1. INTRODUCTION:

1.1 MEDICINAL AND DRUG PROPERTIES:

Drugs are to be anticipated division of our life. We utilize the drugs since the children intuitive till it dies. The reputation of the drugs is an essential quality as it honestly affects the life of end user. The quality of whichever contraption or stuff can be best judges by analyzing it.

Analysis is an element of science and it deals with the quantitative and qualitative level of any area under discussion. Analysis can bestow us the answer what is at hand and how much is nearby in the theme.

Analysis is set up in with reference to every banch whether it is data analysis, advertise analysis or pharmaceutical analysis. Amid these the pharmaceutical analysis deals with the superiority of pharmaceutical harvest and to finish life of the consumer. A different obligation for appeal drug is that the authoritarian and Government agencies became harsh in case of meager reputation drug commodities. Eminence of any drug invention can be notorious by a chain of tests stating from the testing of raw substance, intermediates and finished products etc. Drug analysis deals with the detection, characterization and quantification of drugs in singular or in combination as in dosage forms, biological fluids and bulk drugs. As a pharmaceutical analyst we are fearful in qualitative analysis of individual drugs whether it is at hand in the specified textile and in quantitative analysis we are concerned in the amount of drug at hand in the individual sample.

A long time, man has extended many substances for pharma products depending upon the nature of cure. In fact each country has the record of generating medicines which are native. In few cases permanent relief may be found. These types of drugs are used in case of non infectious diseases. Conformation with the quantity indices is obligatory. The broadly used medicines are in the right place to Allopathic system. Any substance carefully used for changing any structure or purpose of the body is called a Drug1. The preparation, chemical work, physical work, reactive environment, constitution and geometry control on an individual. Quality organize method, storage space environment, living
and the similar to which are prerequistics in the learning of drugs plunge below a probable pasture common laws of chemistry2-7. Each equivalent be put principles nation a legislation8. More than a few pharmacopoeia publications do furnish these policy. IP9, USP10, BP11 and MEP12 are such several standard references. Pharmaceutical analysis13-14 through moreover by way of their precursors. Anything may be the claim for any check, excellence is paramount15. It is therefore essential that a second and substandard quality drugs are kept at arms length. Prevention is also the prime factor here.

Behavior quite a few blood cardiovascular endocrine CNS gastrointestinal emerged the control of blend beleaguered useful device 16-20.

Simple distillation is useful for determination alcohol content of the galanicals or other substances being volatile in current of steam such as methol, thymol and even certain alkaloids such as ephedrine. Moisture contents have been determined by drying in a dedicatar 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 through the most specific and environment procedure being Karl-Fisher titration method, end point being detected manually or by electrometric automatic titration. The flurimeter developed into the sample such as thiamine hydrochloride.

Analytical Chemistry belongs ever authentic part of determine the chemical contain of different materials of nature. The methods of this science are used to determine the substances which may be include in a material and to find out the exact amounts of the determined substances.

Analytical chemistry may be term as the technique and skill of shaping the components of content in terms of the elements or compound contained21. Analytical chemistry is important in nearly all aspects of chemistry. Analytical techniques are important in assuring, maintaining the quality of the content and components of Q.A.

/Q.C. The responsibilities of analytical chemist are the measurement of the utility, reliability, accuracy, interception and specificity.
The universal terms for any pharmaceutical analysis comprise of necessary procedures involved the identity and strength of drugs and chemicals along with quality and purity of drugs and chemicals. The analytical chemistry used for qualitative and quantitative analysis.

a) Qualitative Analysis:
Qualitative analysis deals with the determination of elements, ions or compound present in the sample.

b) Quantitative Analysis:
Quantitative analysis deals with the determination of how much part of one or more component are present in the sample.

Expansion of Analytical process:
The development of process is to be measured accurately, assess the quality and the validity of the products. The development methods have the following parameters:

- As simple as possible, minimal time for analysis.
- Most specific and minimal maintenance problems.
- Most productive, economical and convenient.
- As accurate and precise as required.
- Multiple source of key component (reagents, columns, TLC plates) should be avoided.
- To be fully optimized before transfer for validation of its characteristics such as accuracy, precision, sensitivity, ruggedness etc.

1.2 DIFFERENT ANALYTICAL TECHNIQUES:
The various methods of analysis can be broadly classified into four categories:
The include determination of colour (for liquids), moisture content, melting, freezing and boiling points.

1. Physico-chemical /Instrumental Methods:

A. Optical Methods:
   a) Absorption of EMR: (Eg.UV, Visible, IR Spectroscopy, NMR, Atomic Absorption spectroscopy)
   b) Emission of EMR: (Eg. Fluorimetry, Flame Photometry)
   c) Scattering of EMR: (Eg. Nephlometry, Turbidimetry, X-Ray Diffraction)
   d) Refraction: (Eg. Refractometry)
   e) Rotation of EMR: (Eg. Polarimetry, SpectroPolarimetry)
   f) Other Methods: (Eg. Mass Spectroscopy)

B) Electrochemical Methods:
   a) Conductometry
   b) Polarography
   c) Potentiometry
   d) PH metry

C) Separation Methods:
   a) Chromatography: (Paper Chromatography)
   b) Column Chromatography: (Eg. HPLC, TLC, HPTLC, Gas Chromatography)
   c) Electrophoresis

