Analytical Methods: A Research

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01.INTRODUCTION

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

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

Pharmaceutical analysis, a branch of pharmacy, plays a very significant role in quality control of pharmaceuticals through a rigid check on raw materials used in manufacturing of formulation and on finished products. Analytical chemistry has since long, occupied an important place in the development of science and technology. It is primarily concerned about determining the qualitative and quantitative composition of material under study. The qualitative analysis gives us the information about the nature of sample by knowing about the presence or absence of certain components. The quantitative analysis deals about the content present in the sample. The development in analytical sciences has been more significant and prominent in recent years than the past. This has really broaden our vistas and helped to develop new methods of analysis. In pharmacy analytical chemistry is responsible for developing sensitive, reliable and more accurate methods for the estimation of drug in pharmaceutical dosage form. [3]

1.1 Analytical chemistry

Analytical chemistry is an important part of pharmaceutical analysis. Analytical Chemistry may be defined as the science and art of determining thecomponents of materials in terms of the elements or compound contained. Analytical Chemistry seeks ever improved means of measuring the chemical

composition ofnatural and artificial materials. The techniques of this science are used to identify the substances which may be present in a material and to determine the exact amounts of the identified substances.

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

The discipline of analytical chemistry consists of

- **Qualitative Analysis:**

  Qualitative analysis deals with the identification of elements, ions or compoundspresent in the sample.

- **Quantitative Analysis:**

  Quantitative analysis deals with the determination of how much amount of one ormore constituents are present in the sample.4

Importance of Analytical Chemistry

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

Selection of Analytical Method

Method should be,

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

Classification of Analytical Methods

Analytical methods Classified into two categories; Classical methods and Instrumental methods:

- **Classical Methods:**For qualitative analysis the separated compounds are treated with reagents that could be recognized by either colour, by their boiling or melting points, their solubility.
- **Volumetric Methods:** In volumetric, also called titrimetric, procedures, thevolume or mass of a standard reagent required to react completely with theanalyte is measured.
- **Gravimetric Methods:** In gravimetric measurements, the mass of the analyteor some compound produced from the analyte is determined. The extent of their general application is, however, decreasing with the passage of time and with the advent of instrumental methods to supplant them.
- **Instrumental Methods:**

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

a) Spectroscopic Techniques

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

b) Electrochemical Techniques

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

c) Chromatographic Techniques

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

d) Miscellaneous Techniques

- Thermal analysis
- Mass spectrometric
- Kinetic techniques

e) Hyphenated Techniques

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

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

1.2 Chromatographic techniques: [7-8]

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

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

Classfication:

1) Based on the nature of stationary and mobile phase

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

2) Based on the principle of separation

- Adsorption chromatography
- Partition chromatography

3) Based on the modes of chromatography

- Normal phase chromatography
- Reversed phase chromatography

4) Other types of chromatography

- Ion – exchange chromatography
- Exclusion chromatography

Adsorption chromatography, the analytes interact with solid stationary surface and are displaced with the eluent for active sites on surface. Partition chromatography, result from a thermodynamics distribution between two liquid or liquid like phases on the basis of relative polarities of stationary and mobile phases. Partition chromatography can be divided in to normal phase and reverse phase

chromatography. In normal phase chromatography the stationary bed is strongly polar in nature (e.g., silica gel) and the mobile phase is non polar (such as n-hexane or tetrahydrofuran).

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

1.3 Spectrophotometry:[9-11]

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

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

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

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

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

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

- Mathematically Beer- Lamberts law is expressed as:

  A = a.b.c

  Where,

  A = absorbance or optical density

  a = absorptivity or extinction coefficient

  b = path length in cm

  c = concentration of solute in solution

1.4. High Performance Liquid Chromatography:-[12-17]

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

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

1.4.1Types of High Performance Liquid Chromatography:

a) Normal phase HPLC:

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

decrease the retention time of the analytes while more hydrophobic solvents tend to increase retention times.

b) Reversed phase HPLC:

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

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

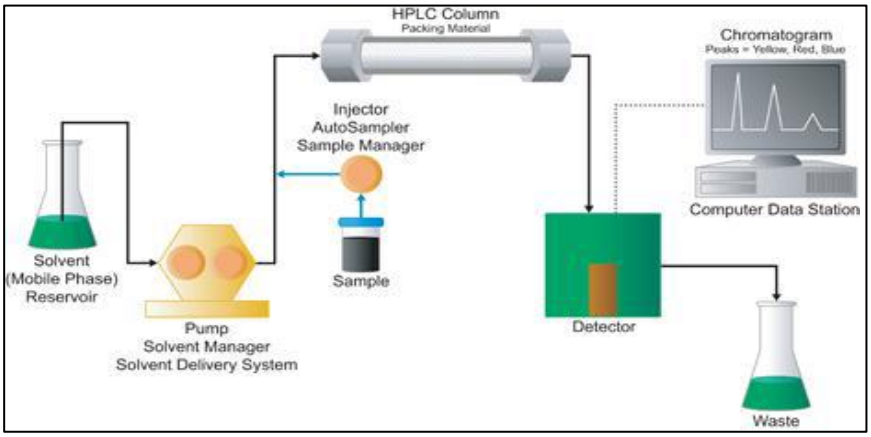


Figure 1.1: Isocratic LC System

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

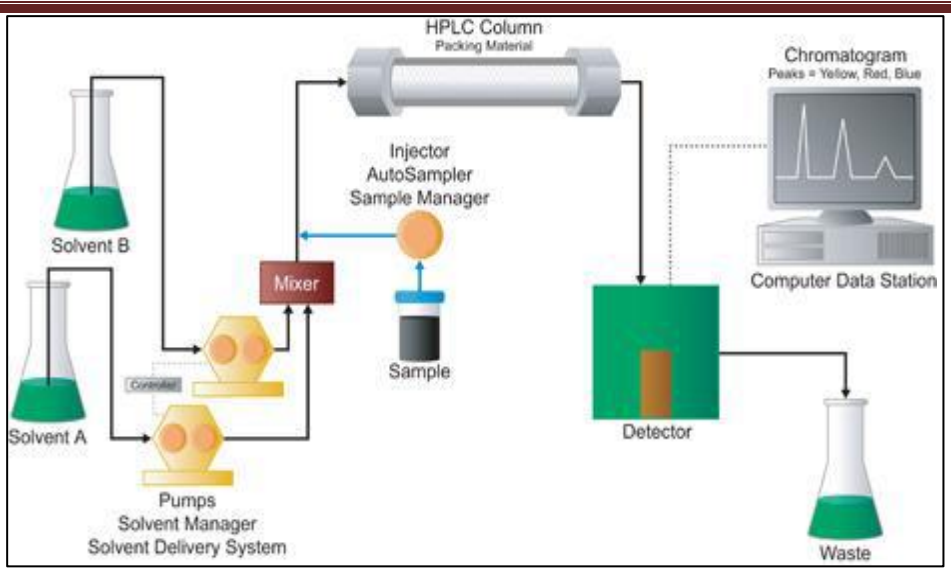


Figure 1.2: Gradient LC System

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

1.4.2Instrumentation and theory of operation of HPLC:

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

a) Solvent delivery system:

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

Figure 1.3: Typical HPLC Waters System

b) Pumps:

1. Pumps are required to deliver a constant flow of mobile phase at pressure ranging from 1 to 550 bar (14.6 to 8000psi).
2. Mechanical pump give a pulsating supply of mobile phase.
3. Flow rate range: 1 to 10ml/min.

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

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

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

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

c) Injection systems:

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

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

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

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

d) Column:

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

Different types of column that are used include:

- **Guard columns:** They are placed anterior to the separating column. They are for protective purpose. They are dependable columns designed to filter or remove particles that clog the separation of the column. These are used in thefollowing cases Compounds and ions that could ultimately cause “baseline drift”, decreased resolution, decreased sensitivity and create false peaks.
- **Preparative columns:** These columns are utilized when the objective is to prepare bulk of sample for laboratory applications. Accessories important to mention are the backpressure regulator and the fraction collector. Back- pressure regulator is designed to apply constant pressure to the detector outlet, which prevents the formation of air bubbles within the system. This, in turn, improves chromatographic baseline stability. It is usually designed to operate regardless of flow rate, mobile phase, or viscosity.
- **Capillary columns:** These are also known as micro columns, capillary columns have a diameter much less than a millimetre and there are three types:

  i) Open-tubular

  ii) Partially packed

  iii)Tightly packed

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

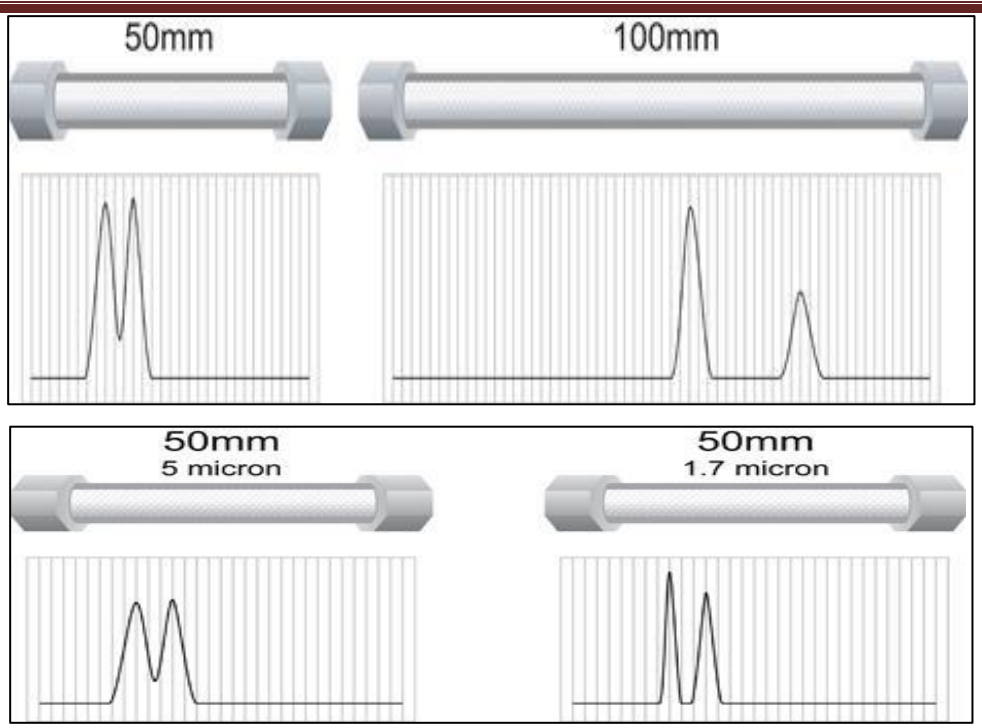


Figure 1.4: Types of Column

e) Detectors:

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

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

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

f) Recorder:

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

1.4.3 Applications of HPLC:

a) **Analytical HPLC:**

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

b) **Preparative HPLC:-**

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

c) Identification:

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

d) Chemical Separation:

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

e) Quantification:

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

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

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

1.4.4Advantages of HPLC:-

a) Capable of separating complex mixtures at low operating temperature.
b) Identification of an unknown solution.
c) Quantification of a compound in a known solution.
d) Capable of separating materials according to size and /or chemical properties.
e) Can be used to separate delicate or heat labile compound.

1.5 Method development by HPLC:[18]

The development of a method of analysis of any compound is usually based on existing literature, using same or quite similar instrumentation. But now days HPLC based method is not similar as compare to existing literature based approaches. The development of new or any improved method should be beneficial in any way than existing method. Method development usually requires selecting the method requirement and deciding the instrumentation to utilize for what purpose.

There are different kinds of reasons for developing new method for analysis:-

- There is no any suitable method for a particular compound or combination of that compound.
- The exiting method may be too error, contamination prone or may be not well suitable.
- The existing method may be too expensive, time consuming or may not be easily automated.
- The existing method may be having less accuracy or precision.

- The existing method may not provide adequate sensitivity or analyte selectivity in the sample.
- There may be need of alternative method to conform a legal or scientific reason, analytical data originally or turned by existing method.

1.5.1Step of HPLC method development: [19-24]

HPLC method development involves several essential steps: sample pre-treatment, detection of sample bands, choosing separation conditions, quantitation and method validation. The wide variety of equipment, columns, eluent and operational parameters involved makes high performance liquid chromatography (HPLC) method development seem complex.

