1.1. INTRODUCTION

Drug [1-8] is defined in many ways as there are distinct meanings of it. In general, drug as a chemical substance finds its usage in the investigation, analysis, treatment, prevention or mitigation of a disease. A natural or artificial substance that influences working or texture of the body [9] of human beings or animals [10-12] is called a drug. In ancient period, most of the drugs are obtained from plant materials. But at present, drugs are synthesised from organic compounds [13]. The central nervous system and brain are affected by drugs. When the drug is used properly, it gives relief from the discomfort but the same is dangerous and addictive if abused. Pharmaceutical chemistry [14-23] contains the detailed study of 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. Mostly, drugs are classified as

1. Pharmacodynamic agents
2. Chemotherapeutic agents

Pharmacodynamic agents provide pain relief and comfort to the body under abnormal conditions by depressing or stimulating functions of body without curing the disease. They are highly advisable in case of non-infectious diseases so as to rectify the abnormality of the body. This group of drugs normally includes 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. Truly speaking, these means are non-reactive towards the infective organisms responsible for various diseases.

To speak about the other side of the coin, chemotherapeutic agents are highly toxic to the invading organisms without causing any damage to the body. These drugs consist of organometallic agents, antimalarials, antibacterials, antiprotozoals, antifungal agents, anthelmentics, antiseptics, antitubercular agents, antineoplastics etc.

There are numerous ways [24] for drug classification. According to pharmacology, drugs perhaps categorized depending upon the chemical activity or by the condition that it treats. Basing on addiction treatment and rehabilitation, drugs can be grouped into five categories, namely narcotics, depressants [25], stimulants [26], hallucinogens [27,28] and anabolic steroids [29]. The term pharmacology is coined from Greek terminology and it means the study of drugs. Pharmacology is a product of science studies which is about the study of the mutual actions that happen between a host body and the chemical substance. It mainly provides information about the preparation, uses and mostly about the action of drugs.

Pharmacology is classified into two types, namely pharmacodynamics and pharmacokinetics. Pharmacodynamics involves the study of chemical interactions with body receptors and pharmacokinetics deals with passage of chemicals through the body in four stages: absorption, distribution, metabolism and excretion. Pharmacology is a different subject and it is quite distinct in its own features and study from pharmacy. Since pharmacy mainly deals with preparation and distribution of medicine while pharmacology deals the interaction and effects between chemicals and biological systems and vice versa.

Pharmaceutical formulations and essential quality indices of every individual drug are presented in a separate article by every country through legislation [30] in pharmacopoeia [31]. Pharmacopoeia literally means drug-making. I.P. [32,33], U.S.P. [34], B.P. [35-37] and Martindale [38,39] are some major pharmacopoeias. Pharmaceutical analysis [40] involves the strategies and approaches of medicaments (drugs and their formulations) and their precursors. The raw material which is responsible for the purity and the quality of drug and its formulations is called the precursor.

Currently, pharmaceutical industries are showing a tendency in further improvement of instrumental techniques for the analysis of pharmaceutical preparations. The production and application of simple methods for analyzing the purity is one of the thrust areas of pharmaceutical analysis. Though quality [41] is essential in every product or service, it is fundamental in medicine as it directly influences life. There should be no

“second quality” in drugs unlike other market products. The main aim of the quality control is to produce a perfect product by numerous trails so as to prevent and eliminate errors at different stages of production.

Quality control [42-44] is associated to sampling, specifications, examining, configuration and testimony and delivery procedures. It ensures that the essential and appropriate tests are strictly processed before release, trade or supply of any product. In pharmaceutical industry, quality assurance is not only restricted to laboratory procedures but requisite to all properties regarding the quality of the output drug and the complete manufacturing process. Without analytical tools the new benchmarks for sample generation and their advantages could not be fully realized in a pharmaceutical industry. Thus, the correlation between sample generation and analysis is of main concern in this industry. Clearly, traditional approaches for analysis are not capable of meeting specialized needs created by dramatic improvements in sample generation.

There are three important stages in the use of a drug as a medicine. First phase is the drug discovery, where the compounds are examined for biological activities. Second phase involves the application of chemistry and safety procedures towards proper manufacturing and analytical practices. Third phase is the usage of the right formula in the preparation of the drug in a convenient dosage. The two basic steps of pharmaceutical analysis [45-49] are separation of the drug and quantitation of the drug and their formulations along with their precursors. In this connection, an analyst plays an essential role for the qualitative and quantitative estimation of the drug in bulk and their pharmaceutical formulations. At present, simple analytical techniques for the determination of drug in bulk and the pharmaceutical formulations are developed by the use of sophisticated instruments and number of varieties of drugs. The availability of advanced techniques with improved equipments has made the latest techniques attractive.

The precision, accuracy, time and economy are a prime factor here. Also many pharmaceuticals need not be analyzed by the same procedure. Unique techniques for analyzing a single drug or combination of drugs in formulation at a single step are adopted basing on advanced knowledge. Various practices in the determination of drugs are sort into physical, chemical, physico-chemical and biological ones. Physical methods imply the study of the physical factors like solubility, colour, density, specific gravity, transparency etc. Chemical methods comprising volumetric, titrimetric and gravimetric involves complex formation, redox reactions etc. Physico-chemical methods [50-54] include the study of the physical phenomena which is the resultant of chemical reactions**.** These methods generally contain spectrophotometric and chromatographic methods (column, paper, thin-layer, gas-liquid [55], HPLC [56]). Methods such as nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) are becoming more and more popular. Mass spectroscopy when combined with gas chromatography, it becomes a powerful tool for the analysis of drug. One of the major tasks of the analyst for the analysis is selection of most effective procedure. This is done well, when the analyst is familiar about theoretical principles and practical details and the analyst must be familiar with conditions under which each method is reliable.

The four major steps involved in the complete analysis of a substance are

1. Sample preparation /sampling
2. Dissolution of the sample and conversion of the analyte into a suitable form in order to facilitate measurement
3. Measurement
4. Estimation and interpretation of the measurement.

Pharmaceutical scientists have explored various approaches to find and develop organic compounds which are available in pharmaceutical formulations for the treatment of diseases. The activity of the drug is greatly influenced by its physical and chemical properties and sometimes, it is influenced by the arrangement, position and size of the groups present in the given molecules. It is the responsibility of manufacturers to produce safe, effective and non-toxic pharmaceutical products.

1.2. SAMPLING TECHNIQUES

Skilful sampling is essential in drug analysis because of great variability in formulation. The extents of variation rely on product and manner of selection. Sampling of pharmaceutical formulation can be adopted through following methods.

Liquids

Liquids are mixed properly and completely without any sediment remained in the solution. The resultant sample solution can be used for the analysis.

Powders

Powders are mixed thoroughly in a suitable mobile phase and the obtained sample solution can be utilized for the complete solution.

