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Method Development and Validation of Methoxsalen

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I. INTRODUCTION

Analytical chemistry more simply Analysis is understood as an examination of a chemical substance with the goal of eliciting information regarding its constituents: their character form, quality, or purity and quantity also known as concentration or content. [1] The Analysis is a key element of the advanced technologies in determining and optimizing the concentration of substances by selecting a suitable analytical method. By this, we can obtain both qualitative as well as quantitative analysis. The analytical method may be spectral, chromatographic, electrochemical, hyphenated or miscellaneous. The analytical instrument plays a significant role in the process to achieve high quality and reliable analytical data. [2]

Analytical method development is the process of developing an accurate assay procedure to determine the composition of a formulation or in a bulk of the specified substance. It is the process of proving that an analytical method is acceptable for use in a laboratory to measure the concentration of subsequent samples. Analytical methods should be used within criteria given as per regulatory authorities and must be developed using the protocols and acceptance criteria as per the regulatory guidelines.

A. Basic Equipment used in Analytical Chemistry

In analytical chemistry different types of the basic equipment used for measuring and transferring of solid, liquid and semisolid object according to the need of analysts. For measuring an accurate solid object the mostly use measuring balance such as analytical balance according to specification. The liquid objects measured in measuring cylinder having high accuracy and for transferring of liquid object different pipettes are used having different dimensions. The basic types of equipment are Analytical balance, Measuring cylinders, volumetric flasks, and pipettes, etc [3]

B. Analytical Techniques

Chemical or Physico-chemical processes that provide the basis for analytical measurement are known as analytical techniques. There are numerous chemical or Physico-chemical processes that can be used to provide analytical information. The Atomic, Molecular spectrometry and chromatography together contain the largest and most widely used groups of techniques. [4]

1) Classification of Analytical Methods

(A) Classical Methods

- a) Qualitative - identification by color, indicators, boiling points, odours, etc.
- b) Quantitative - mass or volume (e.g. gravimetric, volumetric, etc.)

(B) Instrumental Methods

- a) Qualitative - Chromatography, Electrophoresis and identification by measuring physical property (e.g. Spectroscopy, Electrode potential, etc.)
- b) Quantitative - Measuring property and determining relationship of concentration (e.g. Spectrophotometry, Mass spectrometry, etc.)

2) Chromatography

A variety of methods are available for the separation of components from the mixture and to analyze them. There are a few methods for analysis.

- a) Fractional distillation
- b) Extraction
- c) Fractional precipitation
- d) Crystallization

These methods are effective in separation, purification, and identification of many compounds. However, a difficulty arises in case of compounds where individual components have similar physical and chemical properties i.e. the mixture of liquids having very close boiling points, etc. however these methods are not satisfactory in biological materials. Chromatographic methods represent the most useful and powerful technique for these problems. They are used for separation of components of a complex mixture.

Chromatography is a powerful separation technique that is used in all branches of science and these techniques are simple, rapid requires simple apparatus. [5]

3) History

The term chromatography derived from the Greek word “Chroma” means “color” and “Graphe” means “writing”. Chromatography is a physicochemical method for separation of complex mixtures was discovered at the very beginning of the twentieth century by Russian–Italian botanist M. S. Tswett. In 1903 as he produced a colorful separation of plant pigments through a column of calcium carbonate. Chromatography has since developed into an invaluable laboratory tool for the separation and identification of compounds. Although color usually no longer plays a role in the process, the same principles of chromatography still apply. Chromatography technique developed substantially as a result of the work of Archer John Porter Martin and Richard Laurence Millington Syge during the 1940s and 1950s, for which they won the 1952 Nobel Prize in Chemistry. [6]

4) Types of Chromatography

Basically, the chromatography is classified into several classes according to principles of separation.

- a) Adsorption Chromatography
- b) Partition Chromatography
- c) Ion exchange Chromatography
- d) Size exclusion chromatography [7]

Now discussed all the types of chromatography in detailed are as follow,

a) Adsorption Chromatography

Adsorption chromatography has a solid stationary phase and a liquid or gaseous mobile phase. The different solutes travelled a different distance through the stationary phase carried along by the solvent. Each solute has its own equilibrium between adsorption onto the stationary phase and solubility into the solvent. The separation depends upon the affinity of solute towards the stationary phase and mobile phase that solute having a greater affinity towards the stationary phase which elutes slowly compared with solutes having a greater affinity toward mobile phase.

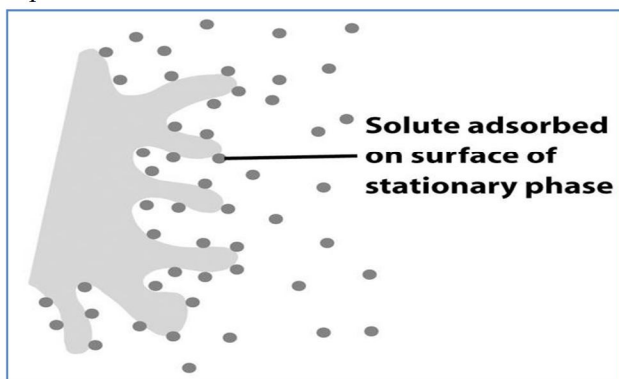


Fig 1.1: Adsorption of solutes over stationary phase

b) Partition Chromatography

Partition chromatography has been one of the most significant classes of separation methods since its development by Martin and Syge in the 1940s. All partition chromatography techniques apply the same principle. There are two phases, one is stationary and other is a mobile phase, and the sample is partitioned between these two phases, based on their greater affinity to either one. The mobile phase can be liquid or gaseous.

c) *Ion exchange chromatography*

Ion-exchange chromatography may be viewed as a type of adsorption chromatography in which interactions between solute and stationary phase are primarily electrostatic in nature. The stationary phase (ion-exchanger) contains fixed functional groups that are either negatively or positively charged. A sample ion (or charged sites on large molecules) can exchange with the counter-ion to become the partner of the fixed charge. Chromatographic separations by ion exchange are based upon differences in affinity of the exchangers for the ions (or charged species) to be separated. [8]

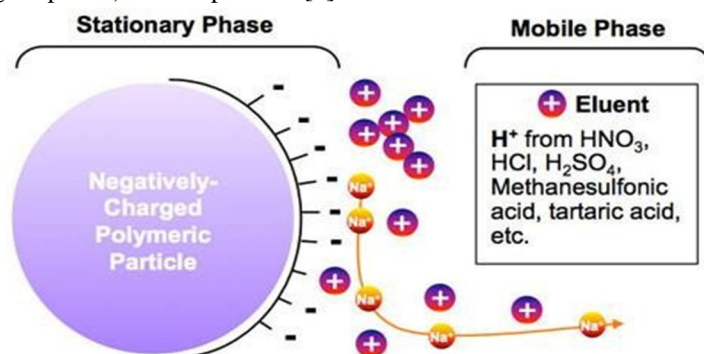


Fig 1.2: Ion exchange chromatography

d) *Size-Exclusion chromatography*

Size exclusion chromatography is also known as Gel filtration. SEC separates molecules in aqueous solution according to their size as they pass through a porous structure. The largest molecule elute first compared to a smaller one. The components which are completely excluded from the gel will not be separated from each other, and similarly, small molecules which completely penetrate the gel will not be separated from the gel. If the substances are of similar chemical type, they are eluted in order of relative molecular mass. [9, 10]

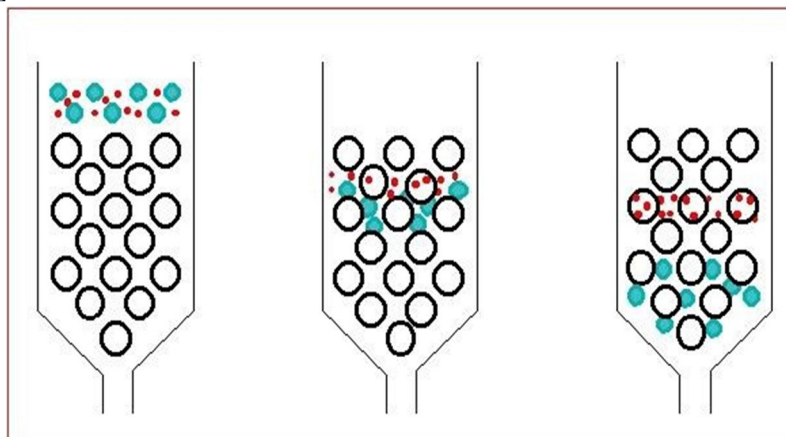


Fig1. 3: Size Exclusion Chromatography

5) *Chromatographic methods*

The different types of chromatographic methods are developed to separate mixtures and identify compounds.

- a. Column chromatography
- b. Thin layer chromatography
- c. Paper chromatography
- d. High-performance thin layer chromatography
- e. High-performance liquid chromatography
- f. Gas chromatography
- g. Ultra performance chromatography
- h. Flash chromatography

6) *High-Performance Liquid Chromatography*

HPLC is also known as High-pressure liquid chromatography, High-performance liquid chromatography, High-price liquid chromatography, High-speed liquid chromatography, High-efficiency liquid chromatography.

HPLC is a modern form of liquid chromatography that uses small particle column through which a mobile phase is pumped at high pressure. This is a chromatographic process, where a mixture of an analyte is separated into its distinct bands as they migrate down the column filled with a stationary phase. HPLC is a dynamic partitioning process of analyte between the flowing liquid and spherical packaging particles. HPLC is used either in the liquid-solid adsorption chromatography mode or the liquid-liquid partitioning chromatography mode, either normal or reverse-phase. Both partition and adsorption chromatography operates on differences in solute polarity since polarity is important in determining both adsorption and solubility. [11]

Modes of HPLC

1) Normal Phase chromatography

The separation by this method based on adsorption of the analyte onto a polar stationary phase. The typical stationary phase includes silica, alumina that has a polar hydroxy group on their surface.

2) Reversed phase chromatography

The separation is based on analyte partition coefficient between the polar mobile phase and non-polar stationary phase. Typically stationary phase includes C18 bonded group on silica. [12]

Table 1.1: Modes of Chromatography

Mode of chromatography	Stationary Phase	Mobile Phase
Normal phase chromatography	Polar	Non-polar
Reverse phase chromatography	Non-polar	Polar

Advantages

- It provides a specific, sensitive and precise method for analysis of different complicated samples.
- There is ease of sample preparation and sample introduction.
- There is the speed of analysis.
- The analysis by HPLC is specific, accurate and precise.
- Provides automated operation.
- Quantitative sample recovery.

C. Instrumentation

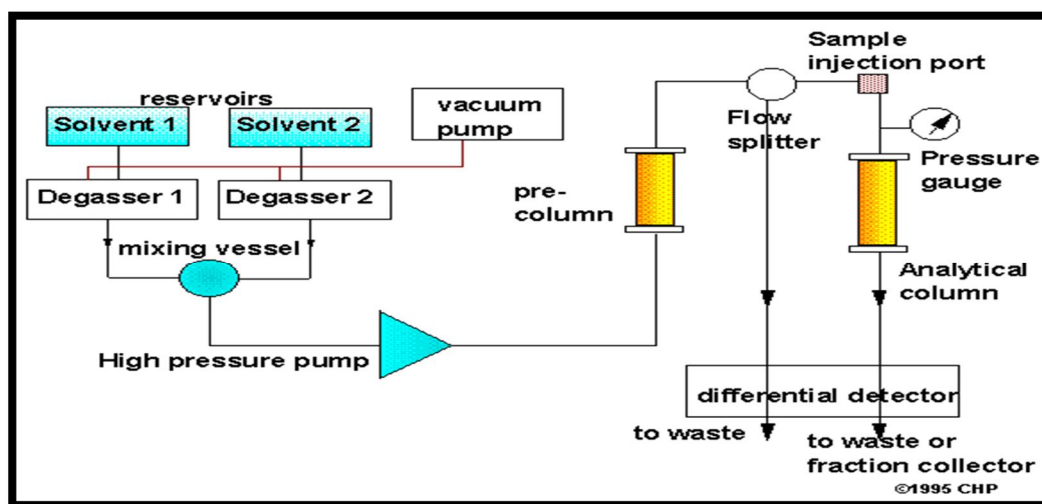


Fig 1.4: Schematic diagram of HPLC system [13]

A schematic diagram of the HPLC system is shown in figure 1.4. To attain high flow rates and yet keep the particle size of packing very low (3-10 μm), pumping pressures of several hundred atmospheres (2000-8000 psi) are required. Thus the equipment for HPLC is quite elaborate though simple.

D. Mobile Phase Reservoir And Solvent Treatment System

A modern HPLC apparatus is equipped with one or more glass or stainless steel reservoirs, containing 500 ml or more of solvent. The reservoir is often equipped with means of removing dissolved gases usually O_2 and N_2 that interfere by forming bubbles in the columns and detector system. These bubbles cause band spreading; in addition, they interfere with the performance of the detector. Hence the degassing of the mobile phase is too much importance in HPLC operation. [14]

E. Solvent Degassing methods

There are four main methods used to degas the mobile phase or solvents. They are: sparging with a less soluble gas, heating, reducing pressure by vacuum and sonication. These methods may be used singly or in combination.

a) Sparging (On-line degassing method)

Sparging or bubbling a gas through a solvent reduces the partial pressure of unwanted gas on the surface of the solvent. This will remove unwanted gas from the solution. But it will saturate with the second gas. Sparging with N_2 or He will remove background absorbance on a UV detector.

b) Heat (Off-line degassing method)

The first is to raise vapor pressure of the solvent at the surface of the solvent, as the partial pressure of the solvent is raised the partial pressure of a gas is proportionally reduced. This will prevent further absorption of gas into solution. Secondly, heat may reduce the solubility of a gas in solution. However, this method is not recommended when organic solvents are present in the mobile phase.

c) Vacuum (Off-line degassing method)

A vacuum reduces pressure on the surface of the solvent. The mass of gas in solution is proportional to the partial pressure of the gas at the surface of the solvent, so, as the pressure is reduced, the mass of gas in solution is reduced. A recent introduction of degassing techniques is the in-line vacuum degassers. Here the mobile phase is degassed and the air free mobile phase enters the pump directly without any possibility of resaturation of air.

d) Sonication (Off-line degassing method)

Sonication with high energy sound waves drives energy into the solvent and seems to cause aggregation of the submicron-sized particles of gas. As the gas aggregates, the bubble becomes large enough to float out of the solvent and dissipate. Sonication alone will degas a gallon of solvent in approximately

F. Pumps

The pumps are used to pass the mobile phase through the column at high pressure and controlled flow rate. These pumps are necessary to force the liquid through a column with finely packed particles. It should be noted that the high pressures generated by the pumps should not lead to an explosion hazard as a liquid is not very compressible.

