purity tests may be useful to show that the analyte chromatographic peak is not attributable to more than one component (e.g., diode array, mass spectrometry).
Accuracy: The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. Accuracy should be established across the specified range of the analytical procedure.

- Accuracy of impurities (quantitation) should be assessed on samples (drug substance/drug product) spiked with known amounts of impurities in cases where it is impossible to obtain samples of certain impurities and/or degradation products, it is considered acceptable to compare results obtained by an independent procedure.

- The response factor of the drug substance can be used. It should be clear how the individual or total impurities are to be determined e.g. weight/weight or area percent, in all cases with respect to the major analyte.

- Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentrations levels covering the specified range (e.g. 3 concentrations /3 replicates each of the total analytical procedure).

- Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals
**Precision:** The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

- The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.
- Repeatability is also termed intra-assay precision. Repeatability should be assessed using a minimum of 6 determinations at 100% of the test concentration.
- Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipments, etc.
- Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).
- Validation of tests for assay and for quantitative determination of impurities includes an investigation of precision.
- The standard deviation, relative standard deviation (coefficient of variation) and confidence interval should be reported for each type of precision investigated.

**LOD and LOQ:** The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated as an exact value. The detection limit is determined by the analysis of
samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in samples matrices, and is used particularly for the determination of impurities and/or degradation products.

- LOD and LOQ can be determined based on the standard deviation of the response and the slope. The slope \( S \) may be estimated from the calibration curve of the analyte.

- The estimate of \( \sigma \) may be carried out based on the calibration curve that should be studied using samples containing an analyte in the range of detection limit (LOD) and quantitation limit (LOQ). The residual standard deviation of a regression line or the standard deviation of Y-intercepts of regression lines may be used as the standard deviation.

- The LOD may be expressed as:

\[
\text{LOD} = \frac{3.3\sigma}{S}
\]

- The LOQ may be expressed as:

\[
\text{LOQ} = \frac{10\sigma}{S}
\]

Where \( \sigma \) = Standard deviation of the response and \( S \) = Slope of the calibration curve
**Linearity:** The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. A linear relationship should be evaluated across the range of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighings of synthetic mixtures of the drug product components, using the proposed procedure. For the establishment of linearity, a minimum of 5 concentrations are recommended. The correlation coefficient, Y-intercept, slope of the regression line and residual sum of squares should be calculated.

**Range:** The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (include these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

**Robustness:** The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

- The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.
If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure.

One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g. resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used. In the case of liquid chromatography, examples of typical variations are influence of variations of pH in a mobile phase; influence of variations in mobile phase composition; different columns (different lots and/or suppliers); temperature; flow rate.

**System suitability or performance testing for LC system**

System suitability testing is an integral part of the LC methods. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such. The parameters like Resolution (R), Number of theoretical plates (n), Tailing factor (T) and system precision (%RSD) to be evaluated to confirm the chromatographic system is adequate for the analysis to be done.

The resolution ‘R’ is a function of column efficiency and is specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the drug. For the separation of the two components in a mixture, R is calculated by the following equation:

\[
R = \frac{t_{2} - t_{1}}{0.5(w_{2} + w_{1})}
\]
\[ R = \frac{2(t_2-t_1)}{W_2 + W_1} \]

Where, \( t_2 \) and \( t_1 \) are the retention times of the two components and \( W_1 \) and \( W_2 \) are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight side of the peaks to the base line.

- The number of theoretical plates \( n \), is the measure of the column efficiency (column efficiency is a measure of peak sharpness, which is important for the detection of trace components). For Gaussian peaks, \( n \) is calculated by the equation:

\[ n = 16\left(\frac{t}{W}\right)^2 \]

Where, \( t \) is the retention time and \( W \) is the width of the peak at its base. The value of \( n \) depends upon the substance being chromatographed as well as operating conditions such as mobile phase, temperature and the quality of the packing and uniformity of the packing within the column.

- The tailing factor, \( T \), a measure of peak symmetry is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced. The tailing factor \( T \) is determined by the equation:

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

\( W_{0.05} \) is the width of the peak at 5% peak height and \( f \) is the distance from the peak maximum to the leading edge of the peak the distance being measured at a point 5% of the peak height from the base line.
Replicate injections of a standard preparation used in the assay or other standard solutions are compared to ascertain whether requirements for precision are met. The data from 5 replicate injections of the analyte are used to calculate the relative standard deviation (RSD), if the requirement is 2.0% or less. The relative standard deviation in % is calculated by the equation:

\[
\% \text{ RSD} = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100
\]
6. REFERENCES


25. NKM Procedure No.4. Validation of chemical analytical methods. NKM Secretariat, Finland, 1996.


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