Tablets

Tablets are primarily ground well into finely divided powder and then properly mixed in a suitable mobile phase before a portion of the sample solution is taken for analysis.

Capsules

Initially, ten capsules are exactly weighed; the contents are discharged into a small beaker and are mixed thoroughly in an appropriate mobile phase to get a sample solution for the analysis.

Appropriate method for analysis is selected basing on following factors,

1. Nature of the information sought.
2. Available size of the sample and proportion of the constituent sought.
3. The purpose of requirement of analytical data.

Four types of analysis are available,

1. Proximate analysis: It involves the determination of amount of all elements present in the sample without giving priority to the major elements present in it.
2. Partial analysis: It involves the determination amount of selected elements present in the sample.
3. Trace constituent analysis: It mainly deals with the determination of specified elements present in minute quantity.
4. Complete analysis: In this analysis, a proportion of each component of the sample is determined.

There are several factors which influence the selection of the analytical methods,

1. The category of analysis.
2. Problem associated with the nature of the material.
3. Interference of elements other than the element of concern.
4. Determination of concentration range.
5. Accuracy of the analysis.
6. Accessible facilities.
7. The time required for complete analysis.
8. Similar type of analysis performed.

1.3. SPECTROPHOTOMETRY

In the recent decades, a number of sophisticated instrumental methods are reported which are rapid and selective with high degree of accuracy. Among these, spectrophotometry is the most frequently used method for variety of materials. Analytical chemists greatly rely on the high accuracy, precision, sensitivity and availability of spectrophotometer. In these techniques colour is basic factor in the recognition of constituents. Coloured solutions exhibit radiation absorption as the significant characteristic, which is responsible for the quantitative or qualitative determination. Yet, addition of chromogenic agents to colourless compounds tends to intensify the colour of the species thereby facilitating the identification of the substance. In the earlier days, measurements are made by using undiffused sunlight or artificial light as the light source and human eye as the detector. To certain level the accuracy and precision of the determinations is enhanced by using optical filters, which isolate specific frequencies of light. Moreover, the utility of prism and grating for wavelength selection, photoelectric detector, phototubes and photomultiplier tubes advanced the measurement.

Modern electronic systems are provided with abundant variety of detector types coupled with the computers based on solid state microelectronics. Latest spectrometer is fitted with microprocessor control and diode array detector for analysis. Most frequently used methods involve the absorption of electromagnetic radiation in the ultra violet and visible region. Of the various spectrophotometric techniques, small scale industries greatly relied on visible spectrophotometry due to low price of the equipment and minimal maintenances.

1.3.1. Principle of UV-visible spectroscopy

The basic principle of spectrophotometry depends on a simple relationship of the colour of the substance to its electronic structure. A molecule with chromophoric groups showed absorption in the UV-visible region due to electronic transitions caused by the

radiations. The fundamental principle behind the absorption is "the number of photons absorbed is directly proportional to the number of atoms or ions or molecules [57] and it is known to be Beer's law.

1.3.2. Deviation from Beer's law

According to Beer's law, a sketch of absorbance against concentration should always yield a straight line passing through the origin [58]. This criterion holds well over a vast area of concentrations when there is no change in the structure of the coloured ion or of the coloured non-electrolytes even in the diffused state. Absorption of light by coloured compounds is not affected by the presence of tracer quantity of colourless electrolytes as they exhibit chemical inertness with the coloured components. If electrolytes are present in large amount, they may cause a shift in absorption maxima and sometimes responsible for a change in the value of extinction coefficient. In concentrated solutions, coloured substances display association, dissociation or ionization and hence Beer's law may not hold well. If there is any possibility for the formation of complexes by the coloured solutes then Beer's law does not hold well. Deviations are more pronounced when monochromatic light is not availed.

1.3.3. Criteria for satisfactory spectrophotometric analysis

Effective and acceptable results are obtained through the conscientious approach of the spectrophotometric methods. In spectroscopy, various reactions are responsible for the colour development hence before applying the method for a specific purpose; a number of points need to be ensured. Several points which should be measured for satisfactory spectrophotometric analysis are discussed below.

1.3.4. Specificity of the colour

Colour is due to definite organic reactions taking place in the material by the presence of small groups and thus operational procedure for a particular analysis should be controlled so that colour is specific for a substance to be determined. This is accomplished by isolating the substance by the normal methods of analysis.

Colour specification is obtained by the addition of complex forming compounds. These are required to suppress the action of interfering substance by the formation of selective complexes.

1.3.5. Stability of the colour solutions

The colour formed must be stable so as to measure accurate readings. Stability of colour is influenced by experimental conditions like temperature, pH etc. Precipitate or turbidity in a solution scatters and absorbs the light. Hence, it should be freed from precipitate or turbidity in order to compare it with a clear standard solution.

1.3.6. Reproducibility

The procedure developed must give reproducible results under specific experimental conditions. A stoichiometric quantitative chemical change need not be the resultant of a reaction every time. The interfering effect of substances even in the minute quantities is more defined in the ultraviolet region than in the visible region, hence it is suggestive that the reaction product exhibits strong absorbing properties in the visible region than in the UV region.

1.3.7. Calibration curve

Calibration is one of the pre-requisites for the analysis of the drugs. Colour development basically depends upon the appropriate quantities of constituents taken and are examined like that of sample solution for the determination of maximum absorption at the optimum wavelength. The absorbance is then plotted against concentration of the constituents. When Beer's law is applicable, it yields a straight line. These calibration curves are highly beneficial to estimate the constituents under the identical experimental conditions. The calibration curves need checking at regular intervals.

1.3.8. Sensitivity

Estimation of pharmaceutical compounds requires the pre-knowledge of sensitivity. Sensitivity is expressed mathematically with the help of the two terms molar absorptivity (ε) at the wavelength (λ_{max}) and maximum absorbance of the coloured species.

$$\text{Molar absorptivity } (\varepsilon) = A / c\, l$$

Where, A is the absorbance

c is concentration and

l is the thickness of the medium.

Monochromatic light is essential to measure the spectrophotometer sensitivity. In general molar absorptivity is inversely proportional to bandwidth. Initially, Savvin [63] gave an expression for sensitivity in terms of molar absorptivity and on later Sandell's expressed it in terms of specific absorptivity [64]. From the above two methods it is clear that, sensitivity is measured as the amount of analyte per unit volume of solution. Sandell's sensitivity [65] is the concentration of the analyte (mcg ml^{-1}) which gave an absorbance of 0.001 in a cell of path length 1.0cm. Organic reagents with high molecular weight are used as chromogenic agents to display maximum sensitivity. As the molar absorptivity also depends on the solvent system it is possible to reduce detection limits to some extent by proper selection of solvent. When analytical wavelength is the wavelength of maximum absorbance, sensitivity of the method and signal to noise ratio is enhanced.