Criteria for selection of pumps

1. Reproducible mobile phase flow rate (variation in flow rate shall affect detector sensitivity and interfere with quantitation)
2. Baseline pulsation minimum (pulseless flow) to minimize detector noise for trace analysis.
3. Suitable for operation at variable pressures (3000-6000 psi).
4. Suitable for a wide range of flow rate both for analytical (0.5-2 ml/min) and preparative chromatography (0-10 ml/min).
5. Provide a constant flow rate.
6. Resistant to chemicals and solvents commonly used in HPLC.
7. Adaptable to gradient analysis.
8. Suitable for use of small volumes of the mobile phase.
9. Protects mobile phase from evaporation.
10. Low maintenance cost.

Type of pumps

1. Syringe pump (Screw driven)
2. Reciprocating pump
 - i. Single piston reciprocating pump
 - ii. Dual piston reciprocating pump
 - iii. Reciprocating diaphragm pump
3. Pneumatic pump
 - i. Direct pressure pump
 - ii. Amplifier pump

Precautions

- Never store the corrosive solvents or buffers in the pump overnight.
- The pumps with electric motors may require periodic oiling.
- Use the degassed mobile phase.
- Avoid overheating in case of motor driven pumps.
- Do not allow the pump to dry.

Table 1. 1: Pump’s Advantages and Disadvantages

Types of pumps	Advantages	Disadvantages
Syringe type	Pulse free delivery at high pressure, flow rate independent of viscosity of MP	Limited solvent capacity
Reciprocating type	Constant flow rate, independent of viscosity of solvent	Detection noise due to pulsating outputs
Pneumatic type	Rugged, inexpensive, easy to operate, pulse free	Flow rate dependent on the viscosity of MP,

Pre-column (saturator column)

One of the factors affecting the life span of an analytical column is the dissolution of base silica due to the condition of pH >8.0 or <2.0, temperature >50°C, concentrated aqueous buffers, and ion-pair reagents. Pre-column fitted between the pump and the injector valve ensures that the mobile phase is fully saturated with silicates ions in pre-column prior to the sample injection. Thus the use of pre-column shall reduce adverse effects of low or high pH mobile phase.

G. Sample injectors

Often the limiting factor in the precision of liquid chromatographic measurements lies in the reproducibility where samples can be introduced into the column packing. It must be noted, that overloading of the sample causes band broadening. Therefore a minimum amount of sample must be introduced.

The sample injectors are of the following types,

a) Syringe injection

This is the simplest technique hence the sample is injected through a self-sealing elastomeric septum and the syringes are designed to withstand pressures up to 1500 psi. The disadvantage is that the reproducibility is poor.

b) Stop flow injection

This is too syringe injection but here the solvent flow is stopped momentarily. After removing the fitting at the column head, the sample is injected directly onto the head of the column packing at atmospheric pressure. Then the fitting is replaced and the system is again pressurized.

c) Solvent flowing

Here sampling valves or loops are used, which inject sample volumes more than 10ul. Currently, this type of injectors is usually used in all automatic system. In the fill position, the sample loop is filled at atmospheric pressure. Actuation of the valve and the sample in the loop occurs at once. Samples in the range of 1-9 ml can be handled without affecting column efficiency.

d) Guard column

The primary purpose of the guard column is to protect expensive analytical columns by removing particulate garbage and strongly irreversible retained sample components which decrease the life span of an analytical column. Guard column is installed between the sample injector and the analytical column. The matching chemistry of guard column with an analytical column is very important. While using RP- C-18 analytical column, it is desirable to use a C-18 guard column.

e) Columns

Columns are often referred to as the heart of HPLC separation process. The stable, high-performance column is an essential requisite for rugged and reproducible method. They are usually constructed from smooth bore stainless steel tubing or heavy walled glass tubing. If prepared from heavy walled glass tubing, then pressure is restricted to lower than 600psi. Occasionally, you may come across coiled columns; but their use is very limited.

Column care

- Do not exceed the pH range from 2-8.5 with silica columns.
- Always avoid dramatic changes in running conditions.
- Always degas and filter the mobile phase.
- Protect your column from contaminants by using a guard column.
- Store the column in pure acetonitrile if possible.
- Increasing back pressure can be taken as a signal to change the guard column.

Table 1.2: Commonly used columns and their Manufacturer [15]

Column	Manufacturer
Adsorbosphere	Alltech
Alltima	Alltech
Chromegabond	ES Industries
Econosil	Alltech
Lichrosorb	E. Merck
Novapak	Waters
Spherisorb	Waters
Zorbax	Hewlett Packard

H. Detectors

A detector is the eye of an LC system and measures the compound after their separation on the column. There are basically two types of detectors,

- a) The bulk property detectors
- b) The solute property detectors

The bulk property detectors function on some bulk property of the effluent, such as refractive index and are not suitable for gradient elution and are usually less sensitive than solute property detectors. The solute property detector performs by measuring some type of physical or chemical property that is specific to the solute only.

Criteria for detectors

- High sensitivity
- Higher linear dynamic range
- Applicable to most of the solutes
- Does not contribute to band broadening
- Non-destructive
- Faster response.[16,]

a) UV- Visible detector

The UV absorbance detector is the most common HPLC detector in use today. The criteria for this detector the analyte should absorb UV light. There are three types of UV detector; fixed wavelength detector and variable and photodiode detector.

b) Fluorescence detector

Fluorescence detector measures the optical emission of light by solute molecules after they have been excited at a higher energy wavelength.

c) Conductivity detector

The conductivity detector is bulk property detector which measures the conductance of mobile phase. The conductance is changed according to the how much solute elutes through mobile phase.

d) Refractive index detector

The refractive index detector is universal bulk property detector and oldest LC detector. RI detector measures the optical refractive index difference between mobile phase and sample. [17]

I. Fundamental Parameters of HPLC

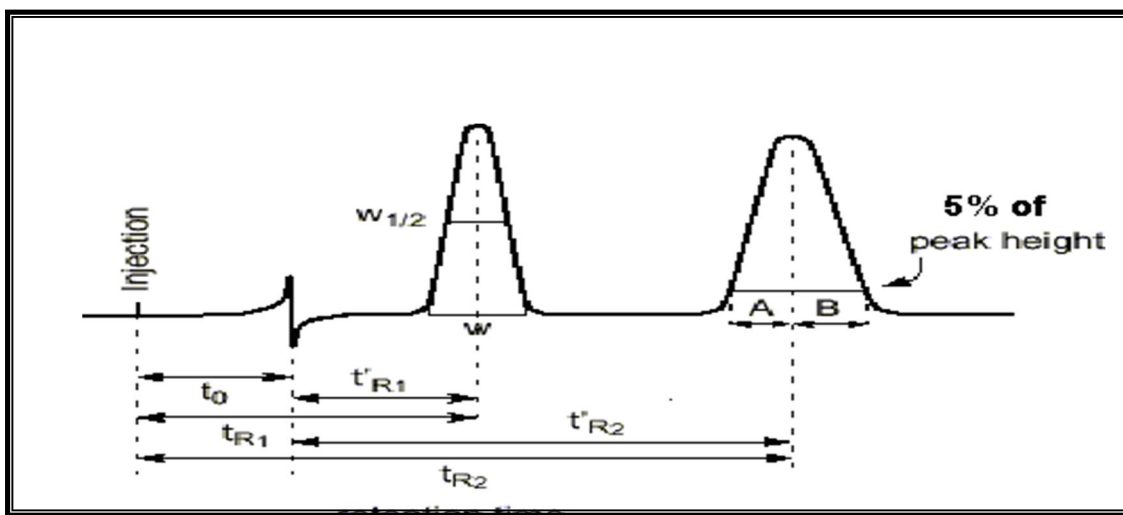


Fig 1.5: Fundamental Parameters of Chromatography

Where,

$w_{1/2}$ = peak width at half height

w = band width of the peak (intersection point of the inflection tangents with the zero line)

A = peak front at 5% of peak height to peak maximum B = peak maximum to peak end at 5% of retention times

t_0 = dead time of a column (retention time of un-retarded substance) t_{R1} , t_{R2} = retention time of components 1, 2.

t_{R1} , t_{R2} = net retention time of components 1, 2.

1) Resolution

The measure of the degree of separation for two closely eluting compounds is called resolution. It is calculated from the width and retention time of two adjacent peaks.

$$R_s = 2(t_2 - t_1) / (w_1 + w_2)$$

Where,

R_s = Resolution,

t_1 = retention time of first peaks, t_2 = retention time of second peak,

w_1 = Baseline band width of first peak,

w_2 = Baseline band width of second peak.

2) Capacity Factor (k')

It is the measure of the position of a sample peak in the chromatogram, being specific for a given compound, a parameter that specifies the extent of the retention of substances to be separated. k' depends on the stationary phase, mobile phase, temperature and quality of column packing.

$$k' = \frac{t_{R1} - t_0}{t_0}$$

Where,

k' = Capacity factor,

t_{R1} = retention time first eluting component, t_0 = dead time of column

3) Separation Factor (Selectivity) (α)

Selectivity (α) is the ability of chromatographic system to discriminate two different analytes. It is defined as the ratio of corresponding capacity factors or it measures peak spacing.

$$\alpha = \frac{t_2 - t_0}{t_1 - t_0}$$

Where,

t_2 = retention time of second eluting component, t_1 = retention time of first eluting component,

t_0 = Dead time of column

4) Tailing Factor (T) or Asymmetric Factor (A_s)

The tailing factor, T, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced. In some cases values less than unity may be observed. As peak asymmetry increases and hence precision becomes less reliable.

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

Where,

$W_{0.05}$ = Width of peak at 5% height,

f = Distance between maxima of two peaks.

5) Therotical plate number (n)

The column plate number is important property of the column. It reflects its quality of separation and its ability to produce sharp, narrow peaks and achieving good resolution of peaks.

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

Where,

n = number of therotical plates,

t = retention time of the component, w = width of the base of the peak.

Factors influencing therotical plate number

- Well-packed column.
- Larger column.
- Less flow-rate (0.5-2.0ml/mm).
- Small packing molecules.[18, 19]

J. Troubleshooting in HPLC

Troubleshooting is a form of problem-solving, often applied to failed products or process. It is a logical search for the source of a problem so that it can be solved and so the product or process can be made operational again.

Troubleshooting strategy,

- 1) Identification of the problem.
- 2) Awareness of the cause (s) of the problem.
- 3) Isolation of exact cause of the problem.
- 4) Rectifying the problem if able.
- 5) Returning the unit to routine use or referring the problem to your maintenancemanager.[19]

K. Problems and solutions

1) High baseline drift

Possible cause: The Detector lamp/ optic temperature is not stable.Solution: allow the detector to warm up

Possible cause: If the mobile phase is not homogeneous.

Solution: After a day or more idle time, gently swirl the eluent bottles to homogenizesolvents already in their reservoir.

2) No peaks or very small peaks

Possible cause: Detector off, Broken connection to the recorder, No sample or wrongsample.

Solution: Check detector, Check connection, verify sample.

3) Change in retention time

Possible cause: Changing mobile phase composition, Loss of bonded stationary phase, Change in flow rate, Contamination builds up, Varying column temperature, etc.

Solution: Ensure system delivering correct composition, Use mobile phase pH between 2-8, Ensure flow rate in the system and Flush column occasionally with a strong solvent, solvent, Use insulated columns or kept constant room temperature.

4) Baseline noise

Possible cause: lack of solvent mixing, detector problem, the air bubble in the mobilephase, pump pulses, etc.

Solution: Use proper mixing device, replace UV lamp, degas mobile phase, service orreplace pump.

5) Negative peaks

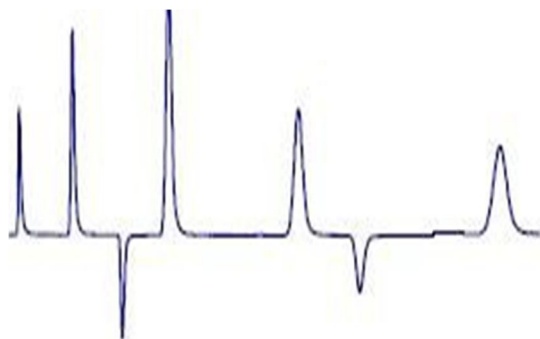


Fig1. 6: Negative peaks

Possible cause: Refractive index of solute less than that of the mobile phase, UV absorbance of solute less than that of the mobile phase, etc.

Solution: Reverse polarity to make positive, use a mobile phase with lower UV absorbance.

6) Peak fronting

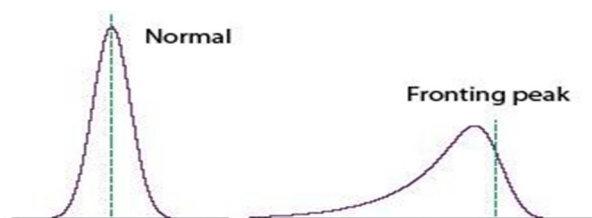


Fig1. 7: Peak fronting

Possible cause: channeling in column, column overloaded, etc.

Solution: replace or repack column, use higher capacity stationary phase, increase column diameter, decrease sample amount.

7) Peak tailing



Fig1.8: Peak tailing

Possible cause: Basic solute-silanol interaction, silica degradation at a higher pH, degradation at higher temperature, etc.

Solution: use stringer mobile phase, use polymeric column, high coverage reversed phase column, and reduce the temperature below 50°C.

8) Broad peak



Fig1. 9: Broad peak

Possible cause: Too long detector response time, too large detector cell,

Solution: Select a response time less than 1/4 of the peak width at half-height of the narrowest peak, Use smaller volume flow cell. [20, 21]

L. UV-Visible Spectrometry

Ultraviolet and visible spectrometers have been in general use for the last 50 years and over this period have become the most important analytical instrument in the modern day laboratory. The alternate title for this technique is ‘Electronic Spectroscopy’ since it involves the promotion of electrons from the ground state to the higher energy state. It is very useful to measure the number of conjugated double bonds and also aromatic conjugation within the various molecules. It also distinguishes between conjugated and non-conjugated systems.

The energy level of the molecule is quantized, the energy required to bring about the excitation is a fixed quantity. Thus, the electromagnetic radiation with only a particular value of frequency will be able to cause excitation. Clearly, if the radiation of a desired or correct frequency is passed or made to fall on the sample of a substance, energy will be absorbed and electron will be promoted to higher energy state. [22]

1) Lambert's law

It states that when a beam of monochromatic radiation passes through a homogeneous absorbing medium, the rate of decrease of intensity of radiation with the thickness of absorbing medium is proportional to the intensity of the incident radiation.

2) Beer's law

This law states that when a beam of monochromatic radiation is passed through a solution of an absorbing substance, the rate of decrease of intensity of radiation with the thickness of the absorbing solution is proportional to the intensity of incident radiation as well as the concentration of the solution.