Step 1: Selection of the HPLC method and initial system

When developing an HPLC method, the first step is to find the chemical composition of the sample which can provide valuable clues for the best choice of initial conditions for an HPLC separation. Depending on this information two approaches to HPLC method development are possible. Some chromatographers try to match the "chemistry" of the sample to a best choice of initial HPLC condition. To do this they rely on their own past experiences (i.e. separation of compounds of similar structure) and/or they supplement this information with data from the literature. Sample preparation is an essential part of HPLC analysis, intended to provide a reproducible and homogeneous solution that is suitable for injection into the column. The aim of sample preparation is to get a sample aliquot that 1) is relatively free of interferences (2) will not damage the column, and (3) is compatible with the intended mobile phase i.e., the sample solution will be miscible with the mobile phase without affecting sample retention or resolution. It may also be desirable to concentrate the analytes and/or derivatize them for improved detection or better separation. e.g.When the peak shape does not improve by lower (1-2) or higher (8-9) pH, then ion-pair chromatography can be used. For acidic compounds, cationic ion pair molecules at higher pH and for basic compounds, anionic ion-pair molecules at lower pH can be used. For amphoteric solutes or a mixture of acidic and basic compounds, ion-pair chromatography is the method of choice.

Step 2: Selection of initial conditions:

This step determines the optimum conditions to adequately retain all analytes; that is, ensures no analyte has a capacity factor of less than 0.5 (poor retention could result in peak overlapping) and no analyte has a capacity factor greater than 10–15 (excessive retention leads to long analysis time and broad peaks with poor detect ability). Selection of the following is then required.

Step 3: Selectivity optimization:

The aim of this step is to achieve adequate selectivity (peak spacing). The mobile phase and stationary phase compositions need to be taken into account.

Step 4: System parameter optimization:

This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved. The parameters involved include column dimensions.

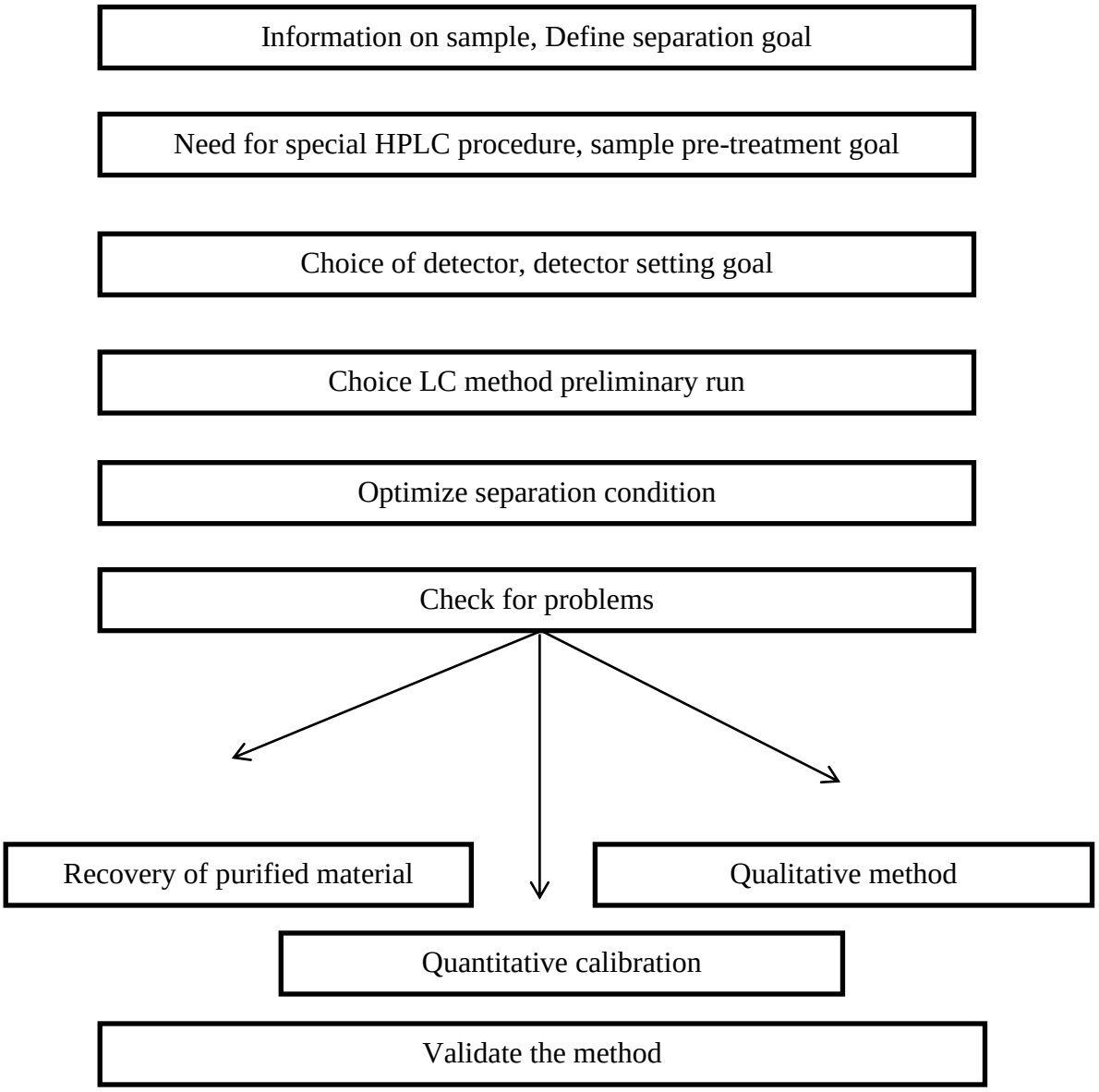


Figure 1.5: Method development by HPLC often follows these series of step

1.6Factor influencing HPLC method development:

Methods for analysing drugs in multi-component dosage forms can be developed, provided one has knowledge about the nature of the sample, namely, its molecular weight, polarity, ionic character and the solubility parameter.

1.6.1Mobile phase composition:

The mobile phase in HPLC refers to the solvent being continuously applied to the column, or stationary phase. The mobile phase acts as a carrier for the sample solution. As a sample solution flows through a column with the mobile phase, the components of that solution migrate according to the non-covalent interactions of the compound with the column. The chemical interactions of the mobile phase and sample, with the column, determine the degree of migration and separation of components contained in the sample. In general one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups and are water soluble. The organic phase concentration required for the mobile phase can be estimated by gradient elution method. For aqueous sample mixtures, the best way to start is with gradient reversed phase chromatography. Gradient can be started with 5-10% organic phase in the mobile phase and the organic phase concentration (methanol or acetonitrile) can be increased up to 100% within 30-45min. Separation can then be optimized by changing the initial mobile phase composition and the slope of the gradient according to the chromatogram obtained from the preliminary run. The initial mobile phase composition can be estimated on the basis of where the compounds of interest were eluted, namely, at what mobile phase composition. If the retention times are too short, the decrease of the organic phase concentration in the mobile phase can be in steps of 5%. If the retention times are too long, an increase of the organic phase concentration is needed. The low solubility of the sample in the mobile phase can also cause bad peak shapes. It is always advisable to use the same solvents for the preparation of sample solution as the mobile phase to avoid precipitation of the compounds in the column or injector. By sight change of the mobile phase composition, the position of the peaks can be predicted within the range of investigated changes. An optimized chromatogram is the one in which all the peaks are symmetrical and are well separated in less run time.

1.6.2 Polarity of mobile phase:

Changing the polarity of mobile phase can alter elution of drug molecules. The elution strength of a mobile phase depends upon its polarity, the stronger the polarity, higher is the elution.

1.6.3 pH of mobile phase:

The pH of the mobile phase has to be selected in such a way that the compounds are not ionized. Ionic samples (acidic or basic) can be separated, if they are present in undissociated form. Dissociation of ionic samples may be suppressed by the proper selection of pH.

1.6.4 Selection of wavelength:

In UV detection, good analytical results are obtained only when the wavelength is selected carefully. This requires knowledge of the UV spectra of the individual components present in the sample. If analyte standards are available, their UV spectra can be measured prior to HPLC method development. The molar absorbance at the detection wavelength is also an important parameter. Even if the compounds exhibit higher λmax, they absorb strongly at lower wavelength. It is not always necessary to detect compounds at their maximum absorbance. It is, however, advantageous to avoid the detection at the sloppy part of the UV spectrum for precise quantitation.

1.6.5 Sample concentration:

When peaks are not detected in the chromatograms, it is possible that the sample quantity is not enough for the detection.

1.6.6 Volume of injection:

An injection of volume of 20µl from a solution of 1 mg/ml concentration normally provides good signals for uv active compounds around 220 nm.

1.6.7 Additionof peak modifiers:

The addition of peak modifiers to the mobile phase can affect the separation of ionic samples. For examples, the retention of the basic compounds can be influenced by the addition of small amounts of triethylamine (a peak modifier) to the mobile phase. Similarly for acidic compounds small amounts of acids such as acetic acid can be used. This can lead to useful changes in selectivity. When tailing or fronting is observed, it means that the mobile phase is not totally compatible with the solutes. In most case the pH is not properly selected and hence partial dissociation or protonation takes place.

1.6.8 Column selection:

The peak resolution can be increased by using a more efficient column (column with higher theoretical plate number, N) which can be achieved by using a column of smaller particle size, or a longer column. These factors, however, will increase the analysis time. Flow rate does not influence resolution, but it has a strong effect on the analysis time.

1.7Parameter used in chromatography: [25-26]

A good resolution of component is achieved by optimization of various parameters such as resolution, number of theoretical plate (N), capacity factor (K), and peak asymmetry factor (AF).

1.7.1 Resolution (Rs):

Resolution is the parameter describing the separation power of the complete chromatographic system relative to the particular components of the mixture. The resolution, Rs, of two neighbouring peaks is defined as the ratio of the distance between two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of Rs is 1.5. It is calculated by using the formula:-

$$Rs = \frac{(RT\ 2 - RT\ 1)}{2\ (W1 + W2)}$$

Where, RT1 and RT 2 are the retention times of components 1 and 2 and

W1 and W2 are peak width of components 1 and 2.

There are three fundamental parameters that influence the resolution of a chromatographic separation:

- Capacity factor (k')
- Selectivity (α)
- Column efficiency (N)

These parameters provide you with different means to achieve better resolution, as well as defining different problem sources.

Capacity Factor (k'):

Capacity factor is the ratio of the reduced retention volume to the dead volume. Capacity factor, k‟, is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. Capacity factor is a measure of how well the sample molecule is retained by a column during an isocratic separation. The ideal value of k‟ ranges from 2-10. Capacity factor can be determined by using the formula,

$$K = \frac{V_1 - V_0}{V0}$$

Where,

V1=retention volume at apex of the peak

V0= void volume of system where an un-retained component elutes

Capacity Factor (k') changes are typically due to:

1. Variations in mobile phase composition
2. Changes in column surface chemistry (due to aging)
3. Changes in operating temperature.

Adjusting Capacity Factor (k')

Good isocratic methods usually have a capacity factor (k') in the range of 2 to 10 (typically between 2 and 5). Lower values may give inadequate resolution. Higher values are usually associated with excessively brood peaks and unacceptably long run times.

Capacity Factor (k') values are sensitive to:

1. Solvent strength
2. Composition
3. Purity
4. Temperature
5. Column chemistry
6. Sample

Selectivity (α):

The selectivity (or separation factor), a, is a measure of relative retention of two components in a mixture. Selectivity is the ratio of the capacity factors of both peaks, and the ratio of its adjusted retention times. Selectivity represents the separation power of particular adsorbent to the mixture of these particular components. This parameter is independent of the column efficiency; it only depends on the nature of the components, eluent type, and eluent composition, and adsorbent surface chemistry.The ideal value of α‟ is 2. It can be calculated by using formula,

$\alpha = V2 – V1 / V1 – V0 = k2’/ k1’$

Where,

V0 = the void volume of the column,

V1 and V2 =the retention volumes of the second and the first peak respectively.

Selectivity (α) values are sensitive to:

- Changes in mobile phase composition (pH ionic strength)
- Purity
- Temperature

Column Efficiency/ Band broadening (N):

Efficiency, N, of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Similar the band spread, higher is the number of theoretical plates, indicating good column and system performance. Columns with N ranging from 5,000 to 100,000 plates /meter are ideal for a good system. Efficiency is calculated by using the formula,

$$N = 16(Rt/W)^2$$

Where,

Rt is the retention time

W is the peak width.

A decline in measured efficiency may be due to:

- Age and history of the column
- Extra column band broadening (such as due to malfunctioning injector or improper tubing ID)
- inappropriate detector settings (for example, time constant)
- Change in flow rate and solvent viscosity.

You can recognize problems in your separation due to a loss of column efficiency when the width and/or shape of all peaks are affected. If the measured efficiency has degraded, either the column has degraded, or system band broadening has increased. At this point, check system band spreading against established benchmarks.

Methods of measuring column efficiency (N) Methods used for the measurement and calculation of column include (in order to sensitivity to abnormal peak shape):

1. Asymmetry-based (Most sensitive to tailing or fronting)
2. 5 sigma
3. 4 sigma

4. Tangent
5. 3 sigma
6. ½ height
7. 2 sigma (infection) (Least sensitive to tailing or fronting)

Choose the method that best suits your operating requirements. It is critical that the same method always be used and executed reproducibly.