1.3.9. Detection limit (DL)

The lowest concentration at which the analyte is identified with at least 95% certainty [66, 67] is called limit of detection. This value is approximately two fold to the value of standard deviation obtained from ten determinations whose concentration is near to the concentration of blank. Various instrumental or non-instrumental procedures are available for the estimation of limit of detection. It is expressed mathematically, with the help of standard deviation of the reagent blank and the slope of the calibration curve of the analyte.

$$DL = (3.3\ \sigma)/\ S$$

Where, σ is standard deviation of the reagent blank,

S is slope of the calibration curve.

The slope S may be estimated from calibration curve of the analyte.

1.3.10. Quantitation limit (QL)

The limit of quantitation is the smallest concentration at which the analyte is not only detected but is analysed with good accuracy [68,69] and precision [70,71]. Limit of quantitation is calculated depending upon the values of standard deviation and slope of the calibration curve of the analyte.

$$QL = (10\ \sigma)/\ S$$

Where, σ is standard deviation of the reagent blank,

S is slope of the calibration curve.

The slope S is estimated from calibration curve of the analyte. The estimate of σ is measured based on the standard deviation of the reagent blank.

Precision describes repeatability of outcomes while accuracy indicates the proximity of a measurement on the agreeable limit. In spectrophotometric method, accuracy and precision depends on three major factors 1) limitation of equipments 2) variability of chemicals and 3) skill of the operator. Equipment limitations are controlled by optical, mechanical, electronic systems and the quality of the instruments. The parameters on which chemical variability determination depends are chromophore stability, purity of standards and reagents, reaction rates, reaction stoichiometry, pH and temperature control. Accuracy or precision of an analytical method is determined by the relationship of the values obtained from a set of results with either (i) the true value or (ii) other sets of values.

1.3.11. Comparative method: There are two common methods for comparing results [72,73]. They are the variance ratio test (F-test) and the student's t-test.

1.3.11a. Variance ratio test (F-test)

By the F-test one can test the significance of the difference in variances of reference and test methods. If the null hypothesis is true then the estimates ST^2 (variance of the test method) and SR^2 (variance of reference method) do not differ much and their ratio should not differ much from unity. F value is determined by using the following equation.

$$F = ST^2 / SR^2$$

It is conventional to calculate the F-ratio by dividing the larger variance by the smallest variance in order to obtain a value equal to or larger than unity. If the calculated F-value is smaller than F-value from the table, one can conclude that the procedures are not significantly different in precision at given confidence level.

1.3.11b. Student's t-test

Some level of confidence is expressed in student's t- test by comparing the mean of the sample with some standard values. By this test, it is also possible to compare the difference between the means of the two sets of data.

$$t = [(\overline{x} - \mu)\sqrt{n}] / S$$

Where, S is standard deviation,

x is arithmetic mean of a series of measurements,

μ is the true value and

n is the number of trials of the measurements.

The minimum level at which the analyte can be quantified is then related to a set of t-tables in which the probability of the t-value falling within certain limits is expressed, either as a percentage or as a function of unity relative to the number of degrees of freedom. This method is also used to compare the values of the mean and precision of the test method with those of the reference method.

1.4. CHROMATOGRAPHY

The term chromatography is the combination of the two words “chromo” (colour) and “graphy” (writing). Even though, most of the organic compounds are enormously recognized through spectrophotometry, it requires high levels of chemical purity. Always it may not be possible to get a sample with high degree of purity. In some cases impurity levels of the sample cannot be controlled by an analyst. Under these circumstances, chromatography gained immense importance for the separation or identification of the components in a mixture. Chromatography is the most extensively used method in different industries. Chromatography technique requires the utilization of two phases namely solid stationary phase and the moving mobile phase. Separation phenomena depend on the rate of adsorption or partition or ion exchange or molecular exclusion. Size of the particles plays a key role in the determination of extent of hydrogen bonding or vander walls forces or electrostatic forces or hydrophobic forces that are major in the development of interactions between the solute and the stationary phase. Whenever the solute is employed, it is retained by the solid stationary phase. On the passage of the mobile phase the solute moves along with the mobile phase. Different solutes exhibit different flow rates due the difference in the distribution of molecules in the mobile phase.

Major chromatographic methods [74-77] comprise of thin layer chromatography (TLC), gas chromatography (GC), liquid chromatography (LC), high performance liquid chromatography (HPLC).

1.4.1. Thin layer chromatography (TLC)

Thin layer chromatography makes the use of a thin-layer plate made up of a glass plate coated with a film of granular material. The plate made up of plaster of paris behaves as stationary phase and the liquid rising due to capillary action acts as mobile phase.

Thin layer chromatography is highly useful for the identification of coloured organic compounds. No separation is provided in the case of colourless compounds but can be achieved by placing the plate under UV light that results a bright spot on a dark back ground. TLC alone may not furnish the complete information but TLC when combined with other testing procedures is beneficial.

1.4.2. Gas chromatography (GC)

Gas chromatography is highly useful for separating and analyzing compounds which can be vapourized easily without decomposition. It consists of a column made up of glass or metal, a stationary phase and a moving phase**.** A microscopic layer of liquid or polymer supported on an inert solid serves as stationary phase**.** Most frequently used carrier of gas is helium or nitrogen and it functions as moving phase. Initially, the sample itself spread on the walls of the column due its interaction with the stationary phase. This originates the elution of every compound at a different retention time. Significant application of GC is found in the field of forensic science. Some other important applications of GC are identification of a compound and preparation of pure compounds from a mixture.

1.4.3. Liquid chromatography (LC)

The mechanism of GC and the liquid chromatography are one and the same. Ions or molecules present in a solvent can be separated into individual components by making use of liquid chromatography. Separation is due the interactions of the sample with the mobile and stationary phases. Different kinds of stationary/mobile phase combinations can be employed for separating a mixture. There are two major differences between the GC and LC. First difference is the nature of stationary and mobile phases; the former utilizes liquid and gaseous phases where as the later employ solid and liquid phases respectively. The second change is with respect to temperature control, in GC it is done by making use of an oven and no such control is possible in LC.

1.4.4. High performance liquid chromatography (HPLC)

The basic function of HPLC is identification, purification and quantification of the individual components from the mixture. Determination of the chemical structure of the compound, identification and determination of impurities in the compound and detection of degradation products in the bulk drug are the other approaches of HPLC. Due to plenty of benefits this technique can be applied to herbal products too. Basing on the relative polarity of the two phases there are two modes of columns in HPLC. Normal phase chromatography consists of silica gel which is polar in nature acts as the stationary phase and non-polar compounds like n-hexane works as the mobile phase. Contradictory functioning takes place in reversed-phase chromatography as it consists of a polar mobile phase like methanol, acetonitrile, water, buffers etc., over a non-polar stationary phase.