3) Chromophore

The term chromophore was previously used to denote a functional group of some other structural features of which gives color to the compound, for example, Nitro group is chromophore because its presence in a compound gives yellow color to the compound. But nowadays the term chromophore is used in a broader sense which defined as "any group which exhibits absorption of electromagnetic radiation in the visible or ultraviolet region and it may or may not impart any color to the compound".

M. Absorption and intensity shift

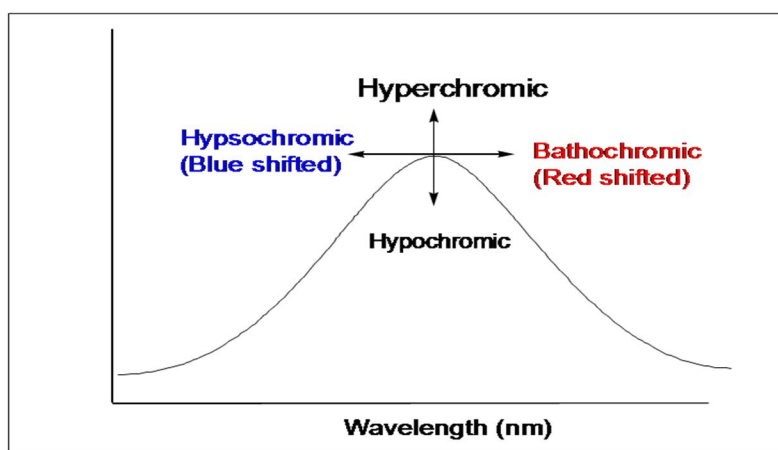


Fig1. 10: Absorption and intensity shift [23] There are four types of shifts observed in the UV spectroscopy-

1) Bathochromic shift

This type of shift is also known as a redshift. In Bathochromic shift the absorption maximum is shifted towards the longer wavelength due to the presence of auxochrome or change in the solvent.

2) Hypsochromic shift

This shift is also known as a blue shift. In Hypsochromic shift, the absorption maximum shifted towards shorter wavelength generally it is caused due to the removal of conjugation or by changing the polarity of a solvent.

3) Hyperchromic shift

Hyperchromic shift is an effect by which absorption maximum increases. The introduction of auxochrome in the compound generally results in the hyperchromic shift.

4) Hypochromic shift

The Hypochromic shift is the effect by which absorption maximum decreases. The Hypochromic effect occurs due to the distortion of the geometry of the molecule with the introduction of a new group. [24]

N. Instrumentation

Several models of various instrument manufacturers are available in the market. A schematic diagram of the Double beam instrument is shown in the figure 1.11.

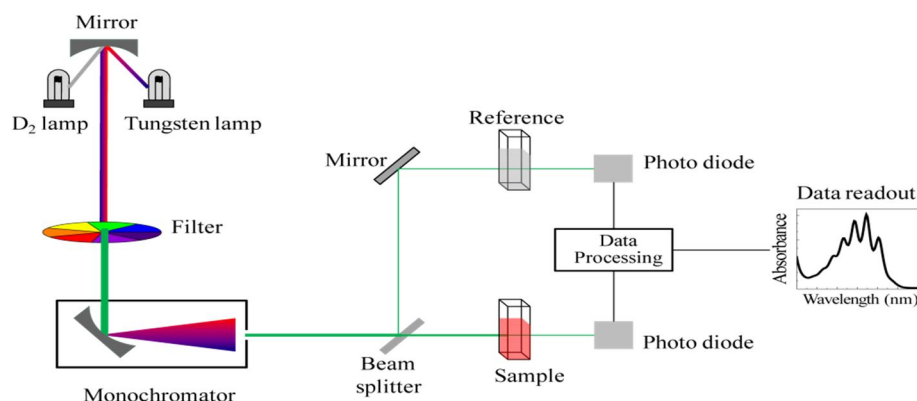


Fig1. 11:1 Schematic diagram of UV-VISIBLE spectrophotometer [25]

1) Source of radiation

For the visible region, tungsten lamp is commonly employed in an instrument it emits continuous, incandescent radiations. For the UV region, a hydrogen or deuterium discharge lamp is used. Some instruments also provide mercury vapor lamps to give intense radiation of specific wavelength both in the UV-visible region.

2) Collimating system

It consists of a lens, mirror, and aperture of entrance slit in the spectrophotometer. This allows a narrow beam of collimated light and directed either on quartz or silica prisms or gratings to render monochromatic radiations.

3) Monochromator

Some instruments make use of prism in the form of a 30° - 60° - 90° triangle with an apical angle of 30° . This prism has its back aluminized which reflects the refracted ray through litrow mounted prism back to the same collimating mirror at a different height.

4) Sample holder

It is a slot in the instrument which holds test tubes, cuvettes of different size and capacity. The sample tubes are made from good quality glass having uniform transmittance. The tubes or cells are of uniform size, shape and internal diameter. For the study in UV region, the cells or cuvettes are made from quartz.

O. Detector

Detector unite is usually a barrier layer cell or a photo-tube. In some instruments, two interchangeable photo-tubes are employed to be useful in the red region and blue region of wavelength. In double beam instruments, two photo-tubes or photomultiplier tubes are employed.

Applications

- 1) Qualitative analysis: UV absorption spectroscopy can characterize those types of compounds which absorb UV radiation. Identification is done by comparing the absorption spectrum with the spectra of known compounds. A record of UV absorption curves is found in certain reference books.
- 2) Quantitative analysis: UV absorption spectroscopy is generally used for the quantitative determination of compounds that absorb UV. This determination is based on Beer's law.
- 3) Detection of conjugation: It helps to show the relationship between different groups, particularly with respect to conjugation.
- 4) Detection of geometrical isomers: in case of a geometrically isomeric compound, the trans-isomers exhibit higher absorption at a slightly longer wavelength and have extinction coefficients than the cis-isomers.
- 5) Detection of functional groups: it is possible to detect the presence of certain functional groups with the help of the UV spectrum. Even the absence of any absorption above 200nm is of some utility since it shows the absence of conjugation, carbonyl group, and benzene rings in the compound. [26]

P. Method Development

Method development is a challenging and time-consuming process requiring much experience, creativity, logical thinking, and experimentation. With all the software and automated system available today, method development is still very much a trial and error approach, expedited by a logical sequence of generic scouting runs and fine-tuning steps to achieve the required resolution and method performance. It is the process of proving that an analytical method is acceptable for use in a laboratory to measure the concentration of subsequent samples. The analytical method should be used within criteria given as per regulatory authorities and must be developed using the protocols and acceptance criteria as per the regulatory guidelines.

Basic criteria for new Method development:

- If the proper analytical procedure for the drug may not be available in the literature.
- Analytical methods for the estimation of the drug in biological fluids may not be available.
- The available method may be complicated or difficult to perform.
- The analytical method for an older drug in a new combination with another drug may not be available.
- The available method analytical procedures require expensive reagents and solvents. It may also involve complicated extraction and separation procedures and these may not be reliable.
- To develop an alternative method to the older method. [27, 28]

Q. Choice of Analytical Method

The analytical method should be chosen considering all the ideal characteristics of drug and applications of the method. The most important is that the method should be less time-consuming. The analytical method should take less time and economical. The accuracy of the analysis in the analytical method must accept the guidelines of Pharmacopoeia. The following fishbone diagram shows the different types of prerequisites.

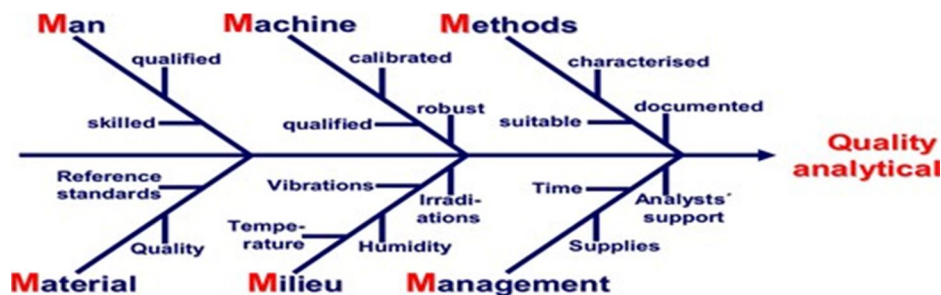


Fig1.12: Prerequisites for analytical method development and validation

R. Development of stability indicating assay method

The main purpose of stability indicating assay method development is to identify the changes in the amount of the active pharmaceutical ingredient in the formulation due to degradation when exposed to extreme storage conditions. The stability indicating method is a validated quantitative analytical procedure used to detect how the stability of the drug substances and formulation changes with a period of time intervals. A stability indicating assay method measures the changes in API concentration without interference from other degradation products, impurities, with accuracy.

Following are the steps involved in carrying out degraded studies.

1) Step I: Method Development

The analytical method development is important during the development of drug substance and drug formulations. Method development should be based on various considerations. It is appropriate to have maximum sample information to make an effective development desired for the intended analytical method application and also on available resources for chromatography. [29]

Following are the steps involved in analytical method development

a) Collection of literature

The primary step of Method development or any type of research is a collection of literature. Literature creates a base for method development. All the information, ideal concepts and knowledge creates in researcher mind due to the collection of literature therefore the literature should have a wide range of area.

b) Solubility Study

Perform the solubility experiments to establish the solubility of the API in a number of aqueous and organic solvents like Water, Buffers, 0.1N sodium hydroxide, Methanol, Acetonitrile, Chloroform, Hexane, and Tetrahydrofuran (THF), etc. covering a range of polarities that are commonly used in the method development. The API should have good solubility in the selected diluent.

c) Selection of Chromatographic Technique

According to the nature of a drug molecule and their solubility researcher choose one method for analysis. Which drug is polar in nature and shows solubility in polar solvents then the researcher selects the Reversed-phase chromatography. The second choice is Normal phase chromatography for the drugs which is soluble in non-polar solvents i.e. Non-polar in nature.

d) Selection of Stationary Phase

HPLC column is the heart of the method and critical in performing the separation. The following parameters of the columns should be taken into consideration while choosing the column for the HPLC method:

e) Column Packing Material

- Size and Shape of the particle
- Column length and diameter
- % carbon load
- Pore Volume
- End-capping

For Reverse phase chromatography, a wide variety of the columns are available like C8, C18, Cyano group –CN and amino group like –NH₂, etc. As column length changes the column efficiency changes in direct proportion to the ratio of the column length. To select the type of column in the method, conduct the experiments using different columns with different mobile phases to get best possible separation. Based on the experimental data, select the column which gives separation of all the possible impurities and principal peak and which is rugged for the variation in the mobile phase.

f) Selection of Mobile phase

Most separations can be achieved by choosing the optimum mobile phase compositions of the aqueous and organic portions. Most widely used solvent for the reverse phase chromatography is Methanol and Acetonitrile. If the sample is eluted with the mobile phase of 100% organic content, and there is no separation, the solvent strength should be decreased to get the retention. Generally, the increase in organic content will shorten the run time but leads to increased band overlap.

g) Selection of Detector

Various types of detector are used in HPLC. Depending upon the nature of API, relates substances and degraded product, selection of detector has been carried out. In UV the selection of the wavelength is a critical step in the method development. To select the wavelength, prepare the standard solution at the required concentration in the selected solvent and scan it on UV-Spectrophotometer and select the wavelength which shows maximum absorbance by the analyte.

h) Optimization of the method

After selection of the entire factor such as Mobile phase, Stationary phase, Detector and other necessities. The analyte goes for trials of HPLC with different conditions from those trials analyte select one trial condition which shows all factor such as tailing factor, Number of Theoretical plates within accepted limits given by guidelines. This selected trial condition is reported as an optimized method.

2) Step 2: Method validation

Validation is defined as “Documented evidence which gives a high degree of confidence that a process, system, facility will consistently produce a product meeting its predetermined specifications and quality attributes.” Validation is a systematic approach for identifying, measuring, evaluating, documenting and reevaluating all critical steps responsible, before establishing the validity of the method. Validation of the analytical method is not only an integral part of the quality system but c-GMP does require assay validation.

Parameters for Method validation:

The parameters for method validation also referred to as “Analytical Performance Parameters” as defined by USP and ICH guidelines are summarized below;

a) Accuracy

The accuracy of an analytical method is the closeness of test results, obtained by that method to the true value. The accuracy of an analytical method should be established across its range. In the case of the assay of a drug in the formulated product, accuracy may be determined by the application of the analytical method to synthetic mixtures of drug product components to which known amount of analyte has been added within the range of the method.

b) Precision

The precision of the analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of a homogenous sample. The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurement.

c) Specificity

The specificity is the ability to assess unequivocally the analyte of interest in the presence of component that may be expected to be present, such as impurities, degradation products, and matrix components. In the case of an assay, demonstration of specificity requires that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substances or product with appropriate levels of impurities or excipients, and demonstrating that the assay result is unaffected by the presence of these extraneous materials

d) Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in a sample within the given range. It should be established across the range of the analytical procedure. Linearity is generally reported as the correlation coefficients, the slope of regression line i.e., $r^2 \geq 0.999$. The range of the analytical method is the interval between the upper and lower level of analyte that has been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using method written.

e) Limit of Detection

The lowest conc. of the analyte in the sample that the method can detect but not necessarily quantify under the stated experimental conditions simply indicates that the sample is below or above a certain level. Limit test prescribed as a percentage or as parts per million. The limit of detection will not only depend on the procedure of analysis but also on type of instrument.

f) Limit of Quantitation

The limit of quantitation (LOQ) is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. It is expressed as the conc. of analyte (e.g., percentage, parts per billion) in the sample.

g) Ruggedness

Ruggedness, according to the USP, is the degree of reproducibility of the results obtained under a variety of conditions, expressed as % relative standard deviation (RSD). These conditions include differences in laboratories, analyst, instruments, reagents, and experimental periods.

h) Robustness

The robustness of analytical method is the measure of its capacity, to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Experiments are performed by changing conditions such as temperature (± 5 °C), buffer pH (± 0.5), and ionic strength of buffers, level of additives to mobile phase. The method must be robust enough to withstand slight changes and allow routine analysis of sample and the %RSD of all the peak areas measure in robustness should not be greater than 2%. [30, 31,32]

Table1.4: Characteristics to be validated in HPLC

Characteristics	Acceptance Criteria
Accuracy/trueness	Recovery 98-102%
Precision	RSD < 2%
Repeatability	RSD < 2%
Specificity / Selectivity	No interference
Detection Limit	S/N > 2 or 3
Quantitation Limit	S/N > 10
Linearity	$r^2 \geq 0.999$