D. Thermo Analytical Methods:
   a) Tharmo gravimetrik Analysis
b) Differential thermal Analysis

c) Differential Scanning Colorimetry

2. CHEMICAL /CLASSICAL METHODS:

A. Volumetric Methods:

a. Acid Base Titration or Neutralization Titrations

b. Oxidation Reduction Titration or Redox Titrations

c. Precipitation Titration

d. Complexation Titration

e. Displacement Titration

f. Non aqueous Titration

B. Gravimetric Methods

3. RADIO ACTIVE METHODS:

Radio Immuno Assay (RIA)

4. BIOLOGICAL METHODS:

a. Biological Assay: (Using Animals)

b. Microbiological Assay: (Using Micro organisms)

In four categories a new analytical method of analysis can be separated:

1. Spectroscopical methods like UV-visible spectroscopy, Infra Red spectroscopy (IR)

2. Chromatographic methods, High Peformance Liquid Chromatography (HPLC), Size exclusion chromatography (SEC), HPTLC, SFC.

3. Radiometric methods like isotopic dilution.

For victorious disconnection and estimation of drugs in combination, chromatographic techniques like HPLC, GC, HPTLC, SEC, SFC etc are in the main worn now days. When the chromatographic separation is not bounty for the complete analysis, two techniques are combined and second-hand concurrently, they are acknowledged as hyphenated techniques. Quite a lot of examples of hyphenated techniques are HPLC-MS, GC-MS, MS-MS, LC-MS-MS etc. The cited methods have their be the owner of recompense and disadvantages. One has to be especially disbelieving for the mixture of analytical method to acquire a hold the obligatory in rank.

1.3 INTRODUCTION TO ABSORPTION SPECTROSCOPY:

Photometric techniques is among the most significant instrumental techniques available to the pharmaceutical analyst. Instruments range from simple flame photometers, which are used to determine the concentration of certain metallic elements to much more expensive spectrometers such as ultraviolet-visible and nuclear magnetic spectrometer which are used in structural and quantitative analysis of molecules. The two parts divided spectroscopy is a electromagnetic radiation matter:

Atomic spectroscopy

This belongs with the reaction of electromagnetic radiations by way of atoms, which are most generally present in their lowly energy state called the opinion state.

Molecular spectroscopy

This belongs with the interface of electromagnetic radiation through molecules.

UV-VISIBLE SPECTROPHOTOMETRY:

The procedure of ultraviolet/visible spectrophotometry is one of the habitually affianced in pharmaceutical.s. It involves the measurement of the quantity of ultraviolet (190-380 nm) or
visible (380-800 nm) emission fascinated by a substance in solution. Light absorption in the UV/Visible region causes the transition of electron from lower state to higher state. The important consequences of rapid relaxation of the excited states are not appreciably distributed by absorption of light energy from any source. Therefore, the fraction of light absorbed from an incident beam is independent of the intensity which is integrated to obtain Beer's and Lambert's law.

Absorption spectrum:

The absorption spectrum is a graphical representation of the amount of light absorbed by a substance at definite wavelengths. To plot a absorption curve, the values of the wavelengths (.) are laid off along the axis of abscissas and the values of the absorbance along the axis of ordinates. A characteristic of an absorption spectrum is a position of the peaks (maxima) of light absorption, which is determined by the absorptivity at definite wavelengths.

Beer Lambert's law: It can be stated that as the intensity of beam of monochromatic light when passed through transparent medium decreases as the width and concentration of absorbing media increases.

\[ A = \log \frac{I_0}{I_t} = abc \]

Where, \( A \) = Absorbance of the solution at particular wavelength of the light beam

\( I_0 \) = Intensity of incident light beam

\( I_t \) = Intensity of transmitted light beam

\( a \) = Absorptivity of atom at the wavelength of beam

\( b \) = Path extent of cell in cm

\( c \) = Concentration of solution in gm/lit

Beer's law is said to be obeyed above a awareness series if a plot of absorption beside absorbance passes through foundation and is a straight line.

Beer's law plot:
A standard sample of the analyte is full and the solutions of it with known concentrations are organized. The maximum calibration curve is plotted by laying off the known concentrations along the axis of abscissas and the absorbances corresponding to them along the axis ordinates. The calibration curve helps to identify the unknown concentration of the analyte in its solutions.

A characteristic of organic drugs is the existence of functional groups in their molecules. Functional groups determine the way of analyzing organic drugs because they are responsible for the properties of substances and conclude the detection reactions and the methods of quantitative determination of drugs. Knowing the reactions for detecting functional groups, one can easily analyze any organic drug with a complicated structure by determine the reactions for identified the functional groups. There are several drug molecules which are polyfunctional in nature i.e., simultaneously contain two or more functional groups. Functional groups in drugs can be classified into three categories.

1) Functional groups imparting an acidic property to a substance:

Phenolic hydroxyl, Carboxyl, Imide, Sulfhydral or thiol, Enol, Nediol.

2) Functional groups imparting basic properties to a substance: Free or substituted primary, secondary amino group (in aliphatic, alicyclic and aromatic system) [The tertiary nitrogen is a necessary element in molecules of alkaloids and heterocyclic compound].