Most of the chromatographic applications make use of reverse phase chromatography. HPLC utilizes two kinds of elutions viz isocratic and gradient. In isocratic elution, eluent is pumped with constant composition into the column during the entire analysis. On the other hand, gradient elution pump eluent with steadily changing composition all over the run. The two kinds of gradient systems include low-pressure gradient mixtures and high- pressure gradient mixtures. In the former the solvents are mixed at 16 atmosphere 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. Mostly the temperature controllers operate best above ambient ($>30^0$ C). For highly ionized drugs, ion-pair chromatography is used.

1.4.4.1. HPLC instrumentation

HPLC instrument includes a pump, injector, column, detector and data system. Column is the chief element of the system through which separation arises. A high pressure pump is necessary to progress the mobile phase throughout the column consisting of micrometer size porous particles as the stationary phase.

Chromatographic process is initiated by injecting the solute onto the zenith of the column. As the analytes and mobile phase are passed across the column, separation of constituents takes place. Ultimately, when all constituents elute from the column narrow peaks are identified on the recorder. Eluting components may be selective or universal basing upon the type of the detector utilized. The rejoinder of the detector to each component is presented on a computer screen as a chromatogram.

1.4.4.2. Mobile phase reservoir

The best solvent reservoir is a glass bottle. Producers supply these bottles with unique caps, teflon tubing and filters to connect to the pump inlet. Helium is used as purge gas to eradicate dissolved air. Complete degassing of aqueous solvents is only possible by the initial application of vacuum for 5-10min and then keeping the solvent under a helium atmosphere.

1.4.4.3. Solvent delivery system

The mobile phase flows through the column at constant rate when pumped under pressure from one or several reservoirs. Eluting power of the mobile phase depends upon three factors such as polarity of mobile phase, polarity of stationary phase and nature of the sample constituents. In case of normal phase separations, eluting power enhances with increasing polarity of the solvent whereas in reversed phase separations, eluting power reduces as solvent polarity enhances. Mixture of two solvents mostly favours optimum separating conditions. Viscosity, boiling point, detector compatibility, flammability and toxicity are the other aspects of the solvents that influence complete separation.

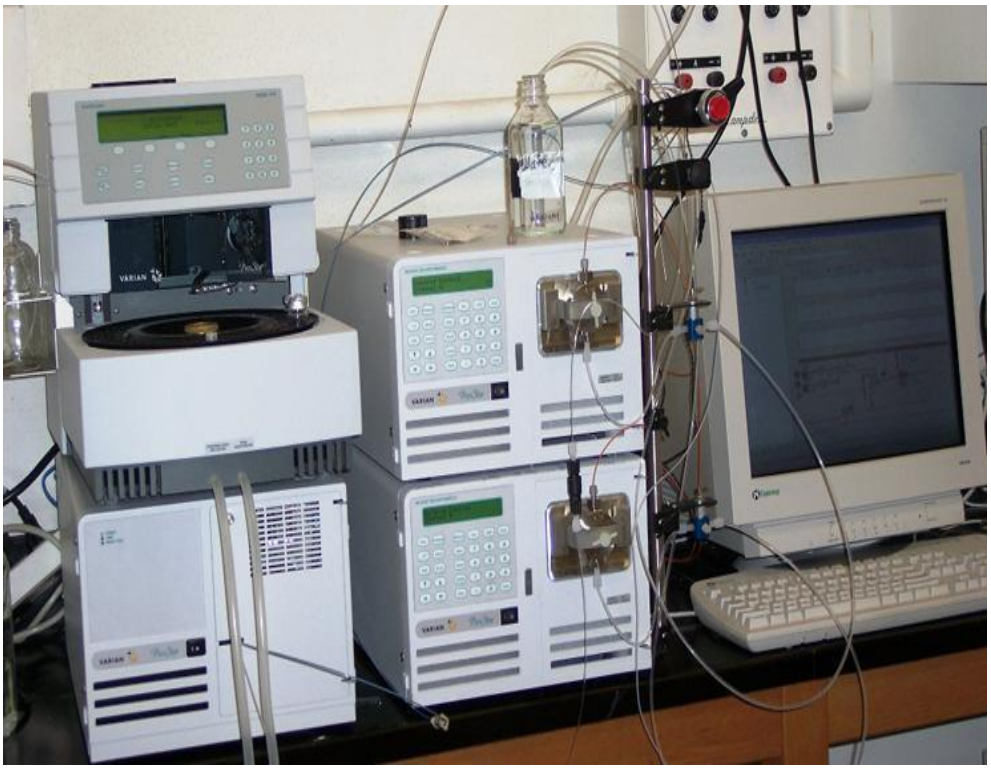

Figure.1.1. Typical high performance liquid chromatography system

1.4.4.4. High pressure pump

The central component of solvent delivery system is the pump since it directly influences the retention time, reproducibility and toxicity**.** High pressure pumps are capable of maintaining a constant pressure of the order 600bar and thus transport the mobile phase with a constant flow at a significant pressure for gradient elution [78]. Reciprocating pump with dual or triple pistons is most extensively used. A twin reciprocating system consists of two phases one for filling the valve and the other for pumping the mobile phase. A dumping device is essential to provide smooth flow and to circumvent critical noise at high level sensitivity.

Modern pumps have the following parameters

Flow rate range	: 0.0 to 5.0ml/min
Flow rate stability	: not more than 1%
For SEC flow rate stability should be	: less than 0.2%
Maximum pressure	: up to 300hPa

1.4.4.5. Injector system

Sample solution can be introduced into the flowing mobile phase near the head of the column with the help of an injector system. These systems generally contain fixed-loop and variable volume devices which can be operated physically or by an auto-sampler. Poorer injection volume precision is due to the partial filling of loops. Valve in the load position indicates the introduction of sample into the loop. At this moment eluent selects another passage to flow from the pump to the column. The loop will be redirected to flow into the column carrying the sample into its destination only when the valve is switched on to inject. In liquid chromatographic systems, liquid samples can be directly injected where as solid samples are required to be dissolved in a suitable solvent. Always the solvent need not be a mobile phase but regularly it is preferred to exit from detector interference, column/component interference and loss in efficiency or all of these. Continuous injections of particulate material may cause blockage in columns or injection devices. It can be avoided by filtering the sample over a 5μm filter or centrifuging. In general, small samples incorporated into a highly sensitive detector yields the highest column performance.

1.4.4.6. Column

Actual separation of components takes place in the column of the HPLC system. It is considered as the heart of the HPLC system. HPLC columns are usually made up of highly polished stainless steel with a column of length 10cm to 30cm and internal diameter 4.5mm to 5mm.