3) Step-3 Forced Degradation

The Forced degradation study is called as stress testing, stress studies, stress degradation studies, forced degradation studies, etc. the forced degradation is a process which involves the degradation of drug substance and formulation at stressed conditions. Thus generates the degradation products that can be studied to determine the stability of the drug molecule. The ICH guideline explains the forced degradation testing is used to identify the degradation products which further helps in the estimation of the intrinsic stability of the drug molecule and establish the degradation pathways and to validate the stability indicating procedures used. [33, 34]

Following are some stress conditions mainly apply to drug and drug products during forced degradation study.

a) Acid Degradation

The hydrolytic degradation of a new drug in acidic conditions can be studied by refluxing the drug in 1N HCl for 1 hrs. If reasonable degradation is perceived, testing can be stopped at that point. However, in case no degradation is seen under these conditions, the drug should be refluxed in the acid of higher strengths and for longer a duration. Alternatively, if total degradation is seen after subjecting the drug to initial conditions, acid strength can be decreased along with a decrease in reaction temperature.

b) Base degradation

The hydrolytic degradation of a new drug in Basic conditions can be studied by refluxing the drug in 1N NaOH for 1 hrs. If reasonable degradation is perceived, testing can be stopped at that point. However, in case no degradation is seen under these conditions, the drug should be refluxed in Base of higher strengths and for a longer duration. Alternatively, if total degradation is seen after subjecting the drug to initial conditions, Base strength can be decreased along with a decrease in reaction temperature.

c) Oxidative degradation

To test for oxidation, it is suggested to use H₂O₂ in the concentration range 3-30% and duration can be from 2-24 hrs. As a hydrolytic degradation, the reaction temperature can be changed according to the requirement for degradation.

d) Photolytic degradation

It should be carried out by exposure to light using either a combination of cool white and ultraviolet fluorescent lamps or one among the xenon and metal halide lamps. Exposure energy should be a minimum of 1.2 million Lux h fluorescent light and 200 W h/m² UV and if decomposition is not see the intensity should be increased by five times. In case no decomposition still takes place, the drug can be declaring photostable.

e) Thermal degradation

Stress testing for thermal degradation can be carried out by heating drug powder at 60°C in a hot air oven or on a water bath. The heating time can be increased if more sufficient degradation is not seen in the initial study as well as the heating temperature also increased to achieve sufficient degradation under thermal stress condition. [35, 36, 37]

II. LITERATURE SURVEY

- 1) Chilukuri Jyothi et., al. - have reported a simple, accurate, precise, and economical method for the estimation of Methoxsalen in tablet dosage form. An Inertsil ODS C18, column (250×4.6 mm×5 μ) in isocratic mode with a mobile phase containing Acetonitrile: Tetra hydro furon: 0.01M NaH₂PO₄ in the ratio of 65:15:25 (v/v/v) was used. The mobile phase flow rate was maintained at 1.0 mL/min and effluents were monitored at 220 nm wavelength. The retention time for Methoxsalen was 4.447 min. The method was validated for parameters give as per ICH guideline. The LOD and LOQ of the developed method were found to be between 0.04 ppm and 0.13 ppm and recovery was about 98.23% from Methoxsalen tablet. This developed method was used for the quantitative estimation of Methoxsalen in tablet formulation.[38]
- 2) Meena Harsahay et., al. - have reported a reliable, accurate and reproducible HPLC method for the simultaneously estimation of four furanocoumarins (Psoralen, isopsoralen, xanthotoxin and bergapten) in *Psoralea corylifolia* and *Ammi majus* plants. The furocoumarins were separated simultaneously on a reverse phase Symmetry C8 (150 × 4.6 mm, 5 μ) column in isocratic method of methanol, acetonitrile and water solution as mobile phase having flow rate at 0.8 mL/min and detected with UV detector. Maximum psoralen and isopsoralen (Angelicin) were recorded in *P. corylifolia*, whereas maximum 8-methoxypsoralen (xanthotoxin) and 5-methoxypsoralen (bergapten) were found in *A. majus*. *P. corylifolia* is a good source of furanocoumarins psoralen and angelicin, whereas *A. majus* is the good source for 5-Methoxypsoralen and 8- Methoxypsoralen. Hence, both plants can be used in the treatment of Vitiligo and Psoriasis. The isocratic HPLC method was found more suitable, accurate, less time consuming and reproducible method for the estimation of above cited four furanocoumarins simultaneously from the *P. corylifolia*, and *A. majus* plants.[39]
- 3) Catherine H. Ketchum et., al. – have reported a simple and rapid procedure for assaying 8-Methoxypsoralen (8-MOP) in plasma by High Performance Liquid Chromatography. A C18 10 cm, Spheri-5 Applied Biosystem column with a mobile phase containing Glass distilled Deionized Water: Methanol: Acetonitrile in ratio 65:25:10 (v/v/v) was used. The mobile phase flow rate was maintained at 0.7 mL/min and effluents were monitored at 300 nm wavelength at 25°C. Absorption maxima also occur at 254 nm and 215 nm in addition to 300 nm. Although sensitivity is greater at 215 nm or 254 nm, we choose 300 nm as our detection wavelength to reduce interference from plasma. The standard curve for the assay is linear for 8-MOP from 15 to 500 μg/L and extraction recovery of 8-MOP was 98%.[40]
- 4) Nagasarapu Rao et., al. - have reported rapid, specific, and economical stability indicating RP-HPLC method for the estimation of pristinamycin in tablet formulation. Pristinamycin was eluted on the ACE-5, C18 HL (250×4.6 mm, 5μ) column with a mobile phase containing 0.2% orthophosphoric acid and acetonitrile 63.37 v/v. The flow rate maintained at 1.5 mL/min. The column was maintained at 40°C and 10 μl of the solutions were injected. The effluent monitored at 206 nm the overall degradation time for eluting the pristinamycin was found to be less than 10 min for its degraded product. The method was then validated according to the ICH guidelines. The forced degradation studies were performed for pristinamycin bulk to demonstrate the stability-indicating HPLC method. The % RSD of system precision and method precision was found to be 0.64% and 1.49% respectively. The procedure provided a linear response over the concentration range of 25–150 μg/ml ($r^2 = 0.9998$). [41]
- 5) Raghabaendra Singh et., al. - have reported a stability indicating high performance liquid chromatographic method has been developed for the determination of norfloxacin. Optimum separation was achieved by using Phenomenex ODS C18 (250× 4.6 mm packed with 5μ) column. The analyte was resolved by using a mobile phase 20 mmol L-1 ammonium formate and acetonitrile (70:30), pH adjusted to 4.0 with formic acid at flow rate 1 mL/min on an isocratic high performance liquid chromatographic system at a wavelength of 280 nm. The method was shown following parameters according to ICH guidelines as specificity, linearity, accuracy, precision, ruggedness, and robustness and can be successfully applied for the determination of this drug in commercial tablets. For stress studies the drug was subjected to acid, alkali and neutral hydrolysis, oxidation, dry heat and photolytic degradation. The degradation studies indicated the drug to be susceptible to acid, alkali hydrolysis and oxidative degradation. The proposed method not required highly sophisticated and expensive instrumentation.[42]

III. AIM AND OBJECTIVES

A. Aim

The aim of present research work was to develop stability indicating RP-HPLC assay method for estimation of the Methoxsalen.

B. Objectives

The objectives of the research work were-

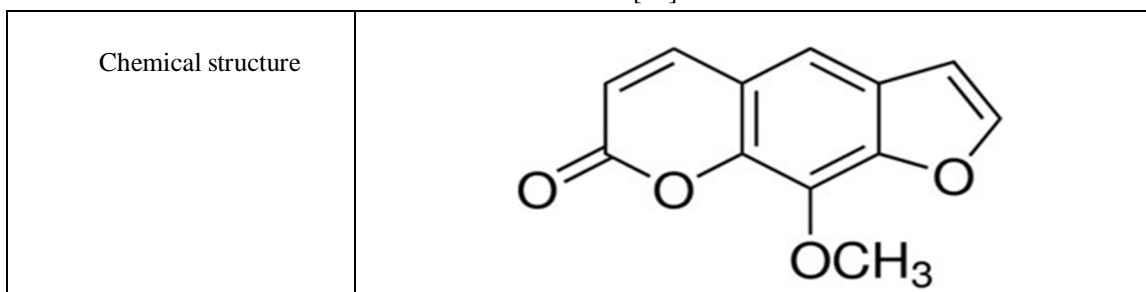
- 1) To develop a new, simple, precise, accurate and economical RP-HPLC method for estimation of the Methoxsalen in the formulation.
- 2) To perform the forced degradation study for the Methoxsalen.
- 3) To estimate the percentage of degraded product from the Methoxsalen.
- 4) To validate stability indicating RP-HPLC assay method as per ICH guidelines.

IV. PLAN OF WORK

- 1) Literature survey
- 2) Selection of Drug
- 3) Procurement of Drug and Marketed formulation
- 4) Method Development
 - Solubility study
 - Selection of wavelength
 - Selection of Mobile phase
 - Trials
- 5) Optimization of chromatographic condition
- 6) Estimation of methoxsalen in marketed formulation
- 7) Method validation
 - Linearity
 - Precision
 - Accuracy
 - Specificity
 - Robustness
 - Ruggedness
 - Limit of Detection (LOD)
 - Limit of Quantification (LOQ)
- 8) Forced Degradation Study
 - Acid Degradation
 - Base Degradation
 - Oxidative Degradation
 - Thermal Degradation

V. DRUG PROFILE

Methoxsalen [43]



IUPAC name	9-methoxyfuro[3,2-g]chromen-7-one
Description	Solid
Molecular Weight	216.192 g/mol
Molecular Formula	C ₁₂ H ₈ O ₄
Melting Point	143 ⁰ C-148 ⁰ C
Category	Antipsoriatic
Mode of Action	The exact mechanism of action of methoxsalen is not known. The best-known biochemical reaction of methoxsalen is with DNA. Then the formation of photo adducts results in inhibition of DNA synthesis, cell division and epidermal turnover.
Solubility	Soluble in chloroform, acetone, hot ethanol Insoluble in water.
Use	To treat Psoriasis and vitiligo
Adverse Effects	Reddened skin, swelling of the skin followed by peeling, skin discomfort.

VI. MATERIALS AND INSTRUMENTS

A. Reference Standard

The following reference standard was used during the project work and is enlisted in Table No.6.1

Table 6.1: Details of Reference Standard Used

Sr. No.	Name of Standard	Gift sample supplier	Purity
1	Methoxsalen USP	Inga laboratories Pvt.Ltd. Mumbai	99.98%

B. Marketed Formulation

The following marketed formulation was used during the project work and is enlisted in Table No.6.2.

Table 6.2: Details of Marketed Formulation Used

Sr.No	Particulars	Details
1	Brand Name	Melanocyl 10 mg Tab
2	Manufactured by	Franco Indian Pharmaceuticals Pvt. Ltd.Mumbai
3	Content	Each tablet contains: Methoxsalen USP.... 10mgExcipients.....q.s.
4	Colour	White
5	Average Weight	0.2013gm

C. Instruments

The following instruments were used and are enlisted in Table No. 6.3

Table 6.3: Details of Instrument Used

Sr.No	Name of Instrument	Make	Model
1	UV-Visible Spectrophotometer	Shimadzu	UV-2450 UV probe v 2.3.3
2	HPLC System	Water's Corp	Waters 1525 (by binary pump) Waters 2489 (UV Visible Detector)
3	Analytical Balance	Shimadzu	AUX 220
4	Ultrasonicator	Citizen pvt. Ltd.	Digital ultrasonic cleaner

D. Chemicals and Reagents

The following chemicals and reagents were used and are enlisted in Table No 6.4.

Table 6.4: Details of Chemicals and Reagents used

Sr.No	Reagent	Grade
1	Methanol	HPLC Grade
2	Water	HPLC Grade
3	Hydrochloricacid	LaboratoryGrade
4	Sodium hydroxide	LaboratoryGrade
5	Hydrogenperoxide	LaboratoryGrade

VII. EXPERIMENTAL WORK AND RESULTS

A. Solubility studies

The solubility of a Methoxsalen was carried out in different solvents such as water, methanol and acetonitrile. An accurately weighed quantity of 0.5 gm Methoxsalen was dissolved in water, methanol, and acetonitrile respectively and the resultant solutions were sonicated for 10 min to dissolve the drug. The results were visually observed. It was found that the Methoxsalen was soluble in methanol, water and acetonitrile.

B. Selection of wavelength

For the selection of wavelength, the 5 ppm of standard Methoxsalen solution was prepared in a mixture of methanol : water (80:20 v/v). It was scanned over the wavelength range of 400-200 nm using double beam UV spectrophotometer with methanol : water (80:20 v/v) as blank. The absorption maxima for Methoxsalen in methanol : water was found to be 218 nm. The spectrum of Methoxsalen is shown in figure 6.1.

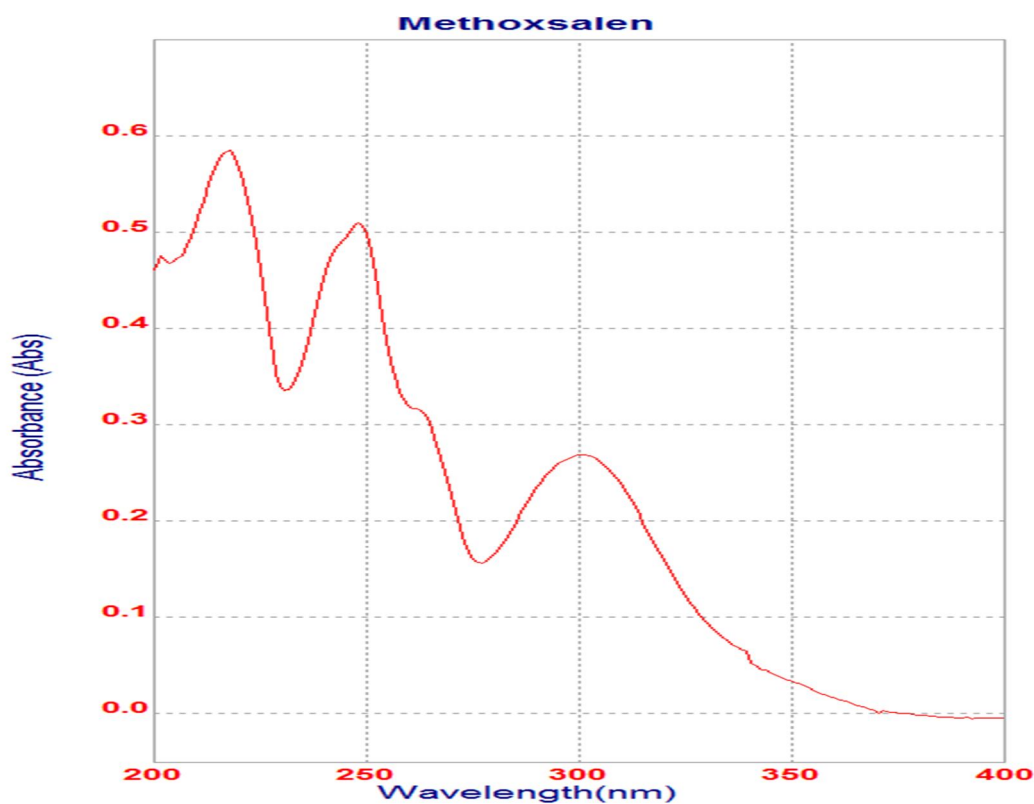


Fig 6.1: UV spectra of Methoxsalen

C. RP-HPLC Method Development and Optimization

The different trials were taken to develop method and optimize the method for the analysis of Methoxsalen. For the trials of the method development, 25 ppm standard Methoxsalen solution was used with the stationary phase Cosmosil C18 (250 mm × 4.6 mm, 5 μ) and 0.8 mL/min flow rate.