3) Functional groups which exhibit neither acidic nor basic properties: Keto, Hydroxymethyl, Nitroso, Nitro, Methoxy, Ether, Lactone, Lactam, Olefinic, Acetylenic. Terms used in absorption spectroscopy:

a) Transmittance (T): It is the ratio of intensity of transmitted light to that of incident light.

\[ T = \frac{I_t}{I_o} \]

b) Absorbance (A): It is the negative base 10 logarithm of transmittance.

\[ A = -\log_{10}T = \log_{10}\frac{I_o}{I_t} \]

\[ A = abc \]
c) Molar absorptivity (e): When concentration, c, in equation A = abc is expressed in mole/lit and cell length in, cm. then Absorptivity is called as molar absorptivity.

\[ e = \frac{A}{bc} \]

Steps to be followed to develop new Multicomponent analysis methods are:

1) Literature Survey:

Existing analytical methods for Multicomponent formulations to be analyzed are scanned to shun copying of the method. Further the information solubility; absorption maxima and the molar absorptivities in various solvents of the individual components of the multicomponent formulation are obtained.

2) Selection of a Solvent:

A solvent or solvent mixture in which all the components in the formulations are soluble and stable is chosen. Another point that needs consideration in selecting the solvent is the difference in absorbance maxima of the component in the particular solvent. Greater the difference in the absorbance better will be the result.

3) Selecting the concentration of each component in mixed standards and Sample analysis:

The concentration of each component in the mixed standard is determined from molar absorptivity values of the component and the ratio in which the different components are present in the formulation to be analyzed. The sample analysis is repeated to confirm the accuracy, reproducibility and repeatability of the method.

4) Selection of the analytical Wavelengths:

Spectroscopic absorbance measurements are ordinarily made at an absorption peak. This absorption peak corresponds to the wavelength because the change in absorbance of wavelength per unit concentration is greatest at that point. The maximum sensitivity is realized at that point. So, analytical wavelengths are selected considering the peaks and valleys in other wavelengths where the various components show the difference in the absorbances.
5) Type of Instrument:

It is the heart of analytical method because more advanced the instrument; greater the sensitivity, greater will be the accuracy of result and confidence with which the results are reported. Instrument used should be calibrated.

Fig: 1.3.1 UV-VISIBLE SPECTROPHOTOMETER

6) Evaluation of reproducibility:

To ensure that proper conditions have been selected and that no important variables have been overlooked, the tentative method should be critically evaluated with respect to Beer’s law.

Quantitative analysis by UV/Visible spectrophotometer:

Single component analysis:

When the absorption of each of a series of solutions of the same substance are measured at the same wavelength, temperature and solvent conditions, a graph of absorbance measured can be plotted against its concentration. If the graph is a straight line passing through the starting point, then it is said to obey Beer’s law.

The slope of the line is equal to “ab”, where “b” is the internal path length of the sample cell in cms and “a” is the absorptivity calculated which is constant. The concentration of a component in a sample which contains other absorbing substances, this may be determined by a simple Spectrophotometric amount of absorbance is described over. If and only if provided that the other components have a sufficiently small or negligible absorbance at the wavelength of
measurement. Once this is resolute, the analysis of known samples of this substance can be easily done under the same identified experimental conditions. Finally absorbance is measured and from the Beer's plot, the unknown concentration can be calculated.

Multicomponent analysis:

Now a day's multicomponent formulation are finding their place in clinical therapy. Hence there is a need. A brief review of the methods used for multicomponent analysis is discussed.

Standard addition method:

This complex samples are within which the likelihood of matrix effects is significant.

1.4 DEVELOPMENT OF A METHOD

The only difference involved is being the concentration of the analyt involving the calculation of an overload of an analytical reagent and the concentration of the reagent. The analytical wavelength can be chosen either from literature search or by experimentally means of scanning a spectrum in the UV-visible region. The preparation of standards and unknowns should always be on a definite time schedule.

Optimization of analytical method:

The basis of the spectrophotometric methods, in the present investigation are (a) oxidation reduction, (b) oxidative coupling, (c) oxidation followed by charge-transfer complex formation, (d) diazotisation and coupling (e) oxidation followed by complex formation reaction (f) condensation with aromatic aldehydes. In every one type of reaction, the colored yield of the species absorbance is calculated. These absorbance states the sensitivity of the method, rate of colour formation and stability. These parameters signify weather they are affected by the concentration of the reagent in the solution. The property of the solvent, the temperature, the pH of the medium, order of addition of reactants and waiting periods also affect the above parameters. For simple systems having no interaction between variables, the one variable at a
time (OVAT) strategy appears to be simple, efficient and effective to establish the optimum conditions. The OVAT approach requires all variables but one to be held constant while a univariate search is carried out on the variable of interest. The details of fixing optimum conditions in different procedures used in present investigations are furnished in subsequent chapters.

The method of least squares:

This method (Least squares) is useful to explain the association between signal and concentration. All models recounting the connection between response (Y) and concentration (X) can be represented by the general function.

\[ Y = f(X, a_1, b_1, \ldots, b_m) \]

where \( a_1, b_1, \ldots, b_m \) are the parameters of the function.