Excellent chromatographic performance is achieved by using silica (SiO_2) as the stationary phase but due to strong adsorption characteristics of unbounded silica these columns are seldom used for analytical work. The stationary phase consisting of a network of siloxane linkages (Si-O-Si) in a rigid three dimensional structure with interconnecting pores is more preferred for the separation of polar compounds. During manufacturing process, the pore size and the amount of silanol groups are controlled for the best performance. In a reverse stationary phase, silica is to be modified by a controlled reaction of organochlorosilanes with the silanol groups or the use of organoalkoxysilanes which alter the surface of the silica. This type of linkage imparts non-polarity to the surface and improves partitioning of compounds for the separation. The best material for making the stationary phase is the (ODS) octadecyl-silica (C18) and the other available materials are octyl (C8), phenyl (C_6H_5), cyanopropyl ($(CH_2)_3$-CN) and aminopropyl ($(CH_2)_3$-NH_2).

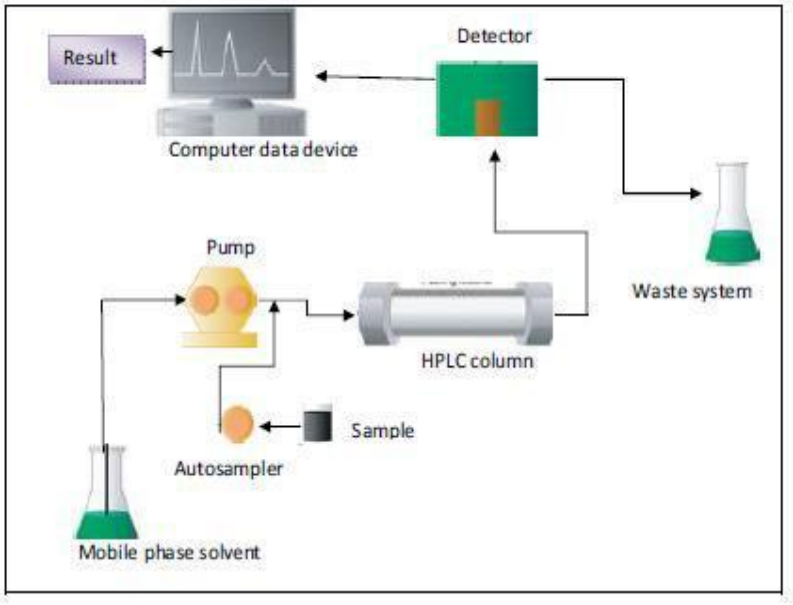

Figure.1.2. Basic instrumentation of HPLC

1.4.4.7. Detector

Today, optical detectors are most frequently used in liquid chromatographic systems. These detectors are capable of passing a beam of light through the flowing column effluent especially when it is passed through a low volume (~ 10µl) flow cell. The variations in light intensity caused by UV absorption, fluorescence emission or change in refractive index or the sample components passing through the cell are monitored as changes in the output voltage. These voltage changes are recorded on a strip chart recorder and frequently are fed into a computer to provide retention time and peak area data.

The most commonly used detector in LC is the ultraviolet absorption detector. Appropriate wavelength range for the detection of most of the samples is from 190 to 400nm and can be monitored by using a variable wavelength detector. HPLC frequently makes use of electrochemical detectors (ECD), fluorescent detector (FLUD), refractive index detector (RID), radioactivity detectors and the ultraviolet visible detectors.

1.4.4.7a. Electrochemical detectors

Compounds which undergo oxidation or reduction reactions use electrochemical detectors for analysis. These detectors are capable of measuring the difference in electrical potential when the sample passes between the electrodes.

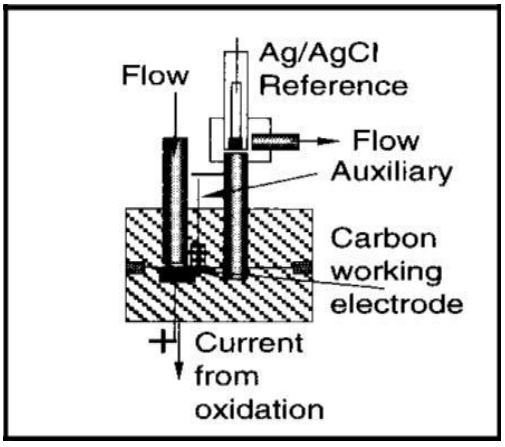

Figure.1.3. Electrochemical detector

1.4.4.7b. Fluorescent detectors

Fluorescence is the characteristic property a compound. At the given wavelength, these detectors are capable of measuring the ability of a compound to absorb and then re-emit light.

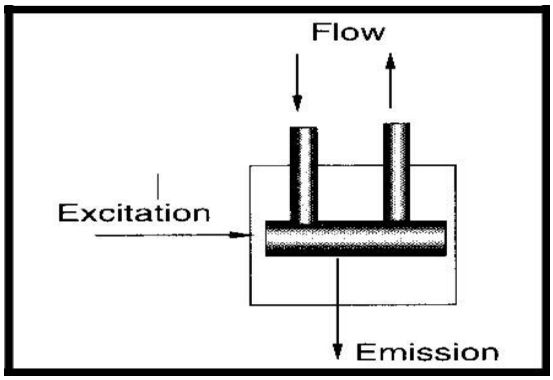

Figure.1.4. Fluorescent detector

1.4.4.7c. Refractive index detector (RID)

Refractive index of the analyte with respect to the solvent is measured by using refractive index detectors. Through refractive index detectors it is also possible to determine the quantity of light bent or refracted and the disparity between the two channels.

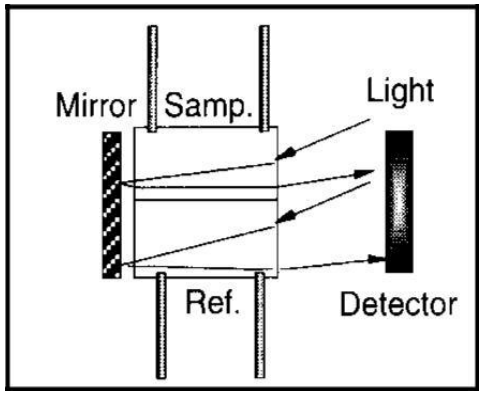

Figure.1.5. Refractive index detector

1.4.4.7d. Ultraviolet detector (UVD)

Among various types of detectors, the most widely used one is ultraviolet visible detectors. The UV detectors are well known for its selectivity, linearity, versatility and reliability for separation of analytes. UV detectors used in HPLC include diode array detector, fixed wavelength detector and the multi-length wavelength detector.

1.4.4.7e. Diode array detector

Diode array detector emits light over the UV spectrum range when deuterium or xenon lamp is used. An achromatic lens makes the light rays coming from the deuterium lamp to move in parallel and focus them on a holographic grating through a detector cell. At this moment, the sample is exposed to light ray of each and every wavelength. The array consists of many diodes and the output from a single diode is sampled by a data system which is stored on a hard disc. The output from the diode is selected and a chromatogram is produced by UV wavelength falling on the diode.