1) Selection of mobile phase

The selection of the mobile phase was carried out on the basis of solubility studies of a drug in different solvents and also on the basis of the literature survey. Various mobile phases were tried for evaluating suitable one. The 100% methanol was suitably selected as a mobile phase.

2) Trial-1

In trial-1, 100% methanol was selected as a mobile phase and the Standard Methoxsalen solution of 25 ppm was prepared in selected mobile phase, then injected the resultant standard solution in HPLC and chromatogram was recorded.

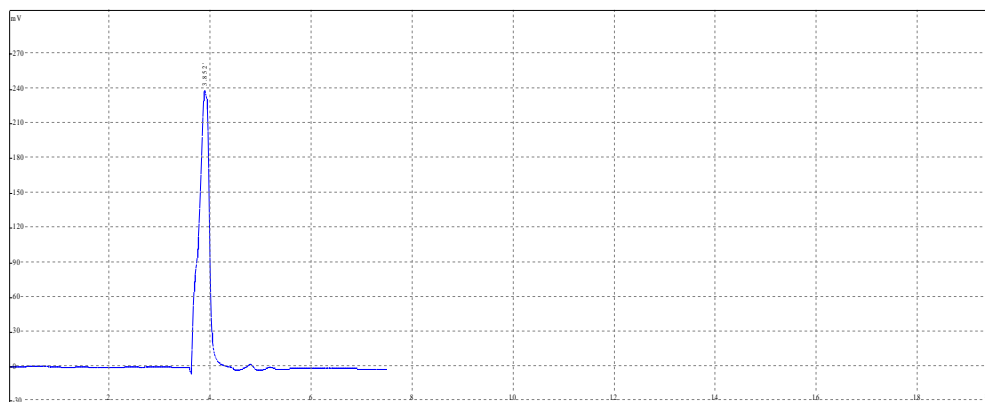
Preparation of 25 ppm standard Methoxsalen solution

An accurately weighed quantity of 5 mg Methoxsalen was transferred into a 10 mL volumetric flask and 7 mL of 100% methanol was added into it, the resultant solution was sonicated for 10 min and made up the final volume with mobile phase upto the mark, from the above solution 500 µL was pipette out in 10 mL of volumetric flask and made up the final volume with methanol.

Table 6.5: Trial-1 chromatographic condition

Column	Cosmosil C18, 250X4.6 mm, 5 µ
Flow rate	0.8 mL/min
Wavelength	218 nm
Injection volume	20 µL
Pressure	10-11Mpa
Mobile phase	methanol (100%)

The chromatogram of the Trial-1 is shown in figure 6.2.



Rt	Peak area	Plate number	Asymmetric factor
3.852	3522490	8588	1.63

Fig 6.2: Typical chromatogram of Trial-1

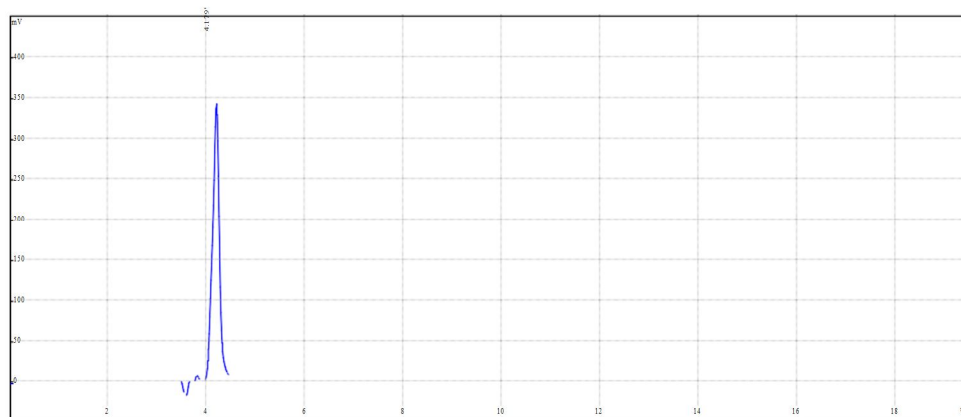
3) Trial-2

In trial-2, the ratio of the mixture i.e. methanol : water of 90:10 v/v was selected as a mobile phase and standard Methoxsalen solution of 25 ppm was prepared in the selected mixture, then chromatogram was recorded after injecting the sample in HPLC system.

Table 6.6: Trail-2 chromatographic condition

Column	Cosmosil C18, 250 X4.6 mm, 5 µ
Flow rate	0.8 mL/min
Wavelength	218 nm
Injection volume	20 µL
Pressure	10-11Mpa
Mobile phase	methanol : water (90:10v/v)

The chromatogram of Trial-2 is shown in figure 6.3.



Rt	Peak area	Plate number	Asymmetric factor
4.179	3587478	8086	1.08

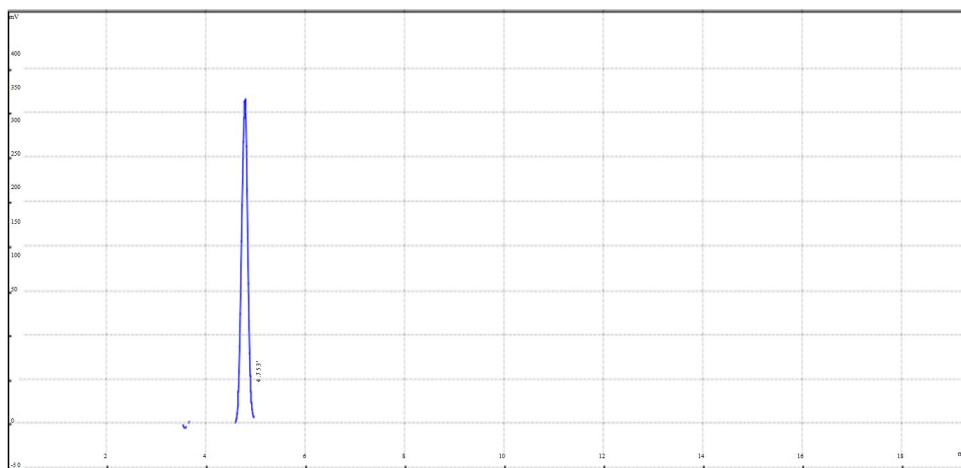
Fig.6.3: Typical chromatogram of Trial-2

4) Trial-3

In trial-3, the ratio of the mixture i.e. methanol : water of 80:20 v/v was selected as a mobile phase and the standard Methoxsalen solution of 25 ppm was prepared in the selected mixture, then chromatogram was recorded after injecting the resultant solution.

Table 6.7: Trail-3 Chromatographic condition

Column	Cosmosil C18, 250 X4.6 mm, 5μ
Flow rate	0.8 mL/min
Wavelength	218 nm
Injection volume	20 μL
Pressure	10-11 Mpa
Mobile phase	methanol : water (80:20v/v)



Rt	Peak area	Plate number	Asymmetric factor
4.753	3566215	8007	1.06

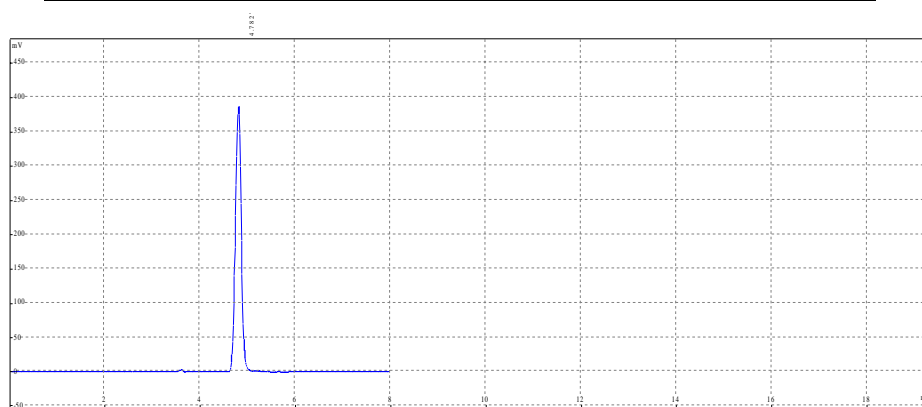
Fig.6.4: Typical chromatogram Trial-3

5) TRIAL-4

In trial-4, same mobile phase and the standard Methoxsalen solution were used (as above in trail-3) and chromatogram was recorded.

Table 6.8: Trail-4 Chromatographic condition

Column	Cosmosil C18, 250 X4.6 mm, 5 μ
Flow rate	0.8 mL/min
Wavelength	218 nm
Injection volume	20 μL
Pressure	10-11 Mpa
Mobile phase	methanol : water (80:20v/v)



Rt	Peak area	Plate number	Asymmetric factor
4.782	3534017	8848	1.09

Fig 6.5: Typical chromatogram Trial-4

D. Optimization Of Mobile Phase Strength

After trials of various proportions of mobile phase and by the observation of resultant chromatograms, the suitable mobile phase for analysis of drug was optimized satisfactorily as shown in Table 6.9.

Table 6.9: Optimization of mobile phase strength

Sr. No.	Mobile phase	Column	Retention time(min)	Peak description
1	Methanol:water (100% v/v)	Cosmosil, C18, 250 mm×4.6 mm, 5μ	3.8	Broad peak
2	Methanol:water (90:10 v/v)	Cosmosil, C18, 25 mm×4.6 mm, 5μ	4.17	Peak is broad at thebase
3	Methanol:water (80:20 v/v)	Cosmosil, C18, 250 mm×4.6 mm, 5μ	4.753	Sharp peak

E. Optimized Chromatographic Conditions

The optimized chromatographic conditions are shown in Table 6.10.

Table 6.10: Optimized Chromatographic Conditions

Column	Cosmosil C18, 250 ×4.6 mm, 5 μ
Flow rate	0.8 mL/min
Wavelength	218 nm
Injection volume	20 μL
Run time	8.21 min
Mobile phase	methanol : water (80:20v/v)

F. Mobile Phase Preparation

An accurately measured quantity of 320 mL methanol and 80 mL of water were transferred in a 500 mL volumetric flask followed by proper mixing of resultant solution, then filtered through 0.45μ membrane filter and sonicated with intermittent shaking for the 15 min in a bath sonicator.

G. Stock Solution Preparation

An accurately weighed quantity of 50 mg standard Methoxsalen was transferred into a 50 mL of volumetric flask and 30 mL of mobile phase (i.e. methanol : water of 80:20 v/v) was added into it, then resultant solution was sonicated for 15 min for complete dissolution of the drug. The solution was again filtered and then final volume was made up to the mark with selected mobile phase.

H. Linearity Study

The linearity was carried out to find out the linear relationship between concentrations of drug to the peak area. The range of 10-50 μg/mL was selected for the linearity of a standard Methoxsalen. The solutions were prepared by diluting the known volume of stock solution with the mobile phase. Then, 10-50 μg/mL solutions were injected sequentially with an intermediate column equilibration and the chromatograms were recorded. The standard calibration curve for linearity was plotted between concentration vs peak area and the 'R²', y-intercept and slope of the regression was calculated.

I. Preparation of Linearity Solutions

The linearity samples were prepared according to Table 6.11

Table 6.11 Preparation of linearity samples

Linearity range (ppm)	Volume taken from Linearity stock solution (μL)	Diluted upto (mL)	Concentration (μg/mL)
10	100	10	10
20	200	10	20
30	300	10	30
40	400	10	40
50	500	10	50

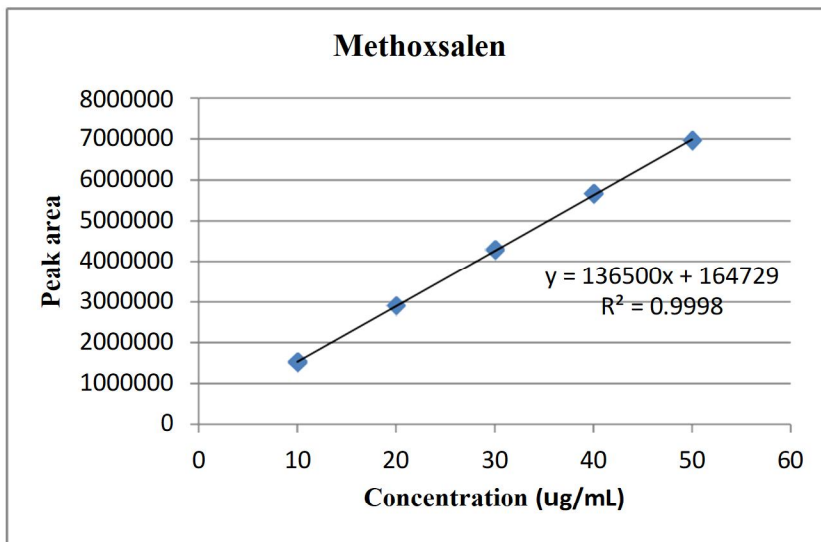


Fig 6.6: Standard calibration curve of Methoxsalen

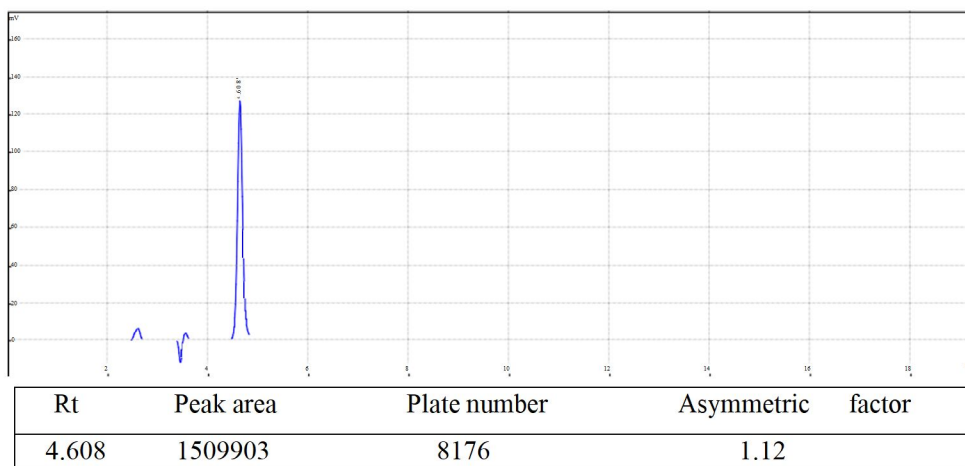


Fig 6.7: Linearity Chromatogram (10µg/mL)