We adopt the convention that the \( X \) values relate to the controlled or independent variable and the \( Y \) values to the dependent variable. The values of the unknown parameter \( a_1 \) \( b_1, \ldots, b_m \) must be estimated in such a way that the model fits the experimental data points as far as possible. The true relationship between \( X \) and \( Y \) is considered to be given by a straight line. The relationship among each study pair \( (X_i, Y_i) \) can be represented as

\[ Y_i = a + \beta X_i + e_i \]

The signal \( Y_i \) now \( a \) and \( \beta \) of the true values and \( a \) and \( \beta \) which are constants. This is done by calculating values of \( a \) and \( \beta \) for which \( e_i^2 \) is minimal. The component \( e_i \) represent the differences between the observed \( Y_i \) values and predicted \( Y_i \) value by the model. The \( e_i \) are called the residuals, \( a \) and \( \beta \) are the intercept and slope respectively. The equations given for slope and intercept of the line are as follows.
Correlation coefficient (r):

The correlation coefficient $r(X,Y)$ is further constructive to convey the bond of the select balance. To attain a correlation coefficient, the covariance is separated by the invention of $X$ and $Y$.

\[
\text{Slope (b)} = \frac{n \sum_i X_i Y_i - \sum_i X_i \sum_i Y_i}{n \sum_i X_i^2 - [\sum_i X_i]^2}
\]

\[
\text{Intercept (a)} = \frac{\sum_i Y_i \sum_i X_i^2 - \sum_i X_i \sum_i X_i Y_i}{n \sum_i X_i^2 - [\sum_i X_i]^2}
\]

\[
r = \frac{\left[ \frac{1}{n} \sum_{i=1}^{n} (X_i - \bar{X})(Y_i - \bar{Y}) \right]}{\left( \frac{1}{n-1} \sum_{i=1}^{n} (X_i - \bar{X})^2 \right) \left( \frac{1}{n-1} \sum_{i=1}^{n} (Y_i - \bar{Y})^2 \right)}
\]

Where $X$, $Y$ are arithmetic means of $X$ and $Y$ respectively.

Selectivity of the method:
An analyte may be disturbed by matrix and interference effects. Some of the Excipients, incipient and additives present in pharmaceutical formulations may sometimes interfere in the assay of drug and in such instances; appropriate separation procedure is to be adopted initially.

In the primary obstruction studies, a fixed concentration of the drug is determined some times by the optimum method in the occurrence of a suitable (1-100 fold) molar surfeit of the unfamiliar compound under examination and its outcome on the absorbance of solution is noticed. These unfamiliar compound is considered to be non-interfering if at these concentrations, it constantly produces an error less than 3% in the absorbance produced in pure sample solution. Sandell.s sensitivity34 refers to the numeral of µg of the treatment resolute, changed to the coloured produce.

Ringbom.s plot:

The relative concentration error depends inversely upon the product absorbance and transmittance. The relative error increases at the extremes of the transmittance (T) scale.

The slope of plot „C. versus T, i.e. Ringbom.s plot35-36 gives relative error coefficient (i.e. plot of log C a T).

The main limitations of Ringbom plot is that it provides no information concerning the concentration range of good precision unless it is combined with ∆T versus T relation. The above expression is valid irrespective of Beer.s law validity.

Precision and accuracy:

The principle of estimation of “true” moving is the purpose to attain elected, generally mentality. It jointly resolve character purpose. It mainly imperative measures.

Precision:

Precision37 extent surrounded by spread or scattering regarding cost. period „,set. is definite sovereign repeat capacity of a few. Frequent provisions people of inspection. entity outcome from outcome. The square of the It is every so often multiplied by 100 and articulated as a percent relation. This is a further exact quantify of the precision.
Accuracy:

Accuracy relates to the variation among a result or mean and the true value. For analytical methods, there are two potential ways of determining the accuracy, absolute method and the comparative method.

Absolute method:

The test for the constituents and proceeding according to the specified instructions. The variation between the means of an adequate number of outcome and the amount of component in reality present, frequently expressed as parts per hundred (%) i.e. % error. The basic in question will typically have to be resolute in the presence of further substances, and it will therefore be needed to know the cause of these upon the determination. This will involve testing the influence of a large number of probable compounds in the chosen samples each in varying amounts. In a few instances, the accuracy of the method is controlled by separations (usually solvent extraction or chromatography technique) involved.

Comparative method:

In the analysis of pharmaceutical formulations or laboratory prepared samples of desired composition, the comfortable of the constituent required has been gritty by two or more (proposed and official or reference) ,,accurate. methods of in essence special character. It can usually be established as indicating the absence of a significant determinate error. The general procedure for the assay of pharmaceutical formulations either in the proposed or reference methods comprise of various operations which include sampling, preparation of solution, separation of interfering ingredients if any and the method for quantitative assay.

Recovery experiments:
Component being determined direction of the sample, which are analysed for the total amount of component at hand. The added component gives the recovery of the amount of added component. If acceptable recovery is observed then the assurance in the accuracy of the method will be superior.

Generally these type of recovery studies are performed while proceeding for pharmaceutical formulations. Then the recognized, which had been previously analyzed.

Evaluation of precision and accuracy by comparison of two procedures:

To evaluate the accuracy of the method, one often compares the method being investigated or test method. With an existing method called the reference method.