1.7.2 Peak asymmetry factor (Tf):

Peak asymmetry factor, Tf, can be used as a criterion of column performance. The peak half width, b, of a peak at 10% of the peak height, divided by the corresponding front half width, a, gives the asymmetry factor.

1.7.3 Mean Value (Average):

It is a measure of the location of the data. It is simply the arithmetic average of data add them all up and divide by the number of data points. The mean of a set of data is usually considered the best estimate of the value.

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n}(x_i)$$

X=mean; n=no of values

1.7.4 Standard Deviation:

It is a measure of the spread of data around the sample mean – a precision measure. It is always in the same units as the mean. Like the mean, it is considered an estimate of the population standard deviation.

$$s(x_i) = \sqrt{\frac{1}{n-1}\cdot\sum_{i=1}^{n}(x_i - \bar{x})^2}$$

Where S (Xi) =Standard deviation X=mean; n= no of values

1.7.5 Relative Standard Deviation:

Relative standard deviation is a measure of the spread of data in comparison to mean of the data. It is simply the standard deviation divided by the mean value.

$$RSD = \frac{s(x_i)}{\bar{x}} \quad (absolute\ or\ \%)$$

Where: - s (xi) = standard deviation; X=mean

1.7.6 Tailing Factor:

Tailing factor establishes the maximum permissible asymmetry of the peak.

$$T = \frac{W}{2F}$$

Where,

T = Tailing factor

W = Peak width at 5% of peak height

F = time from width start point at of peak height to retention time (Rt)

1.7.7 Plate Count:

Plate number (N) is a measure of column efficiency. N is fairly constant for each peak on a chromatogram with a fixed set of operating condition.

N = 16 (tR / Wb) 2 = L / H

1.8Analytical Method Validation: [27-32]

Analytical method validation according to USP is performed to ensure that an analytical methodology is accurate, specific, reproducible, precise and rugged over the specified range that an analyte will be analysed. Validation of method is the process by which a method is tested by a developer or user for reliability, accuracy and preciseness of it intended purpose.

The international conference on harmonization (ICH) of technical requirement for the registration of pharmaceutical for human use has developed suitable text on the validation of analytical procedure. The document includes definitions for eight validation parameter. The United State Food and Drug Administration (US-FDA) have proposed guidelines on submitting samples and analytical data for

method validation. The United State Pharmacopoeia (USP) has published specific guidelines for method validation for compound evaluation.

1.8.1 Objective:-

The objective of a test method is to generate reliable and accurate data regardless of whether it is for acceptance, release, stability or pharmacokinetics study. Data are generated for the qualitative and quantitative testing during development and post approval of the drug product. The testing includes the acceptances of raw material, release of the drug substances and product, in process testing for quality assurance and establishment of the expiration dating period.

1.8.2 Purpose:-

The principle purpose of analytical method validation is to ensure that test methods, which are used for assessing compliance of pharmaceutical products with established specification, will give accurate, reliable and reproducible result.

The real goal of the method validation process is to challenge the method and determine limits of allowed variability for the conditions needed to run the method. It is important to have a well-conceived validation plan for testing the method and acceptance criteria before starting the validation process. Included in this plan should be detailed procedure describing the entire method (including calibration standard and sample preparation, separation, data handling and calculation) that can conveniently be executed by other.

1.8.3 Advantages of Analytical method Validation:-

- The biggest advantage of method validation is that it builds a degree of confidence, not only for the developer but also to the user.
- Although the validation exercise may appear costly and time consuming, it results inexpensive, eliminates frustrating repetitions and leads to better time management in the end.
- Minor changes in the conditions such as reagent supplier or grade, analytical setup are unavoidable due to obvious reasons but the method validation absorbs the shock of such conditions and pays for more than invested on the process.

1.9Parameter for validation of HPLC method:

The validation parameter as per ICH guideline and USP are:-

1) Accuracy
2) Precision

A. Repeatability precision
B. Intermediate precision
C. Reproducibility

3) Linearity
4) Limit of detection
5) Limit of quantitation
6) Selectivity/ specificity
7) Robustness
8) Ruggedness

1. Accuracy:

It is the measure of how close the experimental value is to the true value. It is express as % recovery by the assay of known/added amount of analyte in the linearity range.

2. Precision:

It expresses a degree of agreement among individual test results when procedure/method is applied to the homogenous sample. It is usually express as a variance, SD/COV. It is a measure of degree of repeatability or reproducibility under normal conditions.

A) Repeatability: Repeatability expresses the precision under the same operating condition over a short interval of time. It is also term as intra-day precision.

B) Intermediate precision: Intermediate precision expresses within laboratories variations such as different day, different analyst, different equipment etc.

C) Reproducibility: Reproducibility expresses the precision between laboratories collaborative studies, usually supplied to standardization of methodology.

3. Specificity/Sensitivity: It is the ability of the method to measure accurately and specifically the analyte of interest in presence of matrix and other component likely to be present in the sample matrix and impurities, degradation product and other related substances.

4. Limit of Detection:

The detection limit of an individual analytical procedure is the lowest amount of an analyte in a sample, which can be detected but not necessarily quantitated as an exact value.

Based on visual evaluation: The detection limit is determined by the analysis of sample with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

Based on signal to noise ratio: A signal to noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

Based on standard deviation of the response and slope: The limit of detection (LOD) maybe express as,

$$LOD = \frac{3.3 \times \sigma}{S}$$

Where,

σ = Standard deviation of the response,

S = Slope of calibration curve.

5. Limit of Quantitation:

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. Some approaches listed below may be acceptable.

·**Based on visual evaluation:** The quantitation limit is determined by the analysis of samples with known concentrations of analyte.

·**Based on signal to noise ratio:** Signal to noise ratio between 10:1 is generally considered.

·**Based on Standard Deviation of the Response and Slope:** The limit of quantitation **(**LOQ) may be expressed as:

$$LOD = \frac{10 \times \sigma}{S}$$

Where,

σ = Standard deviation of the response.

S = Slope of calibration curve of analyte.

6. Linearity and Range:

The linearity of an analytical procedure is the ability to obtain test results, which are directly proportional to the concentration of an analyte in the sample. The range of an analytical procedure is the interval between the upper and lower concentration of an analyte in the sample.

7. Robustness and Ruggedness:

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage, determined when one or more operating parameter varied. The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different analysts, and using operational and environmental conditions.

8. Stability and System Suitability Tests:

Stability of the sample, standard and reagents is required for a reasonable time to generate reproducible and reliable results. System suitability test provide the added assurance that on a specific occasion the method is giving, accurate and precise results. System suitability test are run every time a method is used either before or during analysis.

9. Statistical Parameters in Analytical Validation

Statistics deals with effective use of numerical data relating to groups of individuals or experiments. Quantitative results are obtained from devices or instruments that determine the concentration of a chemical in a sample from an observable signal. Always there is some variation in that signal due to noise and/or drift within the instrument. Hence there is always an error, a deviation from the true value, inherent in that measurement. Use of statistics in analytical chemistry provides an estimate of the probable value of that error. The precision or reproducibility of the analytical method was determined by repeating the analysis and the following statistical parameters were calculated.

a) Mean: The mean of the any distribution is a measure of centrality and is obtained by dividing the sum of observed values by the number of observations, n.

b) Standard Deviation: The standard deviation (SD) is a measure of data dispersion or variability. SD is the square of the mean of the sum of the squares of the differences between the values and the mean of those values.

$$SD = [\text{sum of } (x\text{-mean})^2/n\text{-}1]^{1/2}$$

Where,

X = Individual sample reading

n= No. of samples

c) Relative Standard Deviation: The relative standard deviation (RSD) is also called coefficient of variation. This is useful when the SD is proportional to the magnitude of the measurement.

$$RSD = (SD/mean)\ 100$$

d) Regression Equation: A regression is a statistical analysis assessing the relationship between two variables. It is used to find the relationship between two variables.

$$y = mx + c$$

Where,

m --- The slope of the regression line

C --- The intercept point of the regression line and the y axis.

02.REVIEW OF LITERATURE

The literature survey shows that some methods are developed and validated for the analysis of Trifluoperazine

2.2 Reported Methods For Determination Of Trifluoperazine

1) **C.M.Bhaskar Reddy at al (2012)**A new simple, rapid, accurate, sensitive and precise spectrophotometric method for the determination of trifluoperazine hydrochloride in bulk and capsule dosage form .pharmaceutical formulations is described. The method is based on the simple solubility of trifluoperazine hydrochloride in methanol. The absorbance maximum of trifluoperazine hydrochloride measured at wave length 265 nm. The drug obeys Beer's Law in the concentration range 2-45 µg/ml employed for this method. Accuracy and reproducibility of the proposed method was statistically validated by recovery studies. The method is easily be employed in the laboratory for the routine estimation of drug and it's extended to the analysis of trifluoperazine hydrochloride in pharmaceutical formulations.

2)Navya sri D at al (2016) The aim of present research work was to develop and validate UV spectroscopic method for estimation of Trifluoperazine in bulk and tablet pharmaceutical formulation. The method employed simple spectroscopy based on the solubility of Trifluoperazine in water. For determination of wavelength different concentration samples were scanned in 200-400 nm range and the maximum absorbance was found at 257 nm wavelength. Lambert–Beer's law for trifluoperazine were followed in theconcentration range of 1-16 µg/ml (r2 = 0.9999) respectively. The method is validated as per ICH guidelines. Accuracy was assessed by the standard addition method. The recoveries were obtained in range of 98.03-101.64%. The repeatability was determined by RSD for Trifluoperazine and were found to be 0.58% respectively. The intraday precision and interday precision was determined by RSD and were found to be 0.54-0.77% and 0.28-1.00% respectively. The LOD and LOQ value for Trifluoperazine was found to be 0.29 and 0.89 µg/ml.The newly developed method can be used or routine analysis in laboratories and it is suitable for the quality control of the raw material, formulations, and dissolution studies.

3) Suman Pattanayak at al(2015) The present paper describes a reverse phase HPLC method for simultaneous estimation of Trifluoperazine and Isopropamide from their combined tablet dosage form. The proposed RP-HPLC method utilizes an Agilent Zorbax, C18 column (250 × 4.6 mm, 5µm particle

size) using a mobile phase consisting of mixture of Buffer: Acetonitrile (80: 20 v/v) mobile phase flow rate of 0.8 ml/min; and UV detection at 227nm. The retention time for Trifluoperazine and Isopropamide were 2.4 and 3.62 minutes respectively. The method was linear in the range of 40-120µg/ml and 100-300µg/ml and correlation coefficients were 1.00 and 1.00 for Trifluoperazine and Isopropamide respectively. The limit of detection was found to be 2.963 and 2.985µg/ml for Trifluoperazine and Isopropamide respectively. Limit of quantification was found to be 9.877 and 9.9502 for Trifluoperazine and Isopropamide respectively. The percentage recoveries for Trifluoperazine and Isopropamide ranged from 100.03-100.36% and 100.00-100.07 % respectively. The percentage of RSD for precision of the method was found to be less than 2%. The proposed method can be used for the routine analysis for the estimation of these drugs in combined dosage form.

4) **Kanji V. Vaghela at al (2016):** The aim of present research work was to develop and validate UV spectroscopic method for estimation of Trifluoperazine in bulk and tablet pharmaceutical formulation. The method employed simple spectroscopy based on the solubility of Trifluoperazine in water. For determination of wavelength different concentration samples were scanned in 200-400 nm range and the maximum absorbance was found at 257 nm wavelength. Lambert–Beer's law for trifluoperazine were followed in theconcentration range of 1-16 µg/ml (r2 = 0.9999) respectively. The method is validated as per ICH guidelines. Accuracy was assessed by the standard addition method. The recoveries were obtained in range of 98.03-101.64%. The repeatability was determined by RSD for Trifluoperazine and were found to be 0.58% respectively. The intraday precision andinterday precision was determined by RSD and were found to be 0.54-0.77% and 0.28-1.00% respectively. The LOD and LOQ value for Trifluoperazine was found to be 0.29 and 0.89 µg/ml.The newly developed method can be used or routine analysis in laboratories and it is suitable for the quality control of the raw material, formulations, and dissolution studies.

5) **P. SHETTIAT AL at al (2010)** A new, simple, precise, rapid, selective and stability indicating reversed-phase high performance liquid chromatographic (HPLC) method has been developed and validated for simultaneous quantification of trihexyphenidyl hydrochloride, trifluoperazine hydrochloride and chlorpromazine hydrochloride from combined tablet formulation. The method is based on reverse-phase using C-18 (250x4.6) mm, 5 µm particle size column. The separation is achieved using isocratic elution by methanol and ammonium acetate buffer (1% w/v, pH 6.5) in the ratio of 85:15 v/v, pumped at flow rate 1.0 mL/min and UV detection at 215 nm. The column is maintained at 30 °C

through out the analysis. This method gives baseline resolution. The total run time is 15 min. Stability indicating capability is established buy forced degradation experiment. The method is validated for specificity, accuracy, precision and linearity as per International conference of harmonisation (ICH). The method is accurate and linear for quantification of trihexyphenidyl hydrochloride, trifluoperazine hydrochloride and Chlorpromazine hydrochloride between 5 - 15 µg/mL, 12.5- 37.5 µg/mL and 62.5 - 187.5 µg/mL respectively.