1.4.4.7f. Photodiode array detector

Light that suffers wavelength separation by a spectroscopic element can be noticed with the help of photodiode array detector. Photodiode array detector consists of a light receiving element, plurality units which are arrayed in the direction of dispersion of wavelength; and a charge accumulation time setting unit. Light coming out of the sample compartment is illuminated onto the inlet slit of the polychromator. Photodiode initially translates light to electrical signals and finally shows as time-series signals.

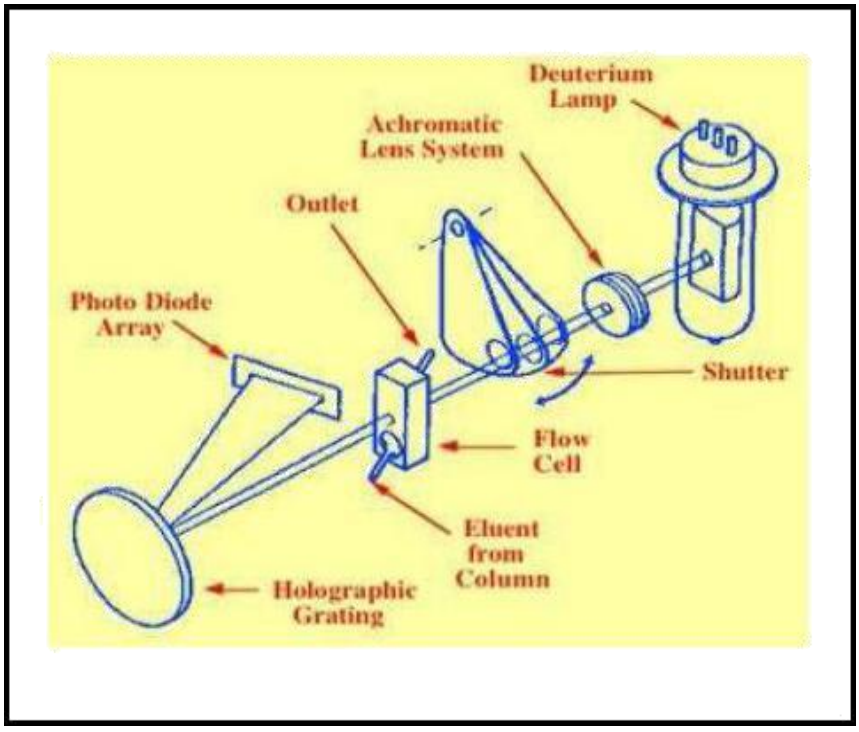

Figure.1.6. Photodiode array detector

1.5. METHOD DEVELOPMENT

Every year, the number of drugs introduced into the market is increasing. All the time these drugs are not the new entities, sometimes already existing drugs with partial structural modifications are introduced. There is a time lag between the date of introduction of drug into the market and its entry into the pharmacopeia. This is due to the development of patient resistance, introduction of better drugs, reports of new toxicities and possible uncertainties in the continuous usage of these drugs. Thus, no standard analytical procedures are available in the pharmacopoeias. Therefore, there is scope for the development of newer analytical methods for such drugs. Hence analytical method development and validation play a significant role in the discovery, development and manufacture of pharmaceutical formulations.

The advancement of any novel or superior technique for the investigation of a component generally depends on tailoring of the existing analytical approaches and instrumentation. Method development [79,80] usually involves selecting the method requirements and on the type of instrumentation. Selection of column, mobile phase, detector and method of quantitation is the primary step in HPLC method development. After the choice of instrumentation, determination of the chromatographic parameters for the analyte of interest is essential. The properties of the analyte(s) are useful to

1). select the nature of the column to be used

2). create proper composition and pH of the mobile phase for the partition of the components,

3). monitor suitable wavelength

4). select the concentration range to be followed and

5). choice of an appropriate internal standard for quantitation purpose.

If the information about the analyte or its related compounds is already available in the literature then it should be followed by optimization and preliminary evaluation of

the method. Optimization criteria must be determined with perception of the goals common to any new method. Initial analytical parameters such as sensitivity (measured as response per amount injected), limit of detection, limit of quantitation and linearity of calibration plots are to be determined. 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, asymmetry of peak, capacity, elution time, limit of detection, quantitation limit and overall ability to quantify the specific analyte of interest**.** Results acquired through optimization should be assessed against the goals of the analysis fixed forward by the analytical records of excellence. This evaluation reveals whether additional improvement and optimization are required to accommodate 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 probable optimization targets are resolution of the analyte from auxiliary components, distinctive peak identification, and on-line demonstration of purity and interfacing of computerized information for regular sample analysis.

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, keeping all the remaining conditions constant and evaluating the changes in response. The variables might include flow rate, mobile or stationary phase composition, temperature, detection wavelength and pH.

This univarient approach of system optimization is usually time consuming and expensive**.** However, it may provide a much superior perceptive of the principle involved and of the interaction of the variables. Experimental inputs are diminished and efficiency is maximized in computerized method development process. This kind of advancement is applicable to several methods. It considerably depress the time of analysis, energy, and cost of analysis.

1.5.1. Systematic approach for separation of pharmaceutical compounds through chromatography

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. Choice of initial conditions can be determined basing on nature of the sample. The inorganic ions, bio-molecules, carbohydrates, isomers, enantiomers and synthetic polymers, etc are called special compounds. When stable and reproducible columns are used for the method development, difficulties arising from irreproducible sample retention can be prevented.

Separation of basic compounds is possible by using definitely designed a C8 or C18 column built up of purified less acidic silica. If temperatures greater than 50^{o}C are used at low pH, sterically protected bonded phase column packing are preferred. A column is preferred, when acceptable resolution, proper pressure drop for different mobile phases and brief run times are achievable. Initial choice of column for various types of mobile phases is a 5µm, 4.6mm X 150mm column with a flow rate of 2ml/min. 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.

Acetonitrile (ACN) is favourable as mobile phase due to low viscosity and preferred UV transmittance and the next option is methanol (MeOH). Tetrahydrofuran (THF), an amine modifier is not advisable as it takes much longer column equilibration times and it creates a problem in method development and routine use of the method. Sometimes, they are responsible for the generation other problems like erratic base line and poor peak shape. However, when poor peak shapes or low plate number are experienced by few samples then utilization of amine modifiers is essential. While selecting the pH of the mobile phase one should follow two important considerations. A low pH that protonates column silanols and reduces their chromatographic activity is generally preferred.

The pH of the mobile phase should be selected with two important considerations. For columns that are stable at low pH, a pH of 2 to 2.5 is recommended as the retention of compounds is greatly rugged at low pH. In case of less stable columns, a pH of 3.0 is a most valuable option.