Student t test:

Student t test is used to evaluate the resources of two related (paired) samples analysed by reference and test methods. It gives answer to the correctness of the null hypothesis with a certain confidence such as 95% or 99%. If the number of pairs (n) are smaller than 30, the condition of normality of X is required or at least the normality of the differences (di). If this is the case the quantity,

\[ t = \frac{d}{Sd\sqrt{n}} \]

where \( di = XR \) (reference method) – \( XT \) (test method) and \( S \) is the standard deviation.

F-test:

By the F-test one can evaluate the significance of the difference in the variances of reference and test methods. Suppose that one carries out \( n \) replicate measurements by using reference methods. If null hypothesis is true, then the estimates \( Sr2 \) (variance of test method) and \( SR2 \) (variance of reference method) do not differ very much and their ratio should not differ much from unity. In fact, one uses the ratio of the variances.
\[ F = \frac{S_r^2}{S_R^2} \]

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

1.5 INTRODUCTION TO HIGH PERFORMANCE LIQUID CHROMATOGRAPHY:

HPLC utilizes diverse types of stationary phases, and analyte from side to side the signal the feature. Its connections with the ration/work of art of solvent(s) used, the mobile phase. The mobile phase can be gaseous or liquid. According to mechanisms of separation, chromatographic methods are divided into two main types. They are adsorption and partition chromatography.

Chromatographic rule of Conduct
Classification of Liquid chromatographic methods according to mechanism of retention:

Fig: 1.5.1 Classification of liquid chromatography
Table 1.5.1 Most commonly used Method in HPLC

<table>
<thead>
<tr>
<th></th>
<th>Reverse Phase Chromatography</th>
<th>Normal Phase Chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mechanism</strong></td>
<td>Retention by interaction of the stationary phase’s non-polar hydrocarbon chain with non-polar part of sample.</td>
<td>Retention by interaction of the stationary phase’s polar surface with polar parts of the sample molecules</td>
</tr>
<tr>
<td><strong>Stationary Phase</strong></td>
<td>bonded siloxane with non-polar functional groups like bonded siloxane with polar functional group like SiO₂, Al₂O₃,</td>
<td></td>
</tr>
<tr>
<td><strong>Mobile Phase</strong></td>
<td>Polar solvents like methanol, acetonitrile, water or buffer (Sometimes with additives of THF or dioxane).</td>
<td>Nonpolar solvents like heptane, hexane, cyclohexane, chloroform, ethyl ether, dioxane.</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------------------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Application</strong></td>
<td>Separation of nonionic and ion forming nonpolar to medium polar substances (carboxylic acids - hydrocarbons)</td>
<td>Separation of nonionic, non polar to medium polar substances.</td>
</tr>
<tr>
<td><strong>Elution Order</strong></td>
<td>Most polar components are eluted first.</td>
<td>Least polar components are eluted first.</td>
</tr>
</tbody>
</table>

**Basic of Separation**

HPLC method development follows a series of steps which are summarized below:
A. Nature of sample

Nature of sample is especially main in the method development. Inclusive description of sample is existing. What are the components present, excipients at hand should be branded. Impurity at hand in sample must be famous.
Some imperative information regarding samples:

a. Number of compounds present.
b. Chemical structure.
c. Molecular weight.
d. PKa value.
e. UV spectra.
f. Concentration range of compound of interest.
g. Sample solubility.

**B. Separation goals**

Goal of HPLC separation necessity to be notorious visibly.

a. Primary goal of HPLC separation is whichever quantitative analysis or detection of impurity.
b. Is it compulsory to determine all sample workings?
c. If quantitativ analysis is required what level of accuracy precisin needed.
d. Sample pre-treatment required or not.
e. For how many samples (different) method should be designed.
f. How various samples resolve be analyzed at one time?

**C. Sample pre-treatment**

Sample pre-treatment is exceptionally imperative in progress of an original method. Most of sample obligatory dilution prior to injection.

a. Solution ready for injection.
b. Solution requires dilution, addition of buffering agent, internal standard.
c. Solid that must be dissolved or extracted removes of interference to protect the column.
d. Direct injection communication precession. Volumetric dilution before injection.

Unsurpassed outcome are achieved when meditation of sample in the chips are same as mobile phase. Nature and awareness as samples are very central since concentrated analytic can break the column.

In HPLC,

There are two types of stationary phases mainly reverse phas and normal phas.

a. **Normal phas HPLC:**
b. **Reversed phas HPLC:**
About 75% of current HPLC analysis is performed using the reverse phase. Mainly silica chemically bonded through a siloxane (Si-o-Si-C) linkage to a low polar function group. These phases are arranged by treating the surface silanol groups of silica with an organochorosilane reagent. The polarity of the column can be changes by varying the alkyl chain length in R.

For our studies mainly we have used C-18 columns

**HPLC Detectors**

RI detectors, UV detectors, Florescent detectors, Electrochemical detectors and Photo diode-array detectors (PDA) are available. For the present study UV and PDA detectors are mainly used.

**UV Detectors:**

It depends of UV rays wavelengths.