6)**Komal V Patel at al (2015)**A simple, rapid, economical, precise and accurate Reverse phase high performance liquid chromatographic (RP-HPLC) method for simultaneous estimation of Chlordiazepoxide, Trihexyphenidyl HCl and Trifluoperazine HCl in Their Combined Dosage Form has been developed. The RP-HPLC method was developed for the simultaneous estimation of Chlordiazepoxide, Trihexyphenidyl HCl and Trifluoperazine HCl in their Combined Dosage Form development method has been achieved. The separation was attained by Column LC- 2010 AT C18 (250mm x 4.6 mm x 5 µm) and Buffer (pH 3.5) : Acetonitrile :TEA (80:20:0.1 v/v/v) as mobile phase, at a flow rate of 1 ml/min. Detection was carried out at Wavelength of 228 nm. Retention time of Chlordiazepoxide, Trihexyphenidyl HCl and Trifluoperazine HCl were found to be 3.807 min, 6.887 min and 4.667 min respectively. The method has been validated for linearity, accuracy and precision. Linearity observed for Chlordiazepoxide 5-15 µg/ml, for Trifluoperazine HCl 0.5-1.5 µg/ml and for Trihexyphenidyl HCl 1-3 µg/ml. The Percentage recoveries obtained for Chlordiazepoxide, Trifluoperazine HCl and Trihexyphenidyl HCl were found to be in range of 99.27 ± 1.09, 99.57 ± 0.56 and 99.22 ± 0.51 respectively. Developed method was found to be accurate, precise and rapid for simultaneous estimation of Chlordiazepoxide, Trihexyphenidyl HCl and Trifluoperazine HCl in their combined dosage form.

7) **Jameel M. Dhabab at al (2013)** A reverse phase HPLC method is developed for the simultaneous analysis of Trifluoperazine (TFP) and prochlorperazine (PCP) in pharmaceutical preparations. HPLC was carried out on a C18 column using acetonitrile as a mobile phase at 1 mL min_1 flow rate and the effluent was monitored at 250 nm. Chlorperazine hydrochloride (CPZ) was used as an internal standard. The retention time of the drugs was 10.879 and 13.708 min, respectively. This method produced a linear response in the concentration range between 5–200 lg ml_1 of trifluoperazine and 10–500 lg ml_1 of prochlorperazine. In this study, a HPLC method was successfully applied for the quantitative assay of

trifluoperazine and prochlorperazine in tablets and ampule of commercial preparations which is simple, rapid and does not require any separation step for each drug.

3. DRUG PROFILE:

TRIFLUOPERAZINE:

Structure:

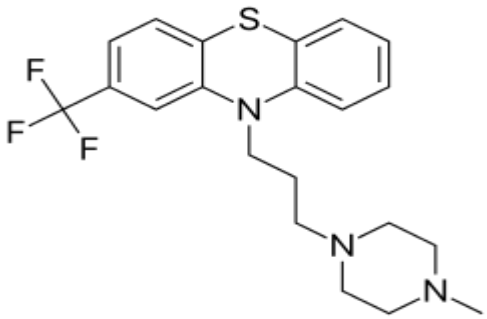


Fig.3.1 TRIFLUOPERAZINE.

Table 3.1 General profile of TRIFLUOPERAZINE

Category	**Antipsychotics**
Chemical Name	10-[3-(4-methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)phenothiazine
Molecular Formula	C21H24F3N3S
Molecular Weight	407.499 g/mol
Odour	Odourless

Description	White crystalline powder.
Solubility	Soluble in Water, sparingly soluble CHLOROFORM
Odour	Odourless
PKa	8.05
Melting point	232°c to 235°c
Storage	Keep container tightly closed in a dry and well-ventilated place.
LOG P-	5.03

Mechanism of Action:

Trifluoperazine blocks postsynaptic mesolimbic dopaminergic D1 and D2 receptors in the brain; depresses the release of hypothalamic and hypophyseal hormones and is believed to depress the reticular activating system thus affecting basal metabolism, body temperature, wakefulness, vasomotor tone, and emesis.

Pharmacokinetics:

The pharmacokinetics for trifluoperazineare the typical of phenothiazines such as chlorpromazine. It is readily absorbed from the gastrointestinal tract with peak plasma levels being reached from 1.5 to 6.0 hours after ingestion. A high interindividual variation in bioavailability has been consistently reported. In the blood, trifluoperazine is highly bound to plasma proteins. It probably penetrates the placental barrier and enters breast milk like chlorpromazine.

The elimination of trifluoperazine from the blood is multiphasic with an α phase elimination half-life of about 3.6 hours and a terminal elimination half-life of about 22 hours. Several metabolites of trifluoperazine have been identified, including an N-oxide, a sulphoxide and a 7-hydroxy derivative. The

N-oxide is thought to be the main metabolite and possibly active. This and the sulphoxide metabolite are thought to be extensively metabolised pre-systemically (i.e. in the gut and/or liver), whilst the 7-hydroxy derivative appears to undergo no such metabolism.

Elimination occurs via bile and urine.

Adverse Drug Reaction: Anticholinergic effects. Weight gainAmenorrhea Erectile dysfunction Insomnia. Restlessness Anxiety, Euphoria Agitation, Depression, Weakness.

Contraindication: Trifluoperazine is contraindicated in CNS depression, coma, and blood dyscrasias. Trifluoperazine should be used with caution in patients suffering from renal or hepatic impairment.

Side Effects: Drowsiness, dizziness, light headedness, dry mouth, blurred vision, tiredness, constipation, weight gain, and trouble sleeping may occur.

Overdose: agitation, coma, convulsions, difficulty breathing, difficulty swallowing, dry mouth, extreme sleepiness, fever, intestinal blockage, irregular heart rate, low blood pressure, and restlessness.

04.AIM AND OBJECTIVE:

Aim: The aim of present study is Development and Validation of RP-HPLC Method for estimation of trifluoperazine drug in pharmaceutical tablet dosage form.

Objectives:

The main objectives of the study are:

- To develop new, simple, sensitive, accurate, and economical analytical method for the estimation of trifluoperazine Tabletby RP-HPLC.
- To Validate the proposed method in accordance with USP and ICH guidelines for the intended analytical application i.e., to apply the proposed method for analysis of the drug in its dosage form

05.PLAN OF WORK:

- Estimation of trifluoperazine Tablet will be done by following methods.

5.1Selection of Drugs and Formulation

- By literature and market survey

Online Journals, chemical and analytical abstracts were studied to find out drugs for which there were no reported RP-HPLC methods. Market survey was carried to check the availability of these drugs and their dosage forms.

5.2. Selection of analytical techniques

- Estimation by UV-Visible spectroscopy.
- Identification by IR Spectroscopy
- HPLC method

5.3.Method development by RP-HPLC.

- Selection of preliminary HPLC conditions.

✓ Selection of mobile phase

✓ Selection of column

✓ Selection of wavelength

- Analysis of laboratory mixture.

5.4. Validation of proposed method.

- ✓ System suitability parameter
- ✓ Linearity and Range
- ✓ Accuracy
- ✓ Precision

a. System precision

b. Method precision

- ✓ Specificity
- ✓ Robustness

5.5. Probable outcomes:

- ➢ A simple and accurate analytical technique can be developed for the determination of trifluoperazine Tablet
- ➢ Method developed can be conveniently used for quality control and routine determination of drug in pharmaceutical industry.

06. EXPERIMENTAL

6.1. MATERIAL:

6.1.1 DRUG:

Table 6.1:Drug and drug product samples suppliers and manufacturers

Name of drug and drug product	Supplier and manufacturer by
Trifluoperazine	Killitch Drugs Ltd.
Trifluoperazine Tablet	Killitch Drugs Ltd.

6.1.2 REAGENTS:

Table 6.2: List of Reagent

Sr.No	Chemical	Make
1	Water	Rankem
2	Acetonitrile	Merck life science
3	Phosphoric acid 88%	Merck life science
4	Potassium dihydrogen phosphate	Merck life science
5	Sodium hydroxide	Merck life science
6	Triethylamine	Merck life science
7	0.45 µ Nylon membrane disc filter	Mdi
8	0.45µ PVDF Syringe Filter	Mdi

6.2.INSTRUMENTS:

6.2.1 HPLC:

Make	Waters
Model	e2695
Pump	Reciprocating Water-510
Detector	Waters 2695 PDA
Software	Empower PRO
Column	X-Bridge

6.2.2. SPECTROPHOTOMETER: Double beam UV-visible spectrophotometer with 10mm Matched quartz cells

Model	UV1700
Make	Thermo scientific

6.2.3 ANALYTICAL BALANCE: Digital Analytical balance

Model	XS205D0
Make	Mettler Toledo

6.2.4 PH METER: Digital pH Meter

Make	Thermo Scientific
Model	Orian Star A211

6.2.5 FTIR Spectrometer:

Make	Thermo Scientific TM
Model	Nicolet TM is5

6.3 METHOD

6.3.1 UV SPECTROSCOPIC METHOD

6.3.1.1 Selection of solvent[26]

Prepare a mixture of 0.1N HCl and Methanol in the ratio 50:50 v/v respectively and mix. Sonicate to degas..Used as solvent for dissolving Trifluoperazine .

6.3.1.2 SELECTION OF WAVELENGTH[40]

Preparation of standard solution

An accurately weighed quantity about 25 mg of Trifluoperazine standard was transferred to 100 mL volumetric flask. Add 70 mL of diluentsonicate to dissolve and dilute up to the mark with diluent and mixed.

6.3.1.3 DETERMINATION OF λ MAX (SELECTION OF WAVELENGTH)

The standard solutions were scanned separately between 400nm to 200nm. From the spectrum at 250 nm show high absorbance, so 250nm λmax of Trifluoperazine were selected for estimation drugs.

6.3.2 IDENTIFICATION BY IR SPECTROSCOPY:

Trifluoperazine30mg of API was mixed properly then carefully triturated in a mortar pestle, At last this mixture was kept in on a plate and IR spectrum was taken using the Diffused Attachment reflectance mode.

6.3.3 REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD DEVELOPMENT AND OPTIMIZATION

The standard solution of Trifluoperazine was used for method development trials to optimize the method for determination of Trifluoperazine.

6.3.3.1 PREPARATION OF SOLUTION[40]

4.1 Preparation of Buffer pH 3.00:

Dissolve 7.0 g of potassium dihydrogen ortho-phosphate in 1000 mL of water and mix well. Adjust to pH 3.00 ± 0.05 using orthophosphoric acid. Filter the solution through a 0.45µm nylon membrane filter.

4.2 Preparation of Mobile Phase:

Prepare a mixture of Buffer pH 3.00 ± 0.05 and Acetonitrile in the ratio 55:45 v/v respectively and mix. Sonicate to degas.

4.3 Preparation of 0.1N Hydrochloric acid:

8.5 ml Concentrated Hydrochloric acid in 1000 mL of water.

4.4 Preparation of Diluent:

Prepare a mixture of 0.1N HCl and Methanol in the ratio 50:50 v/v respectively and mix. Sonicate to degas.

4.5 Preparation of Standard solution:

Weigh and transfer accurately about 30 mg of Trifluoperazine hydrochloride (equivalent to 25.4 mg of Trifluoperazine) standard into 200 mL amber colored volumetric flask. Add about 150 mL of diluent, sonicate to about 2-3 minutes to dissolve and dilute up to the mark with diluent and mix. Further dilute 4.0 mL of this solution to 50 mL with Diluent and mix.

Prepare standard solution in duplicate as standard solution-1 and standard solution-2.

(Concentration of Trifluoperazine standard solution: 10 ppm)

4.6 Preparation of Sample solution:

Sample Solution for 5 mg:

Weigh and transfer 5 tablets in to 250 mL amber colored volumetric flask. Add about 200 mL of diluent, sonicate for 40 minutes with intermittent shaking, at room temperature (25°C) cool and make volume up to mark with diluent and mix. Further dilute 5.0 mL of this solution to 50 mL with Diluent and mix. Filter the sample solution through 0.45µ membrane nylon filter. Discard first 2.0 mL of filtrate.

(Concentration of Sample Solution for 5 mg: 10 ppm)

Note: Prepare Sample in duplicate.

6.3.3.2 REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD DEVELOPMENT AND OPTIMIZATION[37-42]

1) Selection of Stationary phase:

On the basis of reversed phase HPLC mode and number of carbon present in molecule (analyte) stationary phase with C18 bonded phase i.eInertsil ODS-3V, 150 x 4.6 mm, 5µ was selected.