1.5.2. Separation temperature

Temperature controllers work effectively when the operating temperatures are more than 0^0C. At high operating temperatures, mobile phase viscosity diminishes and hence it requires low operating temperature and high plate numbers. 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 significant feature of a column is column plate number (N) and 'N' denotes the potential of the column to achieve sharp, narrow peaks for producing good resolution of band pairs with small values. This configuration provides a large enough N value for most separations and such columns are perfectly reliable. Short columns of 3 particles are useful for carrying out very fast separation (< 5 min). These columns are susceptible to sampling problems, more affected by band broadening effects and more operators dependent. Thus these columns are seldom used.

1.5.3. Peak asymmetry and peak tailing

Peaks with proper symmetry are obtained depending upon the nature of columns and experimental conditions. On the other hand asymmetric peaks results in unreliable plate number and resolution measurement, imprecise quantitation, degraded resolution, poor retention reproducibility. Peak asymmetry factor (A_S) and peak tailing factors are the terms used to measure the peak sharpness. Asymmetric factor of peak is measured at 10% of full peak height. Good columns produce peaks with A_S values of 0.95 to 1.1.

For proper analysis of symmetry, bands should be measured with a magnified time scale because asymmetrical bands also often appear symmetrical when observed in a compressed chromatogram.

1.5.4. Retention time

Retention time can be defined as the time required by an individual compound to show maximum peak height detection when passed across the column. No two compounds exhibit the same retention times. Retention of any compound depends on four factors such as pressure applied on the column, temperature of the column, composition of the solvent and the nature of the stationary phase.

1.5.5. Retention (capacity) factor (k)

Capacity factor is the relative measurement of time taken by the compound to remain in the stationary phase to that of mobile phase. It indicates how longer a sample is retained by stationary phase against its movement through a column with the mobile phase. It can be expressed with the help of the terms adjusted retention volume (time) and the hold-up volume (time).

$$k = \frac{V_R^{'}}{V_M} = \frac{t_R^{'}}{t_M}$$

A compound is extremely retained means it has spent more time in interacting with stationary phase and displays high k value.

1.5.6. Selectivity or separation factor (α)

Separation between two components is only possible if they have different migration rates through column. The measure of retention time of analytes is useful to calculate selectivity or separation factor. It is expressed by the following equation

$$\alpha = (t_2\text{-}t_a) / (t_1\text{-}t_a)$$

Its value is always more than unit. The selectivity factor (α) is greatly utilized to distinguish components chemically. Good separating power and a good separation between the apex of each peak are achieved when selectivity factor shows large values.

1.5.7. Column efficiency or number of theoretical plates (N)

A quantitative measure of the efficiency of the column is known as plate number and it can be determined from the values of retention time (t_R) and the standard deviation of the peak width**.**

$$N = 16 (t_R / W_b)^2 = 5.54 (t_R / W_{1/2})^2$$

Since it is difficult to measure 'W ' (Width at base of the peak), a relationship using width at half height or $W_{1/2}$ is frequently used to calculate 'N' as described in the USP.

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

$$HETP = L/N$$

1.5.8. Resolution (R)

It is the degree of separation between the 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.

$$R = 2 (t_2 - t_1) / (W_2 + W_1)$$

1.5.9. Tailing factor (T)

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

$$T = W_{0.05} / 2f$$

A value of 1.0 indicates a perfectly symmetrical peak. Tailing factor for the majority of peaks lies in between 0.9 and 1.4. Formulae for calculating the different system performance parameters are given in **Table.1.1**

Table.1.1. Formulae for different system performance parameters

Parameter	Formula
Relative retention	$t_R = (t_2\text{-}t_a)\ (t_1\text{-}t_a)$
Capacity factor	$k = (t_2\text{-}t_a) - 1$
Tailing factor	$T = W_{0.05}/2f$
Resolution	$R = 2\ (t_2 - t_1 / W_2+W_1)$
Theoretical plates	$N = 16\ (t / W)^2$
HETP	L/N

Where, t_R - 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 peak1,

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,

L - Column length, in meters.

1.6. VALIDATION OF ENDEAVOR METHODS

Consistent analytical methods are prerequisite in all the areas of analysis which obey national and international regulations. Association of Official Analytical Chemists (AOAC), International Conference on Harmonization (ICH), Pharmacopoeias and Eurochem documents have been prepared numeral protocols and guidelines [81-90] on method validation. A laboratory is internationally recognized when it confirms proper methods in providing quality data. Therefore for any laboratory, method validation [91] is the vital element to produce reliable analytical data [92].Whenever a new analytical method is developed, it is essential to validate it according to standard protocols to certify the suitability of the test for the required purpose. Many International organizations and conferences [93-105] issued several guidance documents on method validations to guarantee patent safety to any analytical procedure.

Validation is the process of certifying that a test procedure is executing within suitable 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.

Validation studies involve the description of final method for the specified sample and testing the appropriateness of samples, sample matrix and data handling. Finally, the same can be conducted in another laboratory having suitable equipment. No single validation approach is available that can be always applied for a new method. Preliminary step in validation is the true approach. A new method is approved in any field depending on the specific validation approaches used by the analysts.

Hence, it is the conscientiousness of the individual analyst to select the correct validation method(s). Validation approaches may be of several kinds such as zero, single, and double-blind spiking methods.

1.6.1. 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 in general is fast, simple and useful, it leads to subjective results and doubt on the part of the unbiased reviewer or end user. Nevertheless, the first approximation and a demonstration of validation potential, requiring minimum 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.

1.6.2. 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, single-blind approach is more precious and authentic 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 had proved successful, but before one decides to involve additional analysts or management.

1.6.3. 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 had 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.

1.6.4. 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. Organizations specialized in preparing, guaranteeing and marketing standard reference materials of various analyte species in different sample matrices are USP and NIST. 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. An analyst should provide accurate and precise measurements of the analyte in a particular sample matrix by using SRMs. Analyst bias becomes an issue, especially when the analyst knows the amounts and levels of the SRM.

1.6.5. 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; sometimes it may require several years to finish the overall process. During that period, the analysts are supposed to exhibit significant effort in coordinating the process, shipping samples and receiving results, statistically analyzing and interpreting the results and then finally interpreting and verifying the data. Even though, the approach is operator dependent (generating laboratory to laboratory variability), when quantitative values obtained from different laboratories are overlapping in comparison with known levels, then the method is fully validated and is approved . If the method is described for the first time in the literature then it is used seldom.

1.6.6. Comparison with a currently accepted method

A new validation approach is comparison of the method with a currently established method. 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 proves that the currently accepted method is indeed invalid, the analyst must then initiate an alternative approach to validate the new method. The question that eventually arises is 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 normally less precise and difficult than one's performed standard reference material (SRM) development.

The main aim of the assay methods is to evaluate the analyte present in a given sample. In this connection, the attempt represents a quantitative measurement of the major component(s) in the drug substance. Analogous validation features are valid to the drug product when assaying for the active or other selective component(s). At the same time these validation attributes can be exercised to assays associated with other analytical procedures (e.g. Dissolution).