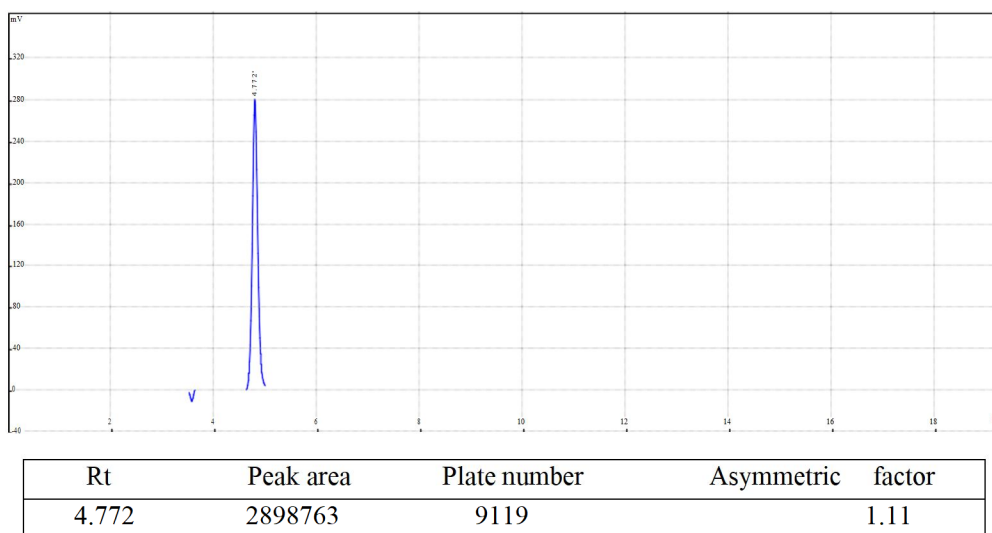
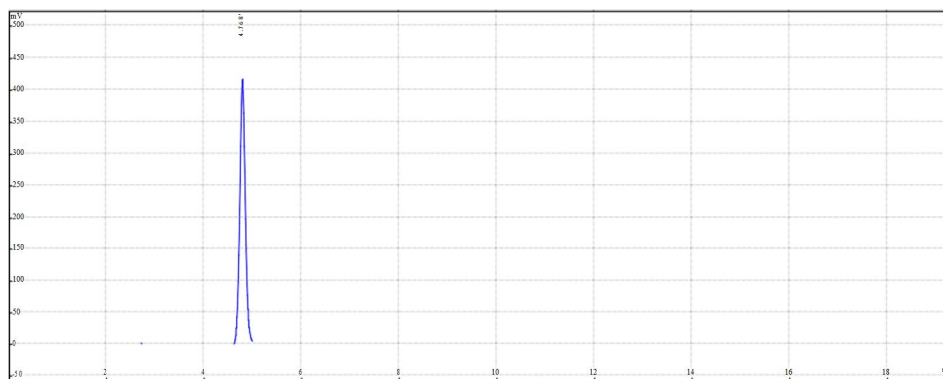


Fig 6.8: Linearity Chromatogram (20µg/mL)



Rt	Peak area	Plate number	Asymmetric	factor
4.768	4275906	8776		1.07

Fig 6.9: Linearity Chromatogram (30µg/mL)



Rt	Peak area	Plate number	Asymmetric	factor
4.767	5659586	8923		1.04

Fig 6.10: Linearity Chromatogram (40µg/mL)



Rt	Peak area	Plate number	Asymmetric	factor
4.836	6954494	8947		1.03

Fig 6.11: Linearity Chromatogram (50µg/mL)

Table 6.12: Results of linearity

Sr.No.	Concentration ($\mu\text{g/mL}$)	Peak Area
1	10	1509903
2	20	2898763
3	30	4275906
4	40	5659586
5	50	6954494
r^2		0.999
Slope		13650
Y-intercept		16472

J. System Suitability Test

The system suitability test is a pharmacopoeial requirement and is carried out to verify whether the analytical system is adequate or not for analysis of drug. The 30 ppm Standard Methoxsalen solution was prepared and injected same solution into five replicates, afterwards chromatograms were recorded and number of theoretical plates with respect to asymmetric factor was also reported. The Chromatograms of system suitability test are shown as follows.

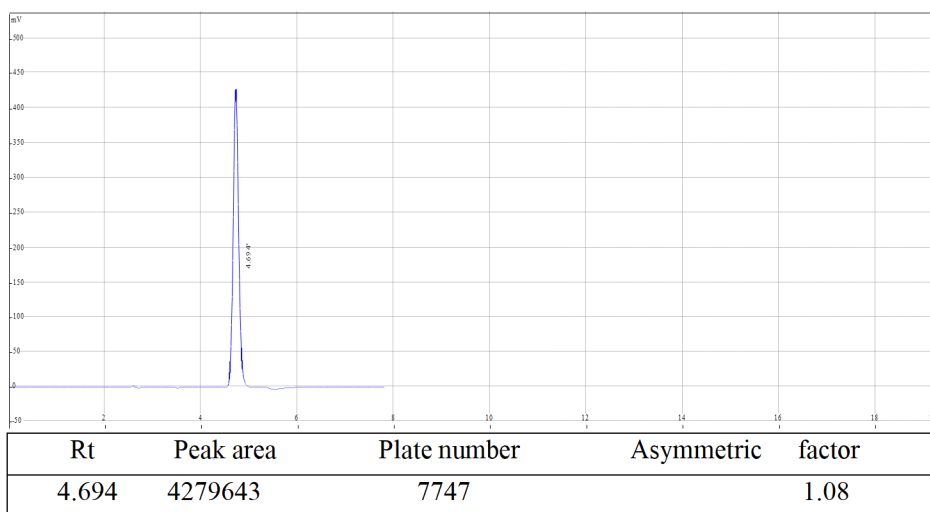


Fig.6.12: Typical chromatogram System Suitability-1

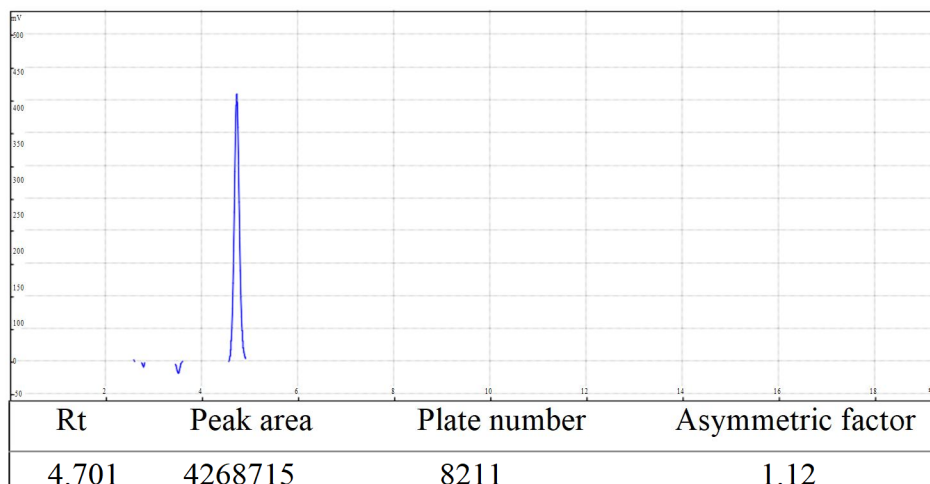


Fig.6.13: Typical chromatogram System Suitability-2

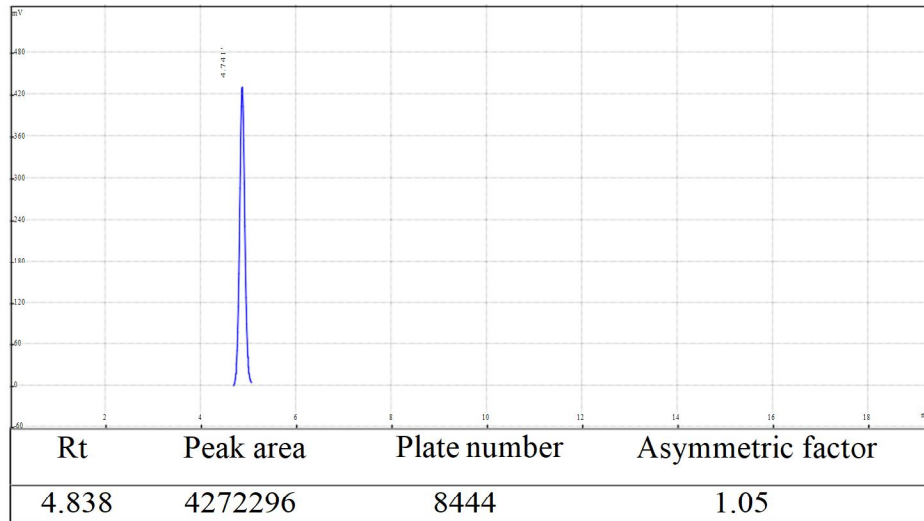


Fig.6.14: Typical chromatogram System Suitability-3

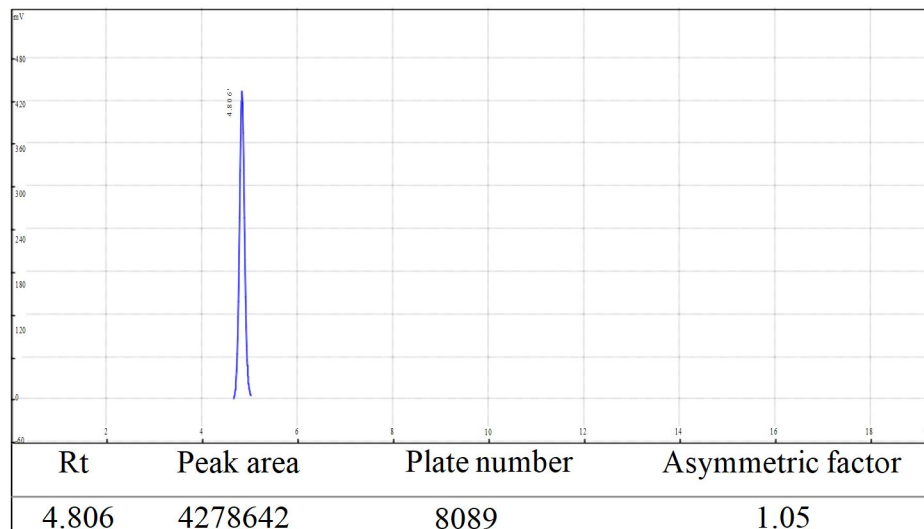


Fig.6.15: Typical chromatogram System Suitability-4

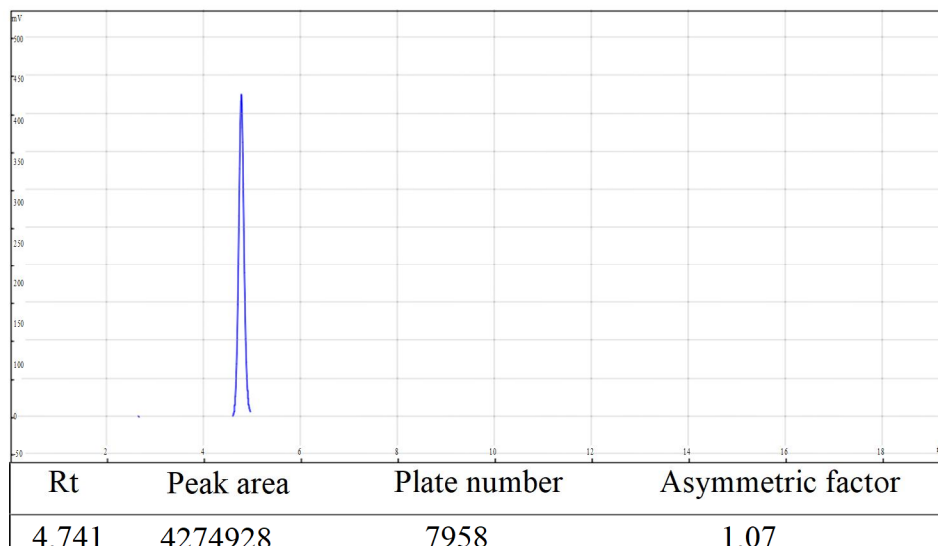


Fig6.16: Typical chromatogram System Suitability-5 Table 6.13: Result of system suitability

Sr.No	Concentration (µg/mL)	Peak area	No. of Therotical plates	Asymmetric factor
1	30	4279643	7747	1.08
2	30	4268715	8211	1.12
3	30	4272296	8444	1.05
4	30	4278642	8089	1.05
5	30	4274928	7958	1.07
		Mean	8089	1.074

K. Analysis of Marketed Formulation Preparation of standard solution

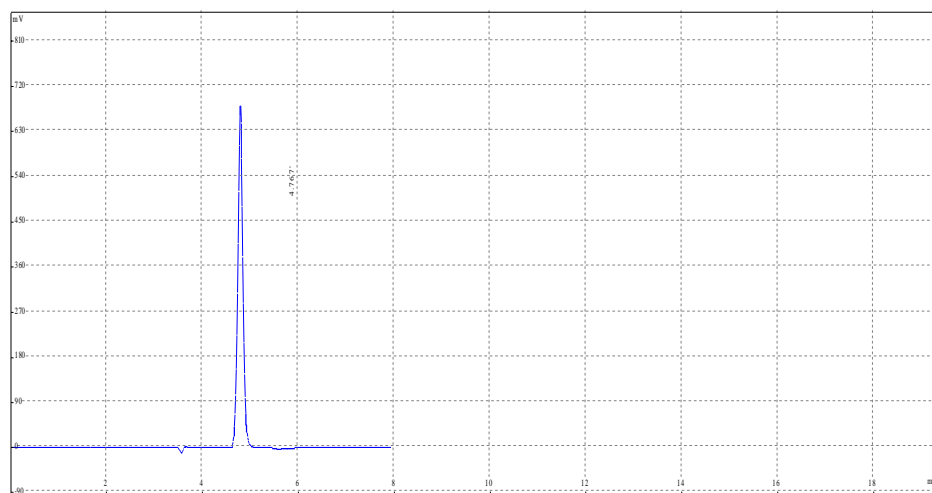
400 µL of stock solution was pipette out and transferred to 10 mL of volumetric flask and then 5 mL of a mobile phase (solvent system) was added into it. The final volumewas made up to the mark with selected mobile phase and the volumetric flasklabelled as a standard solution for assay.