**PDA detectors:**

It measure the range of absorption

**HPLC method development**

The following steps are follows:

Step 1: Initial studies

Step 2: Selection of chromatographic condition

Step 3: Selectivity optimization

Step 4: System parameter optimization

Step 5: Method validation

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**1.6 Validation of analytical method:**

By a good number set of laws and quality standards are mandatory by validation of analytical methods that force laboratories.

ICH guidelines (Q2A and Q2B), The US Food and Drug Administration (FDA), i.e. these methods should be validated as mentioned below:

**1.6.1 Literature from industrial committee and regulatory agencies**

<table>
<thead>
<tr>
<th>Committees and regulatory agencies</th>
<th>Guidelines available</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICH</td>
<td>Q2R1 Guidelines are guidelines for new</td>
</tr>
</tbody>
</table>
Validation Parameters

As per ICH guidelines the following tests are validated:

- Identification test
- Tests for impurities content
- Limit test for the control of impurities
- Quantitative tests of the active ingredient or supplementary chief apparatus of the drug

These various parameters for validation of any analytical method are:

- **Specificity**: It is ability to ensure from excipient and/or impurities.
- **Linearity**: It is curve of calibration was constructing by maneuverings peak vicinity vs concentrations of analyt.
- **Range**: 80% to 120% of the test concentration is required as per ICH course of action for test assay.
- **Accuracy**: Accuracy of the scheme in the present study % improvement was calculated by the following formula

\[
\text{Precision: } \% \text{RSD} = \frac{\text{STD DEV}}{\text{MEAN}} \times 100
\]

Assay values were calculated with the following formula

\[
\% \text{ Assay} = \frac{\text{Area of Sample}}{\text{Area of Standard}} \times \frac{\text{Concentration of Standard}}{\text{Concentration of Sample}} \times \frac{\text{Purity of Standard}}{100} \times 100
\]
At three altitude precision considered: Repetability, Intermediate Precision, Reproducibility

(a) Repetability: Over a diminutive interval of time, it expresses the precision under the same conditions. It is expression intra assay precision.

(b) Intermediate Precision: It communicates, within laboratories variations, different days, different analysts, and different equipment and so on.

(c) Reproducibility: It convey the precision among laboratories.

- Detection Limit (DL): 
  Limit of detection (LOD) was calculated by with the formula
  \[ \text{LOD} = 3.3 \times \frac{\sigma}{S} \]
  Where, \( \sigma \) = Standard deviation of respons
  \( S \) = Slope of regression equation

- Quantitation Limit (QL): Determined suitable and is particulary worn for the purpose of impurites and/or degradation goods.
  \[ \text{LOQ} = 10 \times \frac{\sigma}{S} \]
  Where, \( \sigma \) = Standard deviation of respons
  \( S \) = Slope of regression equation

- Robustnes: It is the degree of reproducibility of consequences acquired by the analysis of the similar.

**System Suitability Tests (SST)**

It is the type of procedure to analyzed sample.

System suitability test parameters are:

- Plate number or number of theoretical plates (n)
- Capacity factor (capacity ratio) k
- The selectivity or Separation Factor (relative retention) a
- Peak Resolution R
- Peak asymmetry factor (A_s).

1. **Plate number or number of theoretical plats (n):**

\[
\text{Number of Theoretical Plates} = 16 \left( \frac{1}{R^2} \right) \\
\text{Or:} \quad \text{N} = 5.5 + \left( \frac{1}{R_{1/2}} \right)^2
\]
Fig: 1.6.1 Plate number or number of theoretical plates

Where, \( t_R \) is the retention time and \( W \) is the peak width.

\( W \frac{1}{2} = \) peak width at peak height

\( W_b = \) peak width at base

\( t = \) retention time of peak

2. Capacity factor (capacity ratio) \( k \)

\[
k' = \frac{t_R - t_0}{t_0}
\]

Fig: 1.6.2 Capacity factor (capacity ratio) \( k \)

Where, \( t_R = \) retention volume at the apex of the peak (solute)

\( t_0 = \) void volume of the system

3. The selectivity or Separation Factor (relative retention) \( \alpha \)

\[
\alpha = \frac{k'_{(2)}}{k'_{(1)}}
\]

Fig: 1.6.3 The selectivity (or separation factor), \( \alpha \)

4. Peak Resolution \( R \) Resolution (\( R_s \)):
Fig: 1.6.4 Peak Resolution R

5. Peak asymmetry factor (Tf):

Asymmetric factor or Tailing factor

Fig: 1.6.5 Peak Asymmetry factor (Tf)

Table: 1.6.2 SYSTEM SUITABILITY TEST PARAMETERS AND RECOMMENDATIONS

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical Plates (N)</td>
<td>should be &gt; 2000</td>
</tr>
<tr>
<td>Tailing Factor (T)</td>
<td>T of ≤ 2</td>
</tr>
</tbody>
</table>

STABILITY INDICATING HPLC METHOD:

Looking for the exterior of extra peaks in chromatogram, a different skill of assessing specificity is to delibrate degrade the sample. Whichever in the existence or lack of added matrix machinery this come close to accepted out, depending on the probable exercise of the method. By a further performance, there will necessitate to be unwavering the concern degradation. No distress for assay of that sample will habitually materialize with sample degradation. Acid, Base,
Heat Light and Oxidation are involved in degradation study. 10-30% of the original samples are degraded by the studies of planned. Secondary chemical harvest that are less to be expected to occur in factual samples generated peaks are pragmatic degradants. Authentic degradation peaks will form when these studies time and again point out.