2) Selection of Mobile Phase:

The selection of mobile phase was done after assessing the solubility of drug in different solvent as well on the basis of literature survey and finally mobile phase was selected for is the mixture of Buffer pH 3.00 ± 0.05 and Acetonitrile in the ratio 55:45 v/v respectively

3) Selection of Detector and Detection wavelength:

UV-visible 2487 detector was selected, as it is reliable and easy to set at the correct wavelength and 250 nm wavelengths was selected as detection wavelength.

4) Optimization of Chromatographic Parameters:

Optimization in HPLC was the process of finding a set of conditions that adequately separate and enable the quantification of the analyte from the endogenous material with acceptable accuracy, precision, sensitivity, specificity, cost, ease and speed.

5) Optimization of Detection Wavelength:

A fixed concentration of analyte was analyzed at different wavelengths. As per the response of analyte, wavelength of 250 nm was selected. This is the optimum wavelength as it shows maximum absorption at this wavelength.

6) Selection of oven temperature:

An inclusion of column temperature (30 °C) minimized day to day variation of retention time due to fluctuations in the ambient temperature; along with this peak sharpening and shortening of run time were observed.

7) Selection of Sample temperature:

An inclusion of sample temperature (10°C) minimized day to day variation of retention time due to fluctuations in the ambient temperature.

Final Reversed Phase High Performance Liquid Chromatographic condition with gradient elution and PDA detection.

mn	sil ODS-3V, 150 x 4.6 mm, 5µ (GL Sciences, 020-01801)
Rate	nL/min
tion Volume	L
elength	nm
mn Temp	
ple Temp	
Time	ninute
ntion Time	ut 4.7 minutes
lle Wash	er : Acetonitrile (10:90)
Wash	er : Methanol (90:10)

ESTIMATION OF TRIFLUOPERAZINE IN SOLUTION DOSAGE FORM BY PROPOSED METHOD:

Procedure:

Separately injected equal volumes of Blank (diluent), 1st standard solution (six replicates), 2nd standard solution (two replicates) and sample solution (single injection). Record the chromatograms and measure Peak areas.

The amount of drug estimated in sample weight was calculated using formula.

Calculation:

$$\text{say} \quad \frac{AT}{AS} \times \frac{WS}{Splwt} \times \frac{P}{100} \times \frac{LC}{Avg.\ wt} \times \frac{1.51}{1.42}$$

Where

AT : Peak area of Trifluoperazine in the chromatogram of Sample solution.

AS : Average Peak area of Trifluoperazine in the chromatogram of Standard solution.

WS : Weight of Trifluoperazine Hydrochloride working standard in mg.

P : % Potency of Trifluoperazine Hydrochloride working standard on as is basis.

LC : Label claim of Trifluoperazine in mg.

Splwt	:	Weight of Sample in mg
Avg. wt	:	Average weight of sample in mg
407.51	:	Molecular Weight of Trifluoperazine
480.42	:	Molecular Weight of Trifluoperazine Hydrochloride

6.4 METHOD VALIDATION

6.4.1. System suitability: Equal volume of Blank (Diluent) and five replicate injections of Standard solution 1 were injected onto the HPLC.

Acceptance criteria:

The relative standard deviation of five replicate injections for Trifluoperazine peak should not be more than 2.0% from standard solution.

The tailing factor should not be more than 2.0 from first injection of standard solution.

The theoretical plates for Trifluoperazine peak should not be less than 2000 from first injection of standard solution.

The relative standard deviation of Trifluoperazine peak area responses obtained from five replicate injections of standard solution and bracketing standard (s) should not be more than 2.0%.

The correlation between 1st standard solution and 2nd standard solution should be range of 98.0% to 102.0%.

6.4.2 Specificity: (Identification, Interference & Peak Purity)

Preparation of Trifluoperazine 4-Isomer stock solution:

Weigh accurately about 2.0 mg of Trifluoperazine 4-Isomer transfer into a separate 20 mL of volumetric flask. Add about 15 mL of Acetonitrile sonicate to dissolve and make up to volume with Acetonitrile and mix. Further dilute 10.0 mL of this solution to 50 mL with diluent and mix.

Identification solution of Trifluoperazine 4-Isomer:

Transfer 5.0 mL of above stock solution to 100 mL volumetric flask and dilute up to the mark with diluent and mix.

Preparation of TrifluoperazineSulphoxide stock solution:

Weigh accurately about 2.0 mg of TrifluoperazineSulphoxide transfer into a separate 20 mL of volumetric flask. Add about 15 mL of Acetonitrile sonicate to dissolve and make up to volume with acetonitrile and mix. Further dilute 10.0 mL of this solution to 50 mL with diluent and mix.

Identification solution of TrifluoperazineSulphoxide:

Transfer 5.0 mL of above stock solution to 100 mL volumetric flask and dilute up to the mark with diluent and mix.

Preparation of 2-Triflurомethylphenothiazine stock solution:

Weigh accurately about 2.0 mg of 2-Triflurомethylphenothiazine transfer into a separate 20 mL of volumetric flask. Add about 15 mL of Acetonitrile sonicate to dissolve and make up to volume with acetonitrile and mix. Further dilute 10.0 mL of this solution to 50 mL with diluent and mix.

Identification solution of 2-Triflurомethylphenothiazine:

Transfer 5.0 mL of above stock solution to 100 mL volumetric flask and dilute up to the mark with diluent and mix.

Preparation of Spike Sample solution:

Weigh and transfer 5 tablets in to 250 mL amber colored volumetric flask. Add about 200 mL of diluent, and add 12.0 mL of each Trifluoperazine 4-Isomer stock-1, TrifluoperazineSulphoxide stock-1 and 2-Ttriflurомethylphenothiazine stock-1 solution sonicate for 40 minutes with intermittent shaking, cool make volume up to mark with diluent and mix. Further dilute 5 mL of this solution to 50 mL with Diluent and mix. Filter the sample solution through 0.45µ membrane nylon filter. Discard first 2 mL of filtrate.

Preparation of Placebo solution:

For 5 mg:

Weigh and transfer Placebo equivalent to 5 tablets into 250 mL volumetric flask. Add about 200 mL of diluent, sonicate for 40 minutes with intermittent shaking, cool and make volume up to mark with diluent and mix. Further dilute 5.0 mL of this solution to 50 mL with Diluent and mix. Filter the sample solution through 0.45µ membrane nylon filter. Discard first 2 mL of filtrate.

Acceptance criteria:

Identification:

Results should be comparable with respect to the retention time.

Interference:

Blank (Diluent) Placebo and known impurity should not show any peaks at the Retention time of Trifluoperazine peak.

Peak Purity:

Standard and Sample peak should be pure for working concentration level.

Purity angle should be less than purity threshold.

6.4.3 Linearity

Evaluate linearity in the range of 50% to 150% of the working concentration level. The working concentration level of Trifluoperazine is about 10 ppm for Trifluoperazine Tablets 1 mg, 2 mg and 5 mg. the range proposed is from 50% to 150% of 10 ppm.

Preparation of Linearity Stock solution:

Weigh and transfer accurately about 29.5 mg of Trifluoperazine hydrochloride (equivalent to 25 mg of Trifluoperazine) standard into 200 mL amber colored volumetric flask. Add about 120 mL of diluent, sonicate to dissolve and dilute up to the mark with diluent and mix.

(Concentration of Linearity Stock solution: 125 ppm)

Table 6.4: Preparation of linearity sample

Linearity Level %	Volume taken from Linearity stock solution (mL)	Total volume (mL)	Concentration (ppm)
50	2.0	50	5
75	3.0	50	7.5
100	4.0	50	10
125	5.0	50	12.5
150	6.0	50	15

Acceptance criteria:

System suitability criteria should be fulfilled.

Response should be linear.

Co-relation coefficient (R) should not be less than 0.999

% Limit of Y- intercept should be within ± 2.0% of the corresponding Y-co-ordinate of the working level.

6.4.4 Accuracy (Recovery):

Evaluate accuracy at three levels, 50%, 100% and 150% of working concentration level for Trifluoperazine Tablets. The working concentration of Trifluoperazine in all the strengths is about 10 ppm. Each level to be prepare in triplicates.

Preparation of Accuracy Standard stock solution:

Weigh and transfer placebo tablets and add Trifluoperazine API as mentioned in above table. Add about 200 mL of diluent, sonicate for 40 minutes with intermittent shaking, cool and make volume up to mark with diluent and mix. Further dilute 5.0 mL of this solution to 50 mL with Diluent and mix. Filter the sample solution through 0.45µ membrane nylon filter. Discard first few ml of filtrate.

Table 6.5: Preparation of accuracy sample

Recovery Level %	Placebo Tablet Added	Amount of API added (mg)	Total volume (mL)	Dilute the solution (mL)	Make up the volume (mL)	Concentration (ppm)
						Trifluoperazine
50%-1	5	15	250	5.0	50	5
50%-2	5	15	250	5.0	50	5
50%-3	5	15	250	5.0	50	5
100%-1	5	30	250	5.0	50	10
100%-2	5	30	250	5.0	50	10
100%-3	5	30	250	5.0	50	10
150%-1	5	45	250	5.0	50	15
150%-2	5	45	250	5.0	50	15
150%-3	5	45	250	5.0	50	15

Procedure:

For accuracy inject each preparation in duplicate and record the responses. Determine the amount of Trifluoperazine recovered from each solution.

Calculate the percent recovery at each level as follows

$$\text{Percent Recovery} = \frac{\text{Recovered Amount (mg)}}{\text{Amount of API added (mg)}} \times 100$$

Acceptance criteria:

System suitability criteria should be fulfilled.

Mean recovery for 50% to 150% should be in the range of 98.0% - 102.0% and individual recovery for 50% to 150% should be in the range of 97.0% - 103.0%.

6.4.5Precision

6.4.5.1 System Precision

7.1.1 **System precision:**
Make five replicate of the standard solution. Record the peak areas and calculate % RSD.

Acceptance criteria:

System suitability criteria should be fulfilled.

The RSD of five replicate injections of standard solutions for peak area of Trifluoperazine should not be more than 2.0%.

6.4.5.2 Method Precision (Repeatability)

Make six independent sample preparations and determine % Assay. Calculate the mean and % RSD.

Acceptance criteria:

System suitability criteria should be fulfilled.

The RSD for % Assay of six independent samples for Trifluoperazine should not be more than 2.0%.

6.4.5.3 Intermediate Precision (Ruggedness)

7.1.2 **Intermediate Precision:**

Evaluate Intermediate precision by performing the method precision as described in section 7.5.2 using same lot on different day, by different analyst, on different make of HPLC system and with different column. Evaluate the reproducibility by comparing the results obtained from Ruggedness with those obtained from Method precision.

Acceptance criteria:

System suitability criteria should be fulfilled.

The RSD for % Assay of six independent samples should not be more than 2.0%.

Absolute mean difference for % Assay from method precision and intermediate precision should not be more than 3.0%.

6.4.6Robustness:

The robustness of an analytical method was carried out to confirm that the method remained unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The standard solution was injected six times for each varied conditions of flow, column temperature, pH, and wavelength and chromatograms were recorded.This parameter was studied by making small, deliberate changes in the chromatographic conditions and Assay parameters, observing the effect of these changes on the system suitability and results obtained by injecting the standard and sample solutions.

6.4.6.1 Change in Flow rate (±0.1mL/min)

Table 6.6: Condition of change in flow rate

Condition	Flow rate (mL/min)
Original flow	1.0
Increased flow	1.1
Decreased flow	0.9

6.4.6.2 Change in wavelength (±2nm)

Table 6.7: Condition of change in wavelength

Condition	Wavelength
Original wavelength	250nm
Increased wavelength	252nm
Decreased wavelength	248nm

6.4.6.3 Change in column temperature (±5 °C):

Table 6.8: Condition of change in temperature

Condition	Temperature (°C)
Original temperature	30
Increased temperature	35
Decreased temperature	25

6.4.6.4 Change in pH of mobile phase (±0.2 unit)

Table 6.9: Condition of change in pH

Condition	pH
Original pH	3.0
Increased pH	3..2
Decreased pH	2.8

Acceptance criteria:

System suitability criteria should be fulfilled.