L. Sample Preparation

Twenty tablets of Methoxsalen were weighed individually and the average weight of Methoxsalen tablet was calculated accordingly (avg. wt. 0.2013 gm). Afterwardthe tablets were crushed and ground to fine powder using mortar and pestle. An accurately weighed quantity of 0.2013g (equivalent to 10 mg of Methoxsalen) tablet powder was transferred into 10 mL of volumetric flask containing about 8 mL of the mobile phase and the resultant solution was sonicated for 20 min with intermittent shaking, the final volume was made upto the mark with selected mobile phase; then final solution was centrifuged at 5000 RPM to settle the undissolved powder and pipette out 0.4 mL of the resultant solution from the above supernatant into a 10 mL of volumetric flask and diluted it with selected mobile phase upto the mark, then the solution was filtered through 0.45µ membrane syringe filter-media to get concentration of 40 ppm solution of Methoxsalen tablet sample.

Procedure

Equal volumes (20 µL) of standard and sample solution were injected separately afterequilibration of stationary phase, the sample solution was injected three times and thechromatograms were recorded. Finally, the percentage of drug in a tablet was calculated. The standard and sample solutions chromatograms are shown as follows.



Rt	Peak area	Plate number	Asymmetric factor
4.767	5659586	8923	1.04

Fig 6.17: Chromatogram of standard solution of Methoxsalen

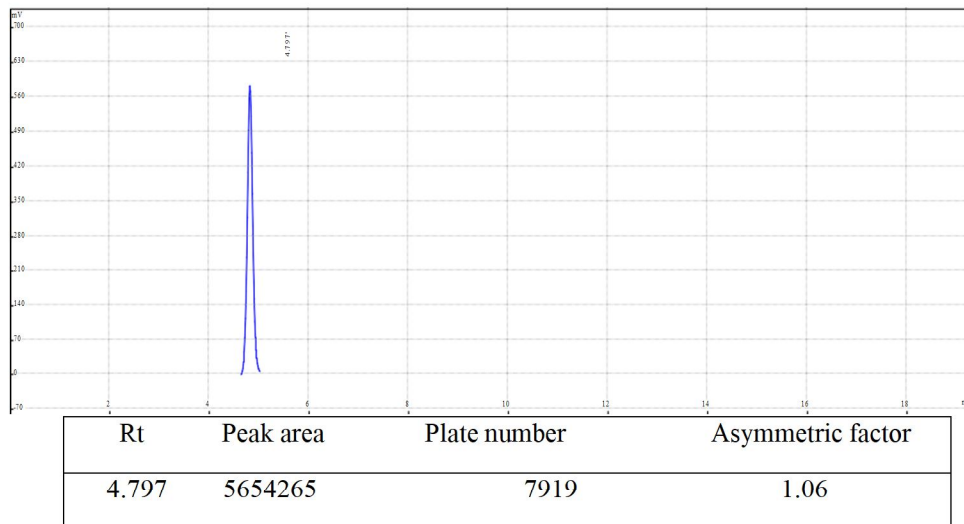


Fig6.18: Chromatogram of sample solution of Methoxsalen (1)

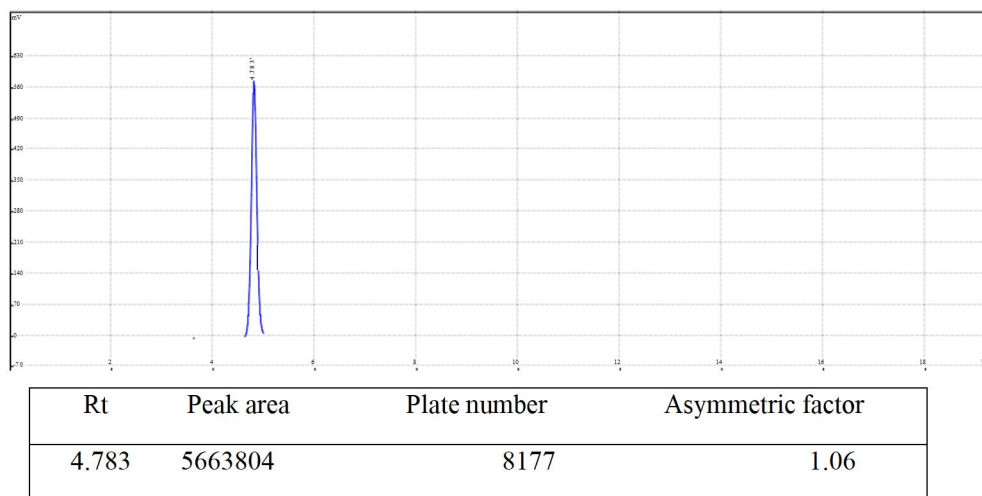


Fig 6.19: Chromatogram of sample solution of Methoxsalen (2)

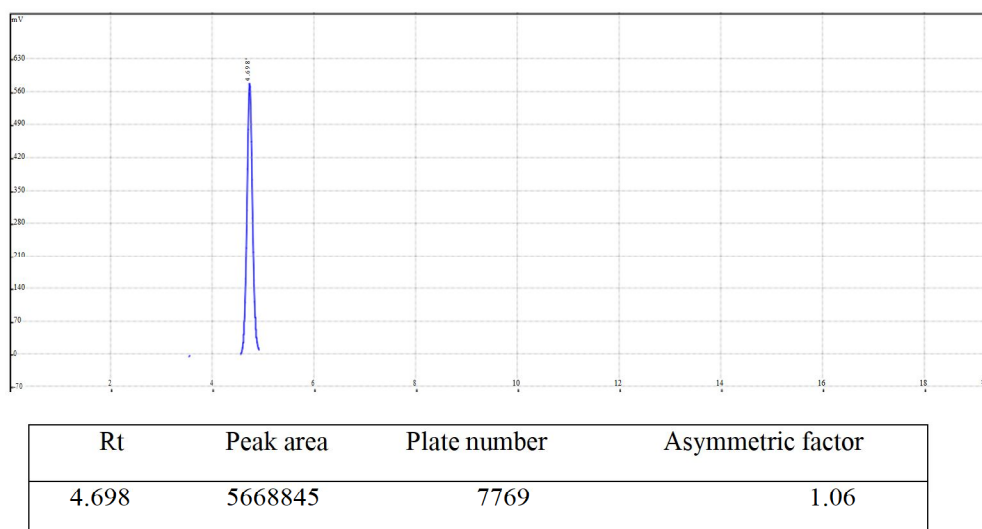


Fig 6.20: Chromatogram of sample solution of Methoxsalen (3)

Table 6.14: Result of assay

Parameters	Methoxsalen
Sample area	5654265
	5663804
	5668845
Mean sample area	5662304
Standard area	5659586
% Assay	99.88%
Label claim found	9.98

M. Accuracy Study

The recovery study was performed to evaluate the developed method was accurate for the analysis of Methoxsalen. The 80%, 100% and 120% levels of recovery study were selected to perform the recovery study.

1) Preparation of test samples

The test samples for recovery study were prepared according to Table 6.15.

Table 6.15: Preparation of % recovery samples

Sr. No	Level of % recovery	Amount of tab taken ($\mu\text{g/mL}$)	Amount of Std added ($\mu\text{g/mL}$)	Total Amount ($\mu\text{g/mL}$)
1	80	20	16	36
2	100	20	20	40
3	120	20	24	44

2) Preparation of standard solution

The solution of standard Methoxsalen in concentration of 36, 40 and 44 ppm were prepared by dilution of stock solution for the evaluation of % recovery.

3) Procedure

Equal volume (20 μ L) of test sample and standard solution of each level of % recovery were injected into three replicates and then chromatograms were recorded. The % recovery of each level was calculated and the standard deviations followed by % RSD of recovery study were reported.

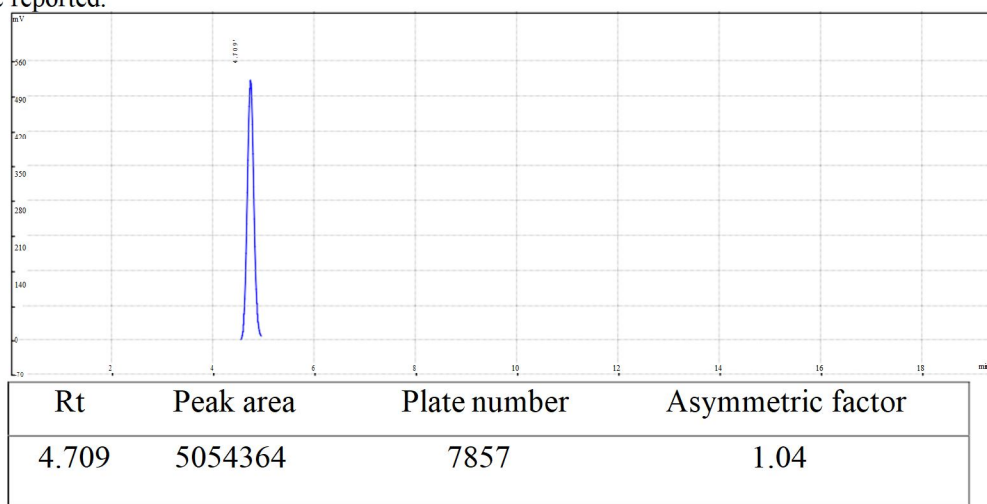


Fig 6.21: Chromatogram of Level of 80% recovery (1)

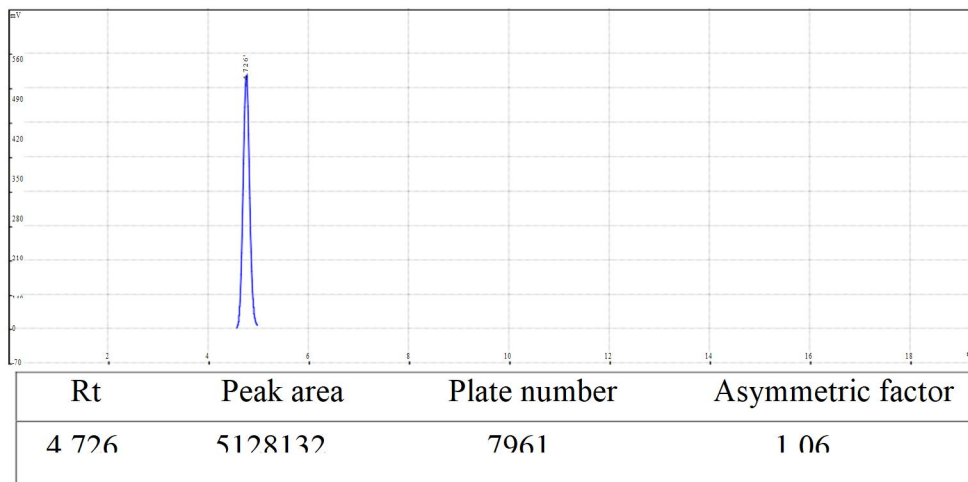


Fig 6.22: Chromatogram of Level of 80% recovery (2)

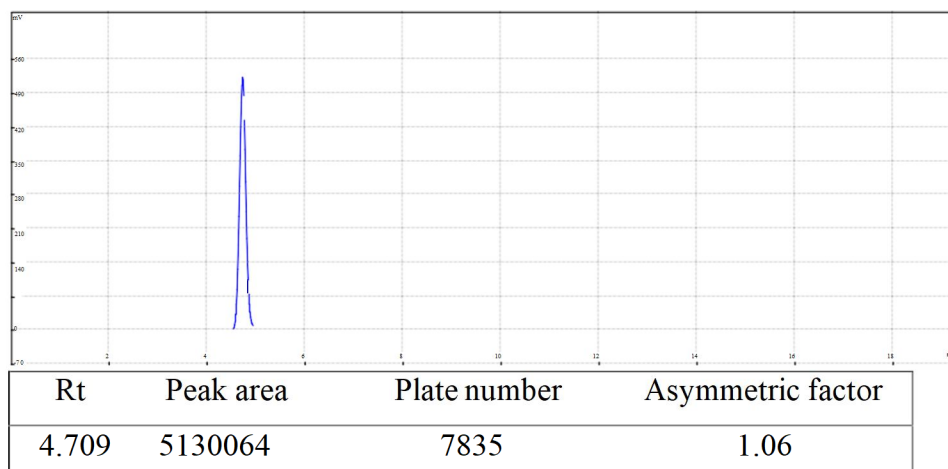


Fig 6.23: Chromatogram of Level of 80% recovery (3)

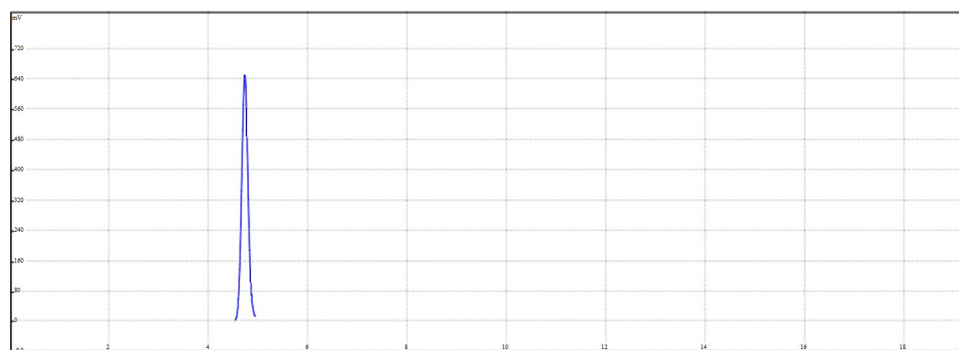


Rt	Peak area	Plate number	Asymmetric factor
4.741	5610694	7922	1.06

Fig 6.24: Chromatogram of Level of 100% recovery (1)



Rt	Peak area	Plate number	Asymmetric factor
4.713	5654733	7799	1.06



Rt	Peak area	Plate number	Asymmetric factor
4.700	5650492	7938	1.06

Fig 6.26 Chromatogram of Level of 100% recovery (3)