The following situation for sample 10-30% for drug substance and tablets form.

a) 0.1 N HCL (acid)
b) 0.1 N NaOH (base)
c) 50°C (heat)
d) 600 foot-candles of UV light
e) 3% hydrogen peroxide solution

<table>
<thead>
<tr>
<th>VALIDATION OF THE DEVELOPED METHOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical Procedure</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Specificity</td>
</tr>
<tr>
<td>Precision</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Accuracy</td>
</tr>
<tr>
<td>Linearity</td>
</tr>
<tr>
<td>Range</td>
</tr>
<tr>
<td>Detection Limit</td>
</tr>
<tr>
<td>Quantitation Limit</td>
</tr>
</tbody>
</table>

VALIDATION PARAMETERS
### Parameter Acceptance criteria

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>No interference of placebo in titration with active drug</td>
</tr>
<tr>
<td>Precision</td>
<td>Repeatability RSD NMT 2.0%</td>
</tr>
<tr>
<td></td>
<td>Intermediate Precision RSD NMT 2.0%</td>
</tr>
<tr>
<td>Accuracy</td>
<td>Recovery (98.0% to 102.0%)</td>
</tr>
<tr>
<td>Linearity</td>
<td>$R \geq 0.999$</td>
</tr>
<tr>
<td>Ruggedness</td>
<td>NMT 2.0%</td>
</tr>
<tr>
<td>Robustness</td>
<td>NMT 2.0%</td>
</tr>
<tr>
<td>System Suitability</td>
<td>RSD for replicate injection NMT 2.0%</td>
</tr>
<tr>
<td></td>
<td>Theoretical Pletes NLT 2000</td>
</tr>
<tr>
<td></td>
<td>Tailing Factor NMT 2.0</td>
</tr>
</tbody>
</table>

**1.7 OBJECTIVES OF THE STUDY**

A patient taking a pharmaceutical product expects the product to be safe and efficacious. Due to abundance of pharmaceutical agents available in the pharmaceutical market in various dosage forms either as a single drug component or in combination by way of further drugs and also due to potency of the most of the drugs, it becomes necessary to quantitate these agents in their formulations in a precise manner.

Today’s planet is a locale where cut-throat antagonism is seen. People are departure on construction new drugs and new formulations of the offered drugs within exceedingly tiny period of time. To search out the authoritarian permission for marketing company has to submit mandatory data together with the examination gossip as to provide evidence that their drug invention is of vital quality for its intended use. For these drugs and formulations there are only some standard official measures to be had for its analysis so, we necessitate to increase several scheme for the identification and estimation of drugs.

The prime consequence of drug analysis to put on in rank on the subject of the qualitative and quantitative masterpiece of substances and species, that is to hit upon out what a core is
serene of and faithfully how much. This information guides advance of the manufacturing function and therapeutic action of drugs. India, a mounting country, with fast industrialization and hasty evolution on all fronts, is making big strides towards global recognition. The flip side is that the trade and industry riches and new way of life is translating. The flip side is that the economic success and recent way of life is translating into an amplify in lifestyle related infection. Home to practically millions of asthma diseases at present, India is fast appropriate the asthamatic capital of the world.

Pharmaceutical regulatory agencies worldwide demand that the product retains its quality, purity, and potency for the time the product is commercially available. Consequently the agencies expect to see stability data supporting the proposed expiry date of the product in the marketing submission. In the broader sense the analytical method validation studies of that are conducted.

Therefore there is always a need to develop validated analytical methods which are precise, accurate, selective and sensitive the drug products. The intention of the here work is develop validated analytical methods with the help of which we can determine quantitate drug.

The specific aim of the work undertaken was:

- Analytical procedure for drugs in biological fluid is not available.
- The presented system for analysis of drugs are lengthy, time consuming or expensive.
- To develop validated analytical method based on HPLC for Montelukast Sodium as a single component in bulk and in a tablet formulation.
- To study applicability of developed HPLC means for the purpose of Montelukast Sodium in occurrence of its degradation goods.
- To develop validated analytical method based on HPLC for Salbutamol Sulphate as a single component in bulk and in a tablet formulation.
- To study applicability of developed HPLC method for the resolve of Salbutamol Sulphate in occurrence of its degradation products.
- To overcome typical challenges encountered while developing and validating methods for pharmaceutical products containing single active ingredients.
• The purpose of the at hand revision is to develop and validate the Analytical method for Anti Asthamatic drugs with healthier selectivity and this method applies in industry to assure the first-class quality of drugs.
• The industrial analytical method will be linear, precise, accurate and robust and will help scientist for standard analysis in pharmaceutical organization by quality control department.
• The validated analytical method can be worn for analysis of stability testing in pharmaceutical industry.
• Methods for both drugs will validated for the future intended analytical studies and applications in drug formulation and production.
• Assay test is chief part in manufacturing of drug substances and drug products.

• Assay value determines the purity of merchandise and slip in quantity of assay will be creating the shock on end user. End users are human being; consequently determination by new performance like HPLC will be gives the accuracy of outcome.