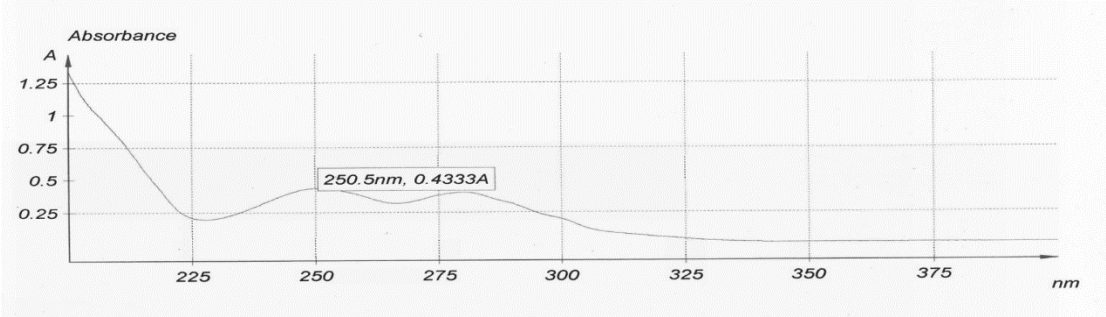
The absolute difference of % assay value in each modified condition should be within ± 2.0 when compared to the original condition

07. RESULTS AND DISCUSSION

A simple, precise and economic RP-HPLC method was developed and validated for estimation of Trifluoperazine Tablet. The method was validated as per ICH guidelines byusing various validation parameters such as Linearity, accuracy, precision, specificity and robustness.

7.1. STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND OPTIMIZATION:

7.1.1 Selection of Wavelength



➢ **Figure 7.1 Spectra showing λmax of** Trifluoperazine

➢ **Table 7.1 Determination of λ max of** Trifluoperazine Tablet

Sr. No.	Wavelength (nm)	Absorbance
1.	250	0.4333 A

7.1.2 Identification by IR Spectroscopy:

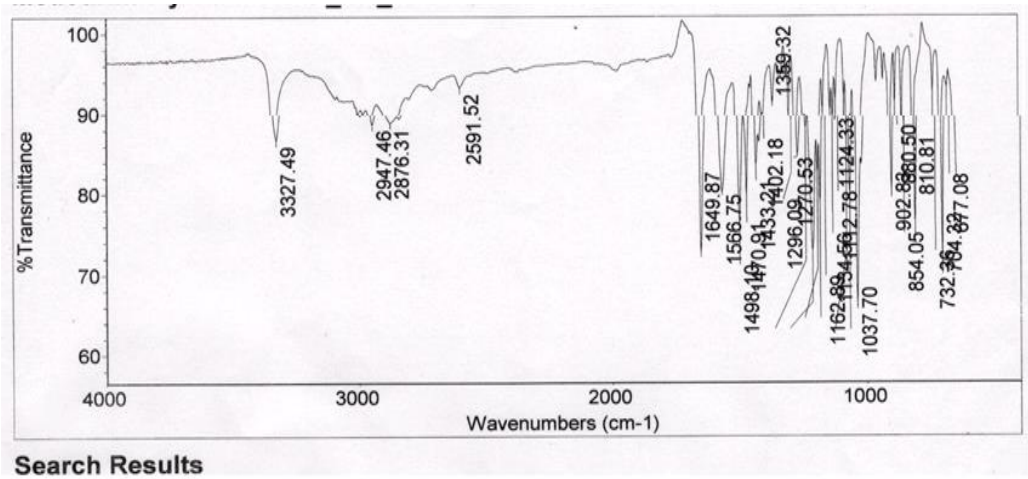


Fig. 7.2 FTIR spectrum of TrifluoperazineStandard Solution

Table 7.2 IR peak Assignment value of Trifluoperazine

Standard IR Ranges (cm^{-1})	IR Ranges (cm^{-1})	Functional Group
3400-3700	3327	O-H Alcohol
2850-2950	2876	C-H ALKANE
1080-1360	1296	C-N STRECH
1400-1600	1484	C=C STRECH OF AROMATIC RING
1000-1300	1162	C-O STRECH
675-1000	854	C-H BENDING OF BENZENE

7.3.3 Reverse Phase High Performance Liquid Chromatography Method Development

Different trials taken were as follows:

TRIAL: 1

Chromatographic Conditions:

mn		, 150 x 4.6 mm, 5µ
ile Phase		er pH 4.00 100%
Rate		nL/min
tion Volume		L
elength		nm
mn Temp		
ple Temp		
Time		minute
ntion Time		bserve
lle Wash		r : Acetonitrile (10:90)
Wash		r : Methanol (90:10)

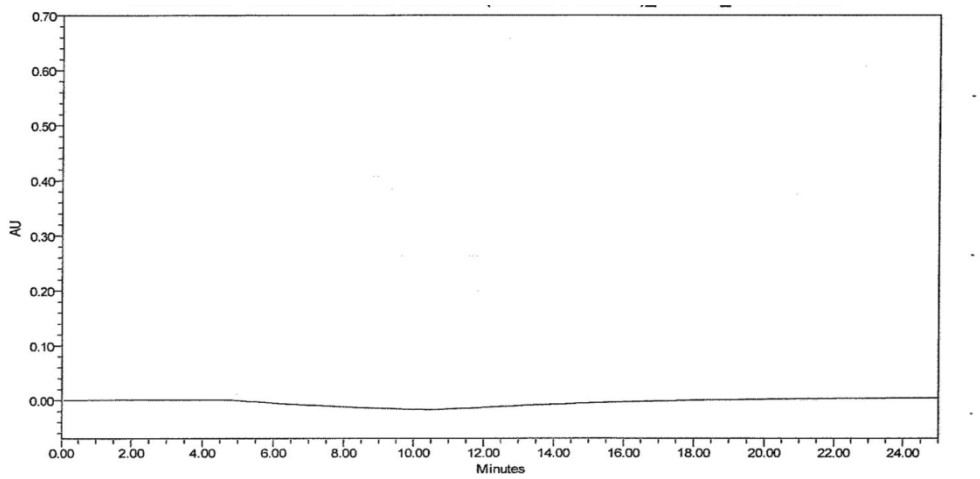


Fig. 7.3Typical chromatogram for Trial- 1

Conclusion:

Trifluoperazinepeak NOT observed

TRIAL: 2

Chromatographic Condition:

mn		, 250 x 4.6 mm, 5µ (GL Sciences, 020-01801)
ile phase		er pH 4.00 ± 0.05 and Methanol in the ratio 90:10 v/v
Rate		nL/min
tion Volume		L
elength		nm
mn Temp		
ple Temp		
Time		inute
ntion Time		Not Observe
lle Wash		r : Acetonitrile (10:90)
Wash		r : Methanol (90:10)

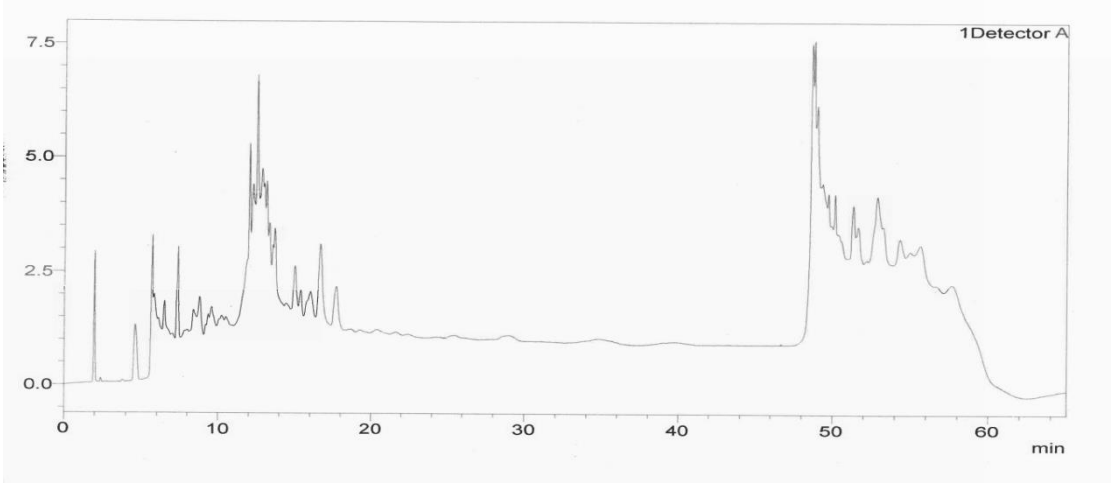


Fig. 7.4 Typical chromatogram for Trial- 2

Conclusion:

Trifluoperazine peak NOT observed. Also Base line not uniform.

TRIAL: 3

Chromatographic Condition:

mn		sil ODS-3V, 150 x 4.6 mm, 5µ
ile phase		er pH 4.00 ± 0.05 and Acetonitrile in the ratio 80:20 v/v
Rate		nL/min
tion Volume		L
elength		nm
mn Temp		
ple Temp		
Time		ninute
ntion Time		ut 4.7 minutes
lle Wash		er : Acetonitrile (10:90)
Wash		er : Methanol (90:10)

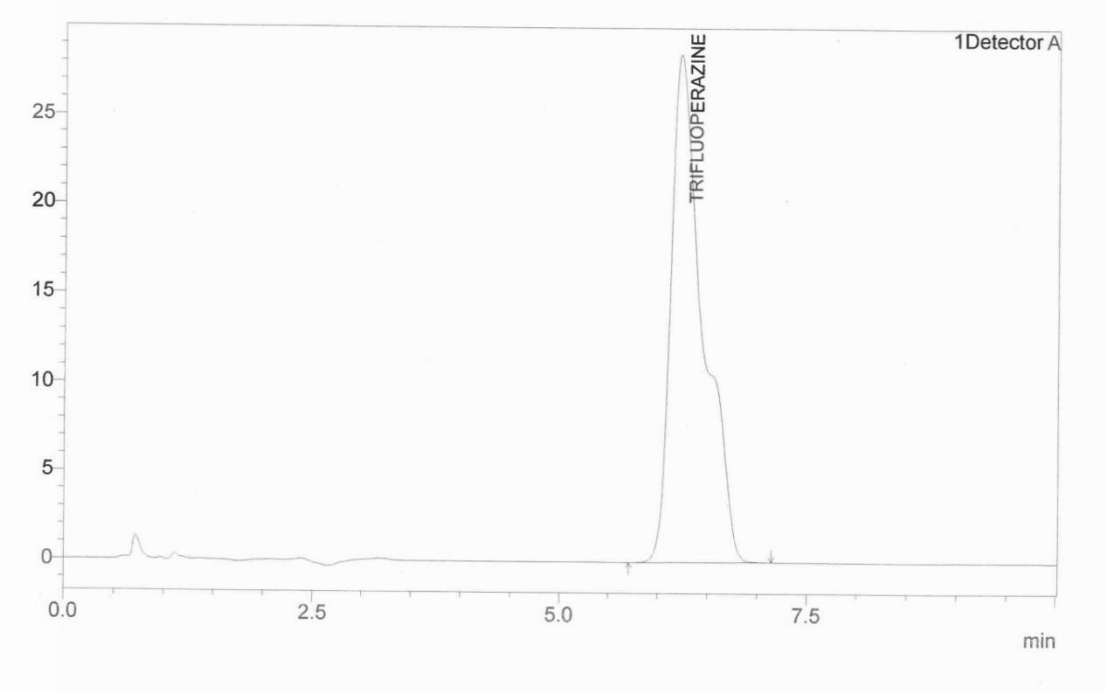


Fig. 7.5 Typical chromatogram for Trial- 3

Conclusion:- Trifluoperazine peak observed at 6.302 min, But peak shape not proper method needs to be optimized.

TRIAL: 4

Chromatographic Condition:

mn		sil ODS-3V, 150 x 4.6 mm, 5µ (GL Sciences, 020-01801)
ile phase		er pH 3.00 ± 0.05 and Acetonitrile in the ratio 80:20 v/v
Rate		nL/min
tion Volume		L
elength		nm
mn Temp		
ple Temp		

Time		hinute
ntion Time		ut 4.7 minutes
lle Wash		er : Acetonitrile (10:90)
Wash		er : Methanol (90:10)

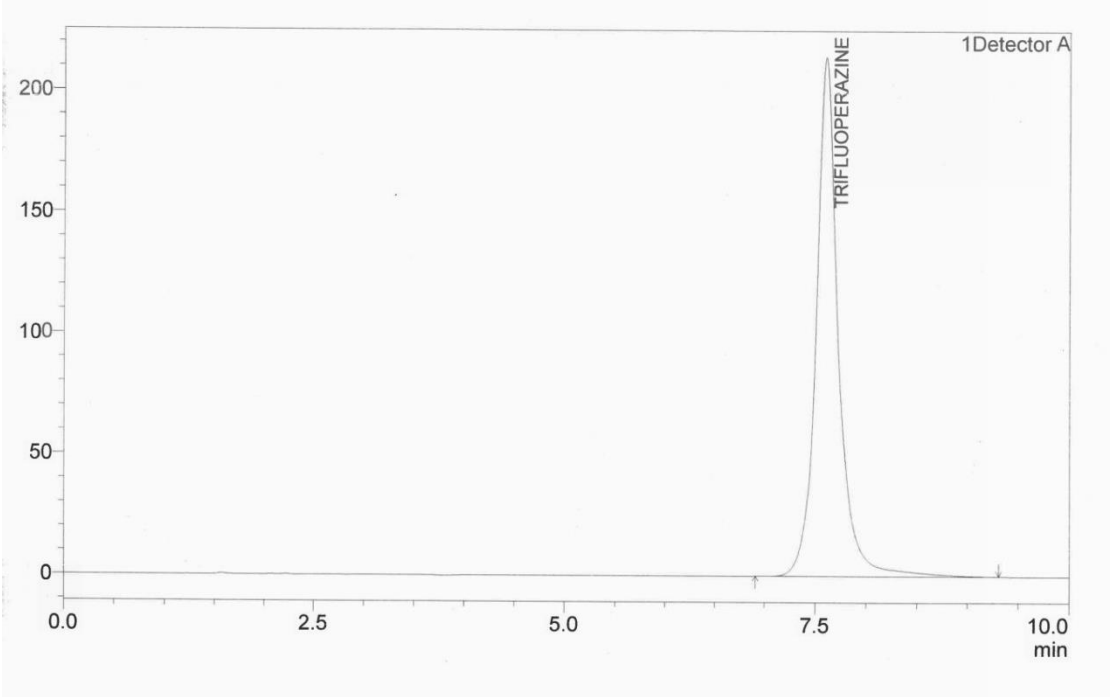


Fig. 7.6 Typical chromatogram for Trial- 4

Conclusion:-Trifluoperazine peak observed at 7.548 min, but reduction of retention time is required method so needs to be optimized.