1.7. METHOD VALIDATION

Validity of the method is determined by making use of linear regression analysis and relative standard deviation. Accuracy, reliability and precision of the method are also examined during validation process. Characteristic analytical parameters which are essential during the validation of the method comprised of accuracy, linearity, range, ruggedness, robustness, limit of detection, limit of quantification, selectivity and specificity.

1.7.1. Precision

The precision of an analytic method is the measure of degree of closeness of individual data values obtained when the process is applied to several homogeneous samples under the given circumstances. Precision is defined as the extent of the repeatability of the entire analytical technique under normal operational conditions. Precision of the method is evaluated by testing a sample for ample number of times to achieve statistically valid results. Precision is generally conveyed in terms of relative standard deviation, variance or coefficient of variation**.** The ICH guidelines state precision on three levels viz repeatability, intermediate precision and reproducibility.

1.7.1a. Repeatability (Intra-day precision)

Repeatability denotes the preciseness of an analytical method under the same experimental conditions by the same analyst measured within a short period of time. Repeatability measurement requires the evaluation of at least six determinations at absolute test concentration. The limit of percentage of relative standard deviation of an assay method should be below 2%.

1.7.1b. Intermediate precision (Inter-day precision)

Intermediate precision of any analytical process assess the validity of the method developed in a diverse environment and certifies that the method will give the same results when the development process is completed. According ICH,

intermediate precision is defined as the long-term variability measurement process, communicating within laboratory variation such as analysis on different day, by analyst and/or different equipment. It is usually examined over a period of days to weeks by relating the outcomes of an analytical method. In general, intermediate precision of an analytical method is calculated on three successive days by injecting in triplicate (n=3) at three different concentrations.

1.7.1c. Reproducibility

In order to enclose an analytical method in an official compendial publication inter–laboratory trials are essential to calibrate the proposed process. It is also possible to transfer the developed process from one laboratory to the other depending upon the extent of reproducibility of proposed method. The reproducibility of developed method can be considered as the check of precision of an analytical procedure in different laboratories i.e. precision between laboratories. Quantitative determination of impurities comprises an analysis of precision.

1.7.2. Accuracy

The accuracy of an analytical process is described as the proximity of an experimental value to true value of the same sample under the investigation. Accuracy of an analytical procedure can be expressed in many ways, consisting of comparison to a reference standard, recovery of spiked analyte and the standard addition of an analyte to a sample. Especially for drug analysis accuracy is measured at 50,100 and 150% of the label claim.

1.7.3. Linearity

The linearity of an analytical technique is its capability (within a given range) to acquire test results within the concentration (amount) of analyte in the sample. Linearity is established by computing the regression line by means of a statistical treatment of least mean squares vs analyte concentration.

1.7.4. Range

The range of an analytical process is the gap between the higher and lower levels of an analyte in the sample with sufficient precision, accuracy and linearity.

1.7.5. Robustness

The robustness is ability of an analytical technique to persevere unaffected by small but considered alterations in the process parameters. Assessment of robustness is mandatory during the developed stage of any analytical process. Under the normal circumstances it provides the sign of reliability of technique. Most commonly used parameters during the measurement of robustness are pH of the mobile phase, mobile phase composition, temperature, flow rate and different columns. Significance of robustness is to ensure the validity of the analytical process over a sequence of system suitability parameters when ever intended. Analytical conditions must be controlled or a counteractive statement must be incorporated in the procedure if the measurements are vulnerable to modifications in an analytical conditions.

1.7.6. Ruggedness

Ruggedness is the extent of reproducibility of outcomes attained by the analysis of the same sample by the analysts, laboratories, instruments, reagents, assay temperature etc.

1.7.7. Limit of detection and limit of quantitation

It is the lowest concentration of a sample at which the sample is detected. Limit of detection is essential for impurity tests. Limit of quantitation is the lowest concentration at which the analyte is detected and analysed with enough accuracy and precision. LOD and LOQ are measured depending upon the response of the standard deviation and slope. Slope is obtained from the calibration curve of the analyte.

Analysis of pharmaceutical products containing a single drug is most familiar and common one. Under these circumstances analysis of pharmaceutical products containing two drugs gained much interest. In addition to this, when degradation studies are coupled it draws the attention of various categories of people as it provides the valuable information about the stability of pharmaceutical products under different stress conditions.

1.8. FORCED DEGRADATION STUDIES

Forced degradation studies are highly useful in the development of formulation and package procedure. The main aim of the degradation studies is determination of stability of molecule [106]. Other advantages are the establishment of degradation pathways and validation of stability indicating procedures [107-111]. Safety, efficacy and quality are the major objectives of forced degradation study. Minimum stress conditions which are applied in forced degradation study are acid and base hydrolysis, oxidation, thermal degradation and photolysis [112-114]. Always it is not necessary to get a degradation product as the resultant of forced degradation studies. In general, degradation of drug products up to 20% is accepted for validation of chromatographic assays [115-116] but in case of small pharmaceutical molecules the allowed degradation limit is only up to 10% [117].

1.8.1. Aqueous degradation

Sample solution for aqueous degradation is prepared by dissolving 300mg of sample in 20 ml of double distilled water and the solution is allowed to stand for about two days. Then the solution is diluted up to the required dilution. The resulting solution is used to get chromatogram from which it is possible to identify the number of degradation products.

1.8.2. Acid degradation

Sample solution to measure the acid degradation is prepared by dissolving 300mg of sample in 20ml of 0.1N hydrochloric acid and the solution is left undisturbed for 48hr. 5ml of sample solution after dilution is neutralized with 5ml of sodium hydroxide solution. 20µl of this solution is utilized to get the acid degradation chromatogram. Chromatogram provides the valuable information about the number of degradation products.

1.8.3. Base degradation

To have the clear idea about the base degradation, sample solution is prepared by dissolving 300mg of sample in 20ml of 0.1N sodium hydroxide solution and kept aside for two days. The solution is neutralized with 20ml of 0.1N hydrochloric acid and then diluted. This solution is used to obtain the base degradation chromatogram.

1.8.4. Peroxide degradation

To study the peroxide degradation, sample solution is prepared by dissolving 300mg of sample in 20ml of 3% hydrogen peroxide solution. The solution is allowed to stand for 48hr and then diluted. The solution is injected into HPLC system to get the oxidation degradation chromatogram.

1.8.5. Thermal degradation

Thermal degradation of the sample is measured by pouring the sample solution into a petri dish which is maintained at 40^0 to 80^0C temperature in an oven and kept undisturbed for two days. After thermal exposure, chromatogram is measured by injecting 20µl of the sample solution into HPLC system. The number of degradation peaks can be easily recognized from the chromatogram.

1.8.6. Light and UV light degradation

Initially, the sample is transferred into a petri dish and is kept under the ordinary light for two days. Then, the sample solution is prepared and is used to measure the number of degradation products. Similarly, the sample solution is placed under UV light for two days to measure the degradation products.

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