Thus new analytical method will be residential on innovative recent instruments reminiscent of HPLC and manner will be facilitate to industry to estimate the potency of drugs accurately.

• The method to be develops for help out in Analytical development lab to present the sustain for tentative revision of route development and formulations development study.
• The method to be develops for analytical prep up in pre-formulation study in pharmaceutical industry.

In nearby analysis we have urbanized simple isocratic RP-HPLC process for quantitative opinion of Montelukast Sodium and Salbutamol sulphate.

Stages of planned work

1. Review literature

2. Select proper solvent system for analysis

3. Developing chromatographic methods for single component formulations

4. Validating the developed methods as per the ICH guidelines.
Plan of work

1. Collection of single component formulation from market

2. Selection of analytical techniques

3. Development of chromatographic method for Montelukast Sodium and Salbutamol Sulphate in bulk and pharmaceutical formulations

- Selection of suitable detection wavelength
- Selection of mobile phase and its composition • Checking system suitability parameters.

1.8 DRUG PROFILE

1.8.1 Montelukast Sodium\textsuperscript{61-99}

**Category:** Antiasthmatic. (add-on therapy for mild to moderate asthma)

**Molecular formula:** C\textsubscript{35}H\textsubscript{35}ClINaO\textsubscript{3}S

**Molecular weight:** 608.2g/mole

**Chemical Structure:**

![Chemical Structure Image]

**Chemical Name:** Monosodium salt of 1-[[[(1R)-1-3-[(1E)-2-(7-chloro-2-quinolinyl)ethenyl]phenyl]-3-[2-(1-hydroxy-1-methylethyl) phenyl] propyl]thio[methyl] cyclopropaneacetic acid

**Physicochemical Properties:**

**Description:** A white to pale yellow powder
**Solubility:** It is freely soluble in water and in methylene chloride, freely soluble to very soluble in ethanol

**Official Status:** Montelukast Sodium is official in United States Pharmacopeia, Indian Pharmacopeia & British Pharmacopeia

<table>
<thead>
<tr>
<th>Marketed Formulations</th>
<th>Name of Manufacturer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MONTAIR-10</td>
<td>CIPLA LTD.</td>
<td>Each film-coated tablet contains Montelukast Sodium IP equivalent to Montelukast 10mg</td>
</tr>
<tr>
<td>B/N: D22600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mfg Dt. Nov/2012</td>
<td></td>
<td></td>
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<tr>
<td>Exp. Dt. Oct/2015</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 1.8.2 Salbutamol Sulphate^{100-113}

**Category:** Beta-adrenoceptor agonist

**Molecular formula:** \((C_{13}H_{21}NO_{3})_2H_2SO_4\)

**Molecular weight:** 576.7g/mole

**Chemical Structure:**

![Chemical Structure](image)

**Chemical Name:** (RS)-1-(4-hydroxy-3-hydroxymethyl-phenyl)-2-(tert-butylamino) ethanol sulphate

**Physicochemical Properties:**

**Description:** white or almost white, crystalline powder

**Solubility:** It is generously soluble in water, basically indiscernible or especially faintly soluble in ethanol and in methylene chloride
**Officeal Status:** Salbutamol Sulphate is official in Indian Pharmacopeia, British Pharmacopeia, United States Pharmacopeia and European Pharmacopeia

<table>
<thead>
<tr>
<th>Marketed Formulations</th>
<th>Name of Manufacturer</th>
<th>Composition</th>
</tr>
</thead>
</table>

### 1.9 IMPORTANCE OF WORK

In this era of medicinal chemistry, there are numbers of new molecule treat as medicine and developed the molecules as to complete the property of medicinal chemistry. In the way of new development of drug molecule, there is very important to check their medicinal property on primary base or in the form of clinical trials. In the process of clinical trials the new molecule of compounds undergoes the different phases starting from phase zero to phase IV and in this method, there must be the development of molecule in the process of method development and method validation. On the basis of that the molecule passed out from the process of the clinical trials. After that the new molecule passed out the different phases then it gives the permission to form the medicine and give it to permitted to form in bulk process in the form of tablet or capsules in this both form after the formation of tablets and capsule formation there is must be assay the percentage of the active molecules by to development of method of two different medicine are montelukast and salbutamole to form and find out percentage assay of the active molecules.

In this novel work there has been tried to developed the method to find out the method of assay to form the percentage of the active molecules of active ingredients. In this method there is a development of the method and also to validate it. This method is validated for 10mg tablets of montelukast drug which is used for the patients of asthma and also the other formulation 4mg tablets of salbutamole is also validated for their assay as per ICH guidelines as per given in the formulation of different assay. In this method development and validation there is effort to develop the cost effective method compare to the other methods mentions for to check the assay of formulations. There is also one benefit of when there is unavailability of other chemical this method will used as optional methods. All the assay of method validation performed as per the guidelines of ICH which is very useful for the future intended analytical operations as well as
production of new methods of the drugs so, the material and reagents for the development and validations of the method are very phesible and also the reagents which are used in the method development and validation are also available also in the financially cost effectiveness of industrial strategy so, all the factors are very useful for the depicted in the method development and validation of future intended analytical operations of the methods.