TRIAL: 5

Chromatographic Condition:

mn		sil ODS-3V, 150 x 4.6 mm, 5µ (GL Sciences, 020-01801)
ile phase		er pH 3.00 ± 0.05 and Acetonitrile in the ratio 55:45 v/v
Rate		nL/min
tion Volume		L
elength		nm
mn Temp		
ple Temp		
Time		ninute
ntion Time		ut 4.7 minutes
lle Wash		er : Acetonitrile (10:90)
Wash		er : Methanol (90:10)

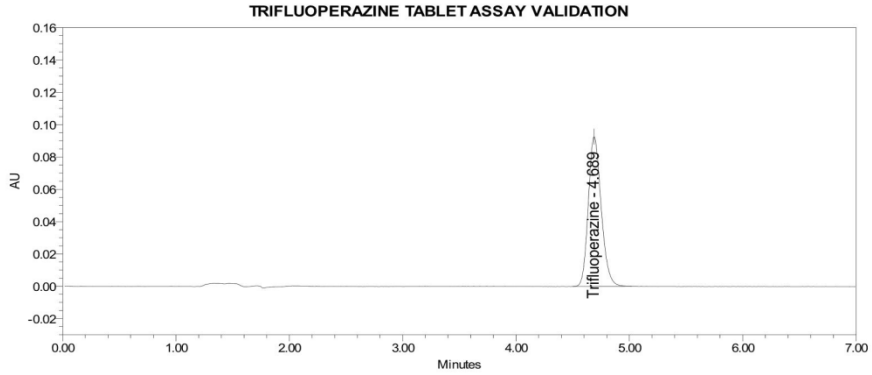


Fig. 7.7 Typical chromatogram for Trial- 5

Conclusion:- Trifluoperazine peak observed at 4.689 min.

7.2. METHOD VALIDATION

The following parameters were considered for the analytical method validation of title ingredients.

- System Suitability.
- Specificity.
- Linearity.
- Accuracy.
- Precision.
- System Precision.
- Method Precision.
- Intermediate Precision.
- Robustness.

7.2.1 SYSTEM SUITABILITY: System suitability test is a pharmacopoeial requirement and is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done.

Table 7.3 system suitability test ofTrifluoperazine

Component	**Trifluoperazine**
USP Tailing	1.2
Theoretical Plates	8221
S. No.	**Peak Area**
1	764915
2	765245
3	766067
4	767967
5	765739
Mean Area	**765986.6**
%RSD	**0.16**
Correlation	**98.8**

The tests were performed by collecting data from Single injection of blank (Diluent) and five replicate injections of Standard solution were injected into the chromatograph. The data obtained is summarized in Table 7.3

Conclusion:

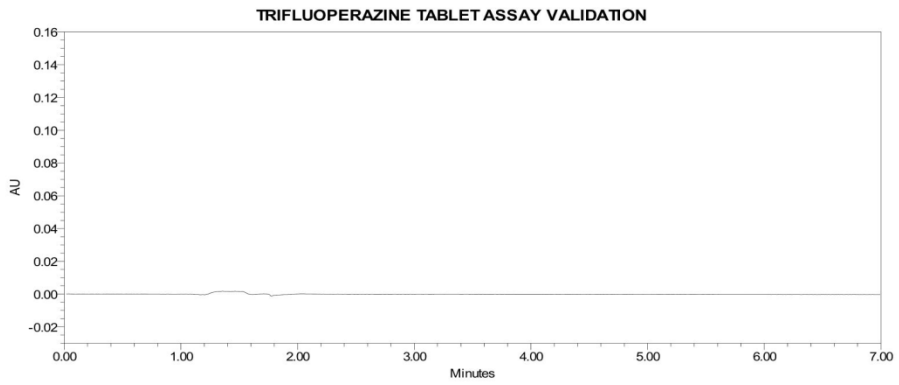
- The data demonstrates that the system suitability is within the acceptance criteria thus the system is suitable.

7.2.2 Specificity: (Identification, Interference & Peak Purity)

Inject Blank (Diluent), standard solution, impurity Solution, placebo solution and sample solution .The data obtained is summarized in Table 7.4

Table 7.4 Specificity (Identification and Interference)

Component	Retention time (min)	Purity angle	Purity threshold
Blank (Diluent)	-	-	-
Placebo Solution (Strength – 5 mg)	-	-	-
Trifluoperazine 4-Isomer	3.75	0.522	0.796
Trifluoperazine Sulphoxide	2.360	2.991	6.564
Standard solution	4.690	0.191	0.391
Sample solution	4.689	0.178	0.354



Chromatogram of Blank

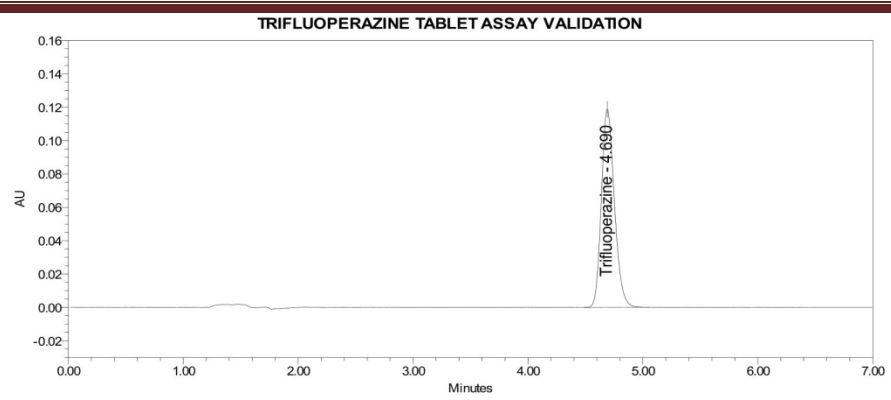


Fig 7.9 Chromatogram of Standard

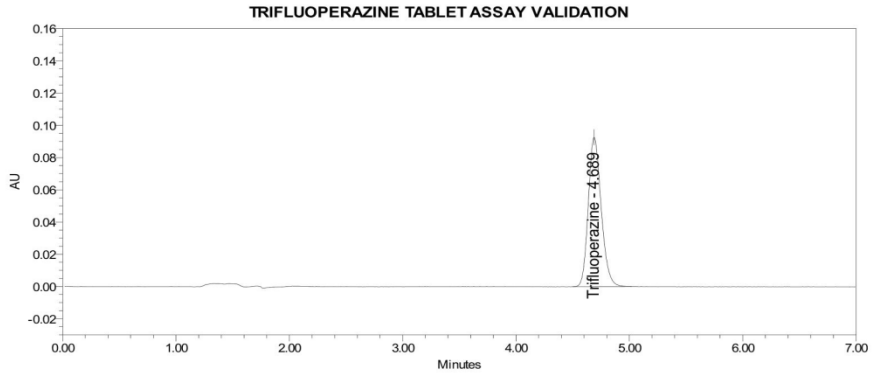


Fig 7.10 Chromatogram of Sample

Fig 7.11 Chromatogram of Placebo

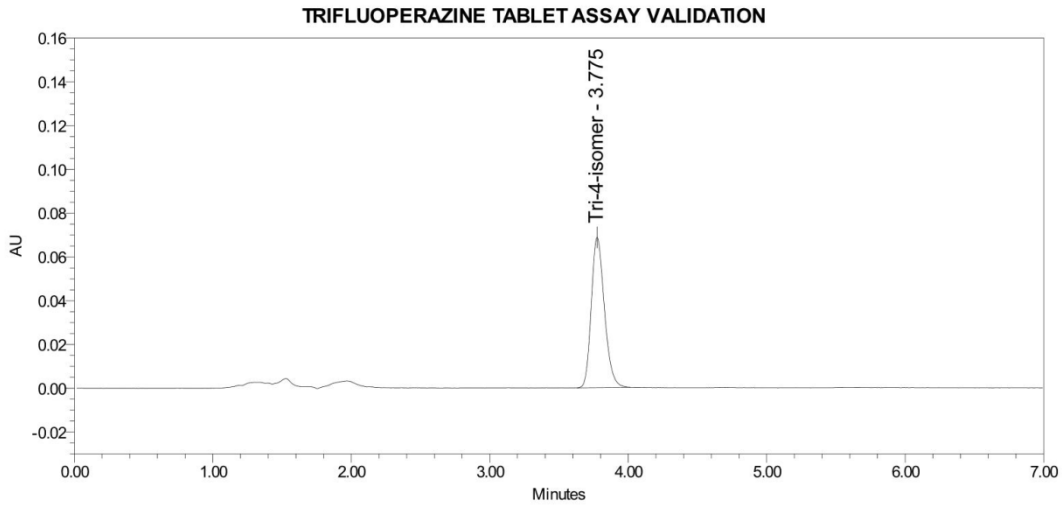


Fig 7.11 Chromatogram of tri- isomer

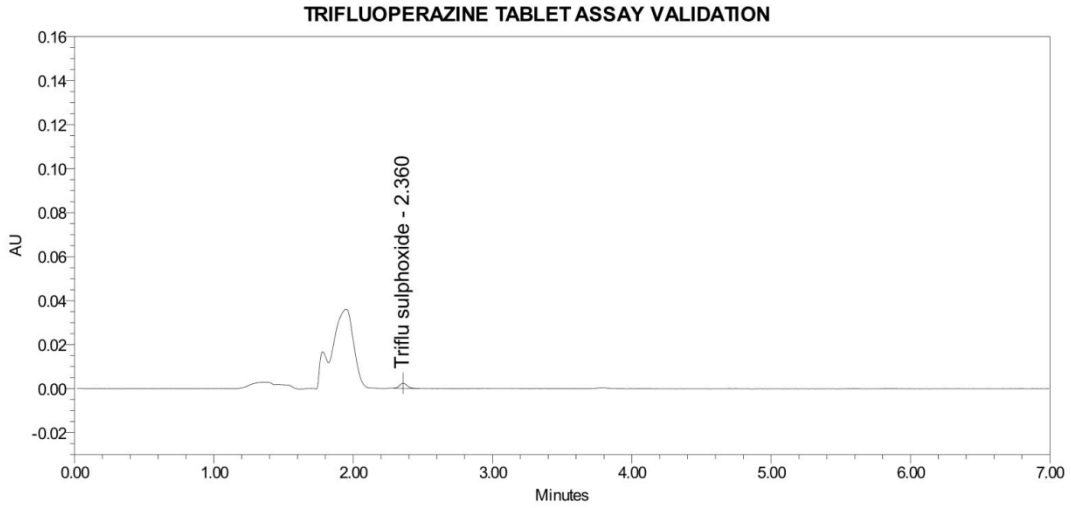


Fig 7.12 Chromatogram of Triflusulphoxide

Conclusion:

The data demonstrates that retention time in standard and sample is same for Trifluoperazine peak.

The data demonstrates that there is no interference in Blank and Placebo at the retention time of Trifluoperazine peak.

Peak Purity match in both chromatograms obtained from Standard and Sample solution.

6.2.3. LINEARITY:

Linearity was evaluated in the range of 50% to 150% of Trifluoperazine for working concentration. The working concentration of Trifluoperazine is 10 ppm

Level (%)	Concentration w.r. sample (mg/mL)	Peak Area Injection - 1	Peak Area Injection - 2	Mean Peak Area
50	5.07	479487	479915	479701
75	7.61	706597	709138	707868
100	10.15	958790	957741	958266
125	12.69	1187021	1187294	1187158
150	15.22	1424035	1425047	1424541
Correlation Co-coefficient (R)				**1.000**
Slope of regression line				**93368.874**
%Y- intercept				**0.41**
Regression (r^2)				**1.000**

Table 7.5: Linearity ofTrifluoperazine

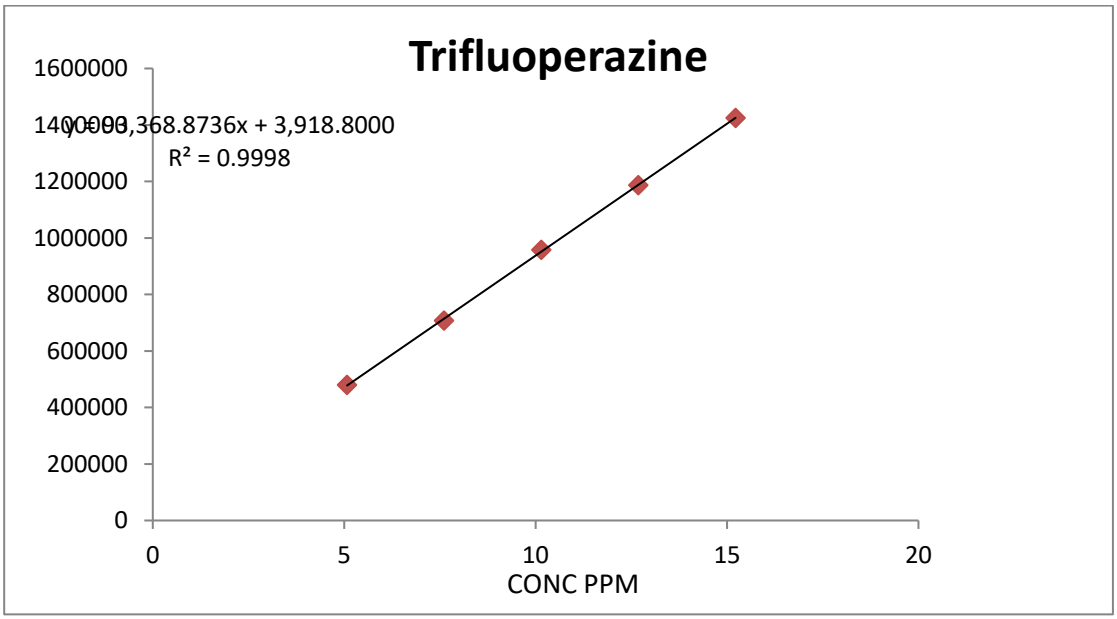


Fig 7.13 Linearity plot of Trifluoperazine

Conclusion:

The data shows that system suitability is fulfilled.

The data shows that the response is found to be linear.

% Limit of Y- Intercept is within ± 2.0% of the corresponding Y-co-ordinate of the working level.

7.2.4 Accuracy (Recovery):

Evaluated accuracy at three levels 50%, 100% and 150% of the working concentration for Trifluoperazine. The working concentration level of Trifluoperazine is 10 ppm. Each level prepared in triplicates.

Table 7.6: % Recovery for Trifluoperazine

Level %	Conc added (µg/mL)	Peak Area	Conc recovered (µg/mL)	% Recovery	% Mean recovery	% Overall Mean recovery
50%	5.121	469841	5.007	97.8	**98.2**	**98.5**
	5.089	470742	5.017	98.6		
	5.088	469092	4.999	98.3		
100%	10.346	955465	10.183	98.4	**98.2**	
	10.346	959902	10.230	98.9		
	10.346	943822	10.059	97.2		
150%	15.264	1418491	15.118	99.0	**99.2**	
	15.366	1436652	15.311	99.6		
	15.400	1430714	15.248	99.0		

Conclusion:

The data shows that the Mean recovery for 50% to 150% is in the range of 98.0%-102.0% and individual recovery for 50% to 150% is in the range of 97.0% - 103.0%.

7.2.5 Precision:

7.2.5.1 System Precision:

Single injection of Blank (Diluent) and five replicate injections of Standard solution were injected into the chromatographic system. The data obtained is summarized in Table 7.7

Table 7.7 System precision

Component	Trifluoperazine
USP Tailing	1.2
Theoretical Plates	8221
S. No.	**Peak Area**
1	764915
2	765245
3	766067

4	767967
5	765739
Mean Area	**765986.6**
%RSD	**0.16**
Correlation	**98.8**

Conclusion:

- Observed %RSD for five replicate of standard injections meet the system suitability requirement, hence system is precise

7.2.5.2 Method Precision:

Single injection of blank (Diluent), Standard solution (five replicates) and sample solution (six preparations) was injected on the system.

Table 7.8 Method precision

Independent Sample	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
%Assay	97.7	96.3	97.5	97.4	98.6	96.9
Mean	**97.4**					
%RSD	**0.80**					

Conclusion:

- The data shows that system suitability is fulfilled.
- The data shows that % RSD for % Assay is within the acceptance criteria and hence the method is precise.

7.2.5.3 Intermediate Precision:

Five independent sample preparations were prepared on different day and by different analyst and injected on the HPLC.

Table 7.9Intermediate Precision

Parameter	Method Precision (Analyst-I)	Intermediate Precision (Analyst-II)
HPLC NO.	AD/I-029	AD/I-050
Column No.	C18-740	C18-530
Date of analysis	15 SEP 201	
Sample No.	**% Assay**	
1	97.7	97.2

2	96.3	99.6
3	97.5	98.4
4	97.4	99.2
5	98.6	98.2
6	96.9	99.3
Mean	**97.4**	**98.7**
Absolute Mean difference % Assay	**1.3**	

Conclusion:

- The data shows that system suitability is fulfilled.
- The data shows that % Assay is of six samples is not more than 2.0
- The data shows that % Assay is within the acceptance criteria and hence the method is rugged.

7.2.5 Robustness:

This parameter was studied by making small, deliberate changes in the chromatographic conditions and Assay parameters, observing the effect of these changes on the system suitability and results obtained by injecting the standard and sample solutions.

Table 7.10 Robustness for Trifluoperazine

Changes in parameters	Values	Retention time of Trifluoperazine	Theoretical Plates	Tailing factor	% RSD of standard area	% Assay	% Difference
Control	**As per method**	-	-	-	-	97.4	NA
Change in Flow rate (± 0.2 mL/min)	**+ 0.2 mL/min**	3.8	6975	1.2	0.38	97.1	0.3
	- 0.2 mL/min	5.3	7383	1.1	0.15	97.0	0.4
Change in wavelength (± 2 nm)	**+ 2 nm**	4.7	8174	1.2	0.17	97.3	0.1
	- 2 nm	4.7	8202	1.2	0.13	97.3	0.1
Change in Column temperature (± 5°C)	**+ 5°C**	4.6	7760	1.2	0.10	97.9	0.5
	- 5°C	3.9	5403	1.1	0.38	95.4	2.0
Change in Buffer pH (± 0.2 units)	**+ 0.2 units**	5.1	7687	1.2	0.17	97.6	0.2
	- 0.2 units	4.6	7778	1.2	0.39	96.9	0.5
Change in Mobile	**+ 0.2% Organic**	3.8	7399	1.2	0.26	98.9	1.5

phase composition (± 2% absolute for Organic solvent)	**solvent**						
	- 0.2% Organic solvent	5.3	8001	1.2	0.19	98.6	1.2
Change in sonication Time of Sample	**30 mins**	4.5	7761	1.2	0.10	97.9	0.5
	50 mins	4.5	7761	1.2	0.10	98.5	1.1

Conclusion:

- System suitability criteria were fulfilled.
- The difference of % assay value in each modified condition is within acceptance criteria.
- % Assay obtained in each Robustness condition when compared to the original condition was found within the acceptance criteria

08. CONCLUSION

8.1 RP-High Performance Liquid Chromatography (HPLC) Method:

HPLC has gained the valuable position in the field of analysis due to ease of performance, specificity, sensitivity and the analysis of sample of complex nature. This technique was employed in the present investigation for estimation of trifluoperazine Tablet formulation. HPLC Water2469 with GL-Science, Inertsil ODS 3V C18, 5µ, 4.6 x 150mmcolumn andUV/PDA detector with empower pro Software was used for the study. The standard and sample solution of Trifluoperazine tabletwhere prepared in diluent.Different pure solvents of varying polarity in different proportions were tried as mobile phase for development of the chromatogram.

During selection and optimization of the mobile phase it was observed that the sharpness of the peak is achieved pH change.

The mobile phase that was found to be most suitable was Buffer and ACN, thewavelength 250nm were selected for the evaluation of the chromatogram oftrifluoperazine Tabletrespectively. The selection of the wavelength was based on the λmax obtained by scanning of standard laboratory mixture in 0.1 n HCL: Acetonitrile. This system gave good resolution and optimum retention time with appropriate tailing factor (<2).

After establishing the chromatographic conditions, standard laboratory mixture was prepared and analysed by procedure described under Materials and methods. It gave accurate, reliable results and was extended for estimation of drugs in tabletformulation.

The results from table clearly indicate that the RP-HPLC technique can be successfully applied for the estimation of above-mentioned drugs in their formulation.

From the studies it can be concluded that RP-HPLC technique can be successfully used for the estimation of the Trifluoperazine tabletFormulations.

The method shows good reproducibility; moreover the RP-HPLC method is accurate, precise, specific, reproducible and sensitive. The analysis of single dose formulation of Trifluoperazine tabletcan also be successfully performed by the RP-HPLC method. No interference of additives, matrix etc. is encountered in these methods. Further studies on other pharmaceutical formulations would throw more light on these studies. Suitability of these methods on biological samples needy also studies

09.SUMMARY

The results of analysis in this method were validated in terms of accuracy, precision, ruggedness, linearity. The method was found to be sensitive, reliable, reproducible, rapid and economic also.

Table 9.1 Summary of System suitability

S. No.	Parameters	Acceptance criteria	Result obtained		
1.0	System suitability The relative standard deviation of six replicate injections	NMT 2.0%	0.16		
	Tailing factor	NMT 1.5	12		
	Theoretical plates	NLT 2000	8221		
2.0 2.1	Specificity Identification	Results should be comparable with respect to the retention time.	RT of Standard: 4.690		
			RT of sample	4.690	
			Trifluoperazine 4-Isomer	3.750	
			Trifluoperazine Sulphoxide	2.360	
2.2	Interference	Blank (Diluent), Placebo and known Impurities should not show any peak at the retention time of Pomalidomide peak	Complies		
2.3	Peak purity	Standard and Sample peak should be pure at working concentration level. Purity angle should be less than purity threshold.		**Purity angle**	**Purity Threshold**
			Standard	0.191	0.391
			Sample for	0.178	0.391

<table>
<tr><th>Sr. No</th><th>Parameters</th><th>Acceptance criteria</th><th>Result obtained</th></tr>
<tr><td rowspan="2">3.0</td><td>Linearity and Range
Correlation coefficient</td><td>NLT 0.990</td><td>1.000</td></tr>
<tr><td>Y- intercept</td><td>Intercept y <± 2.0% of standard response</td><td>0.41</td></tr>
<tr><td>4.0</td><td>Accuracy (Recovery)</td><td>Mean and Individual recovery for 25% to 150% should be in the range of 95.0%- 105.0%.</td><td>Pomalidomide<table><tr><td>Level %</td><td>% Mean Recovery</td></tr><tr><td>50</td><td>98.2</td></tr><tr><td>100</td><td>98.2</td></tr><tr><td>150</td><td>99.2</td></tr></table></td></tr>
<tr><td>5.0
5.1</td><td>Precision
System Precision</td><td>System suitability criteria should be fulfilled.</td><td>Complies</td></tr>
<tr><td>5.2</td><td>Method precision</td><td>The RSD for % assay of six independent samples preparations: NMT 2.0%.</td><td><table><tr><td>% Mean Assay</td><td>97.4</td></tr><tr><td>% RSD</td><td>0.80</td></tr></table></td></tr>
<tr><td rowspan="2">5.3</td><td rowspan="2">Intermediate Precision (Ruggedness)</td><td>The RSD for % Assay of six independent samples preparation should not be more than 2.0%.</td><td></td></tr>
<tr><td>The cumulative % RSD for % assay of twelve independent samples preparation of two analysts should not more than 2.0%.</td><td><table><tr><td>% Mean Assay</td><td>98.05</td></tr><tr><td>% RSD</td><td>1.3</td></tr></table></td></tr>
</table>

Sr.No	Parameters	Acceptance criteria	Result obtained
6.0	Robustness Change in Flow rate (± 0.1 mL/min) Change in Column temperature (± 5°C) Change in Wavelength (±2 nm)	System suitability criteria should be fulfilled. The cumulative % RSD for % assay obtained in each modified condition should not be more than 2.0 when compared to the method precision.	Complies