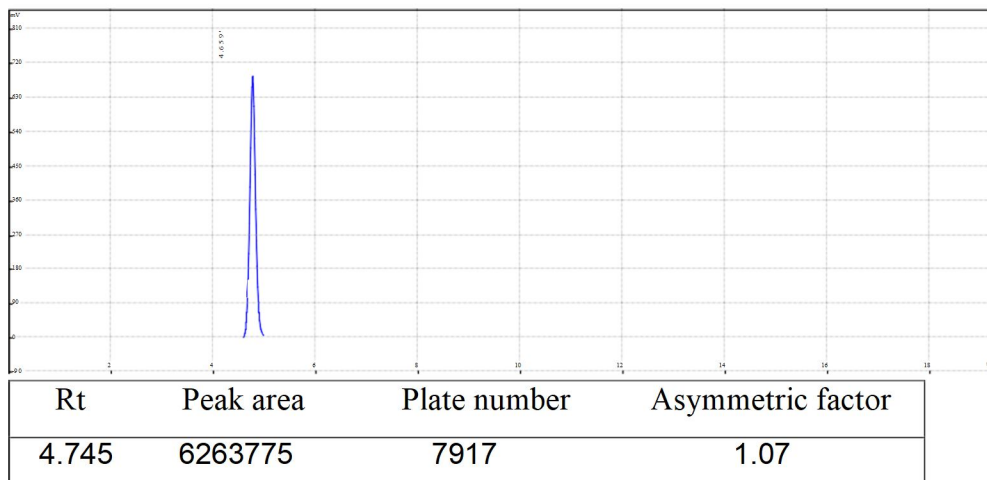


Fig 6.27: Chromatogram of Level of 120% recovery (1)

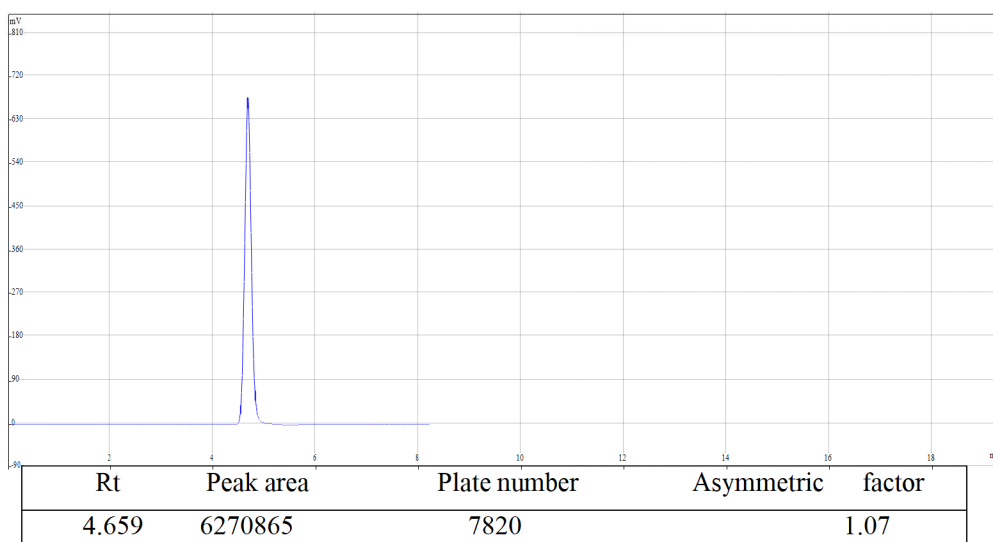
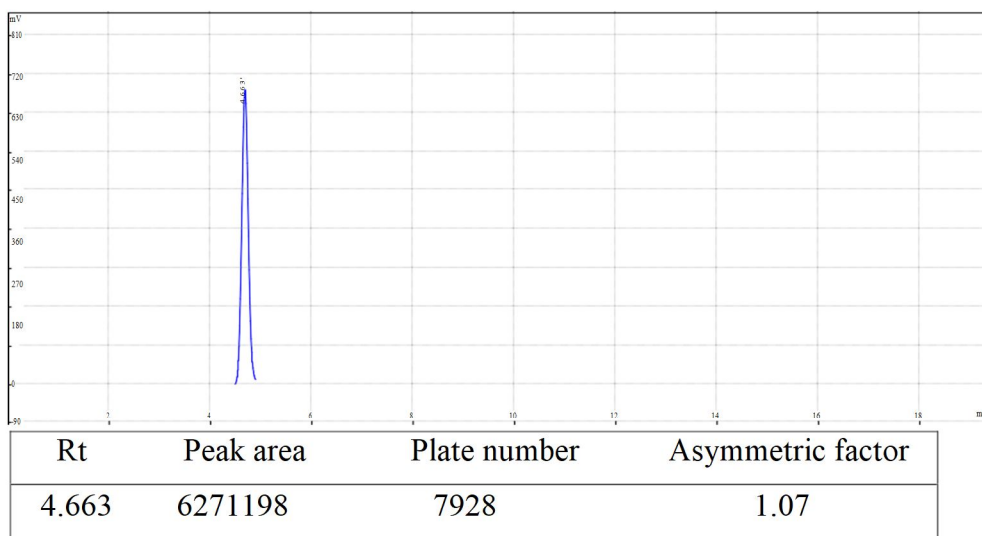


Fig 6.29: Chromatogram of Level of 120% recovery (3)

Table 6.16: Result of % recovery test

Level of % recovery	Amt of sample taken($\mu\text{g/mL}$)	Amt of STD added ($\mu\text{g/mL}$)	Total amount ($\mu\text{g/mL}$)	% recovery	Total Amount Found ($\mu\text{g/mL}$)
80%	20	16	36	98.61	35.49
	20	16	36	99.97	35.98
	20	16	36	99.95	35.98
100%	20	20	40	99.29	39.71
	20	20	40	100.04	40.01
	20	20	40	99.94	39.97
120%	20	24	44	99.87	43.94
	20	24	44	99.93	43.96
	20	24	44	100.01	44.00

Table 6.17 Statistical result of accuracy

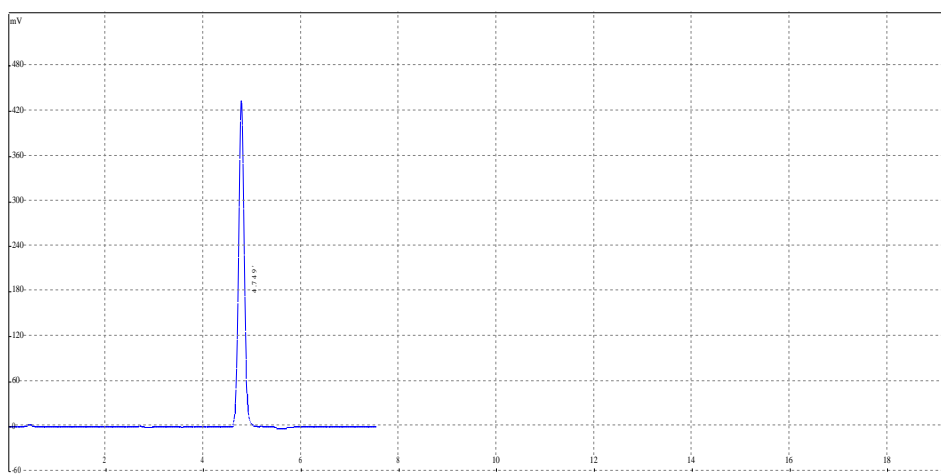
Level of % recovery	Mean % recovery	SD	%RSD
80%	99.51	0.77	0.78
100%	99.75	0.407	0.408
120%	99.93	0.7023	0.7028

N. Precision

The precision of a method was carried out in a two parts such as Interday precision and Intraday precision. The 30 ppm standard Methoxsalen solution was suitably selected for method repeatability.

1) Interday precision

The Interday precision was carried in two different consecutive days. In day 1, three replicates of standard solution were injected and the chromatograms were recorded. In day 2, injected the same standard solution in three replicates which was carried out in day 1, the % RSD of peak areas of the six chromatograms were reported for the fulfilment the accepted criteria. The results were shown as follows:



Rt	Peak area	Plate number	Asymmetric factor
4.749	4278925	8178	1.07

Fig 6.30: Chromatogram of Interday precision Day 1

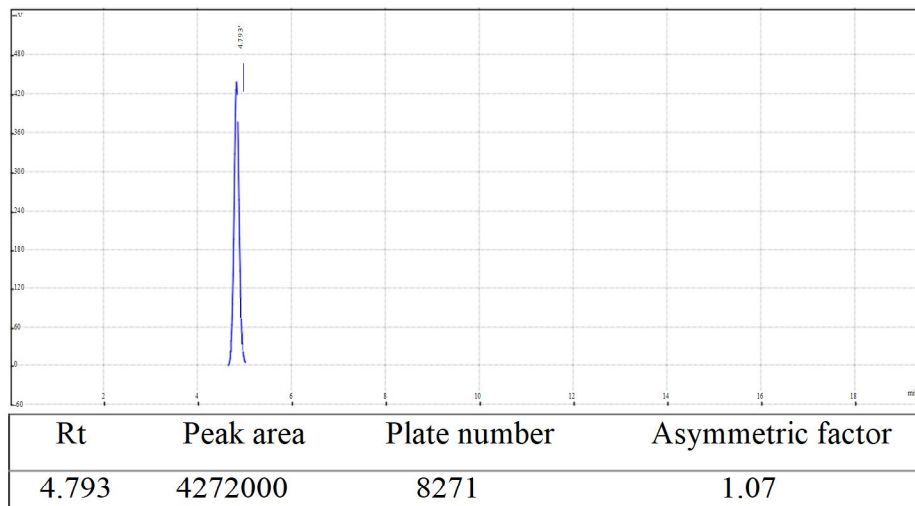


Fig 6.31: Chromatogram of Interday precision Day 1

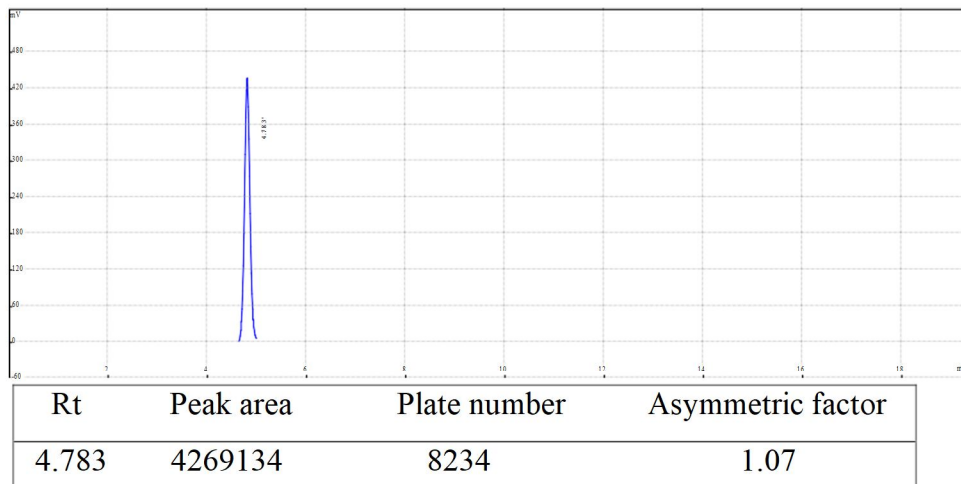


Fig 6.32: Chromatogram of Interday precision Day 1

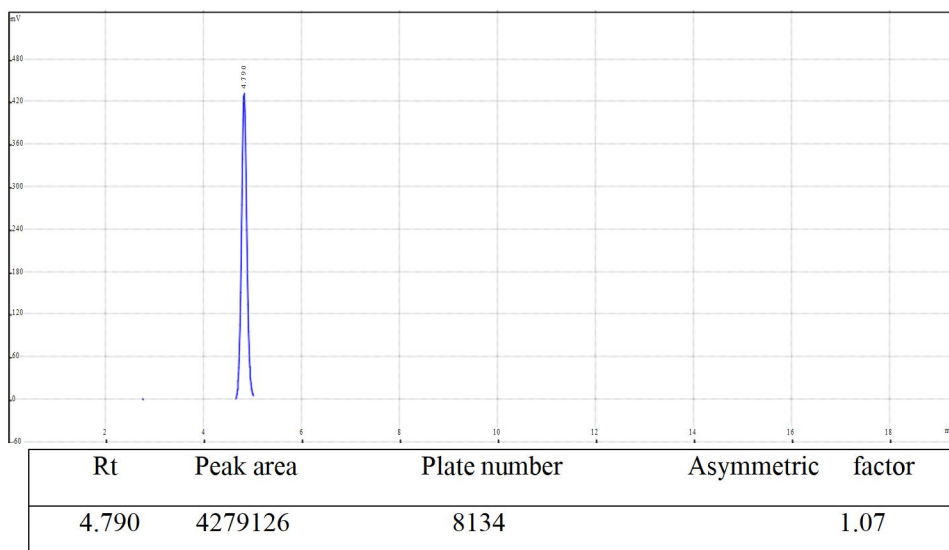
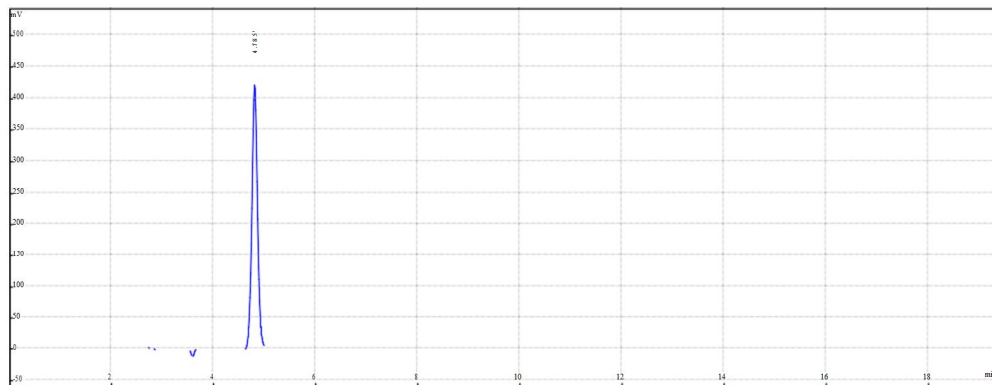


Fig 6.33: Chromatogram of Interday precision Day 2



Rt	Peak area	Plate number	Asymmetric factor
4.785	4286916	8926	1.04

Fig 6.34: Chromatogram of Interday precision Day 2



Rt	Peak area	Plate number	Asymmetric factor
4.777	4281753	7949	1.05

Fig 6.35: Chromatogram of Interday precision Day 2

Table 6.18: Result of Interday precision

	Concentration (µg/mL)	Peak area
Day 1	30	4278925
	30	4272000
	30	4269134
Day 2	30	4279126
	30	4286916
	30	4281753
	Mean	4281753
	%RSD	0.15

2) *Interday precision*

The Intraday precision was carried in two different time period. In Morning, three replicates of standard solution were injected and the chromatograms were recorded, In Evening, injected the same standard solution in three replicates which was carried out in Morning, the % RSD of peak areas of the six chromatograms were reported for the fulfilment the accepted criteria. The results were shown as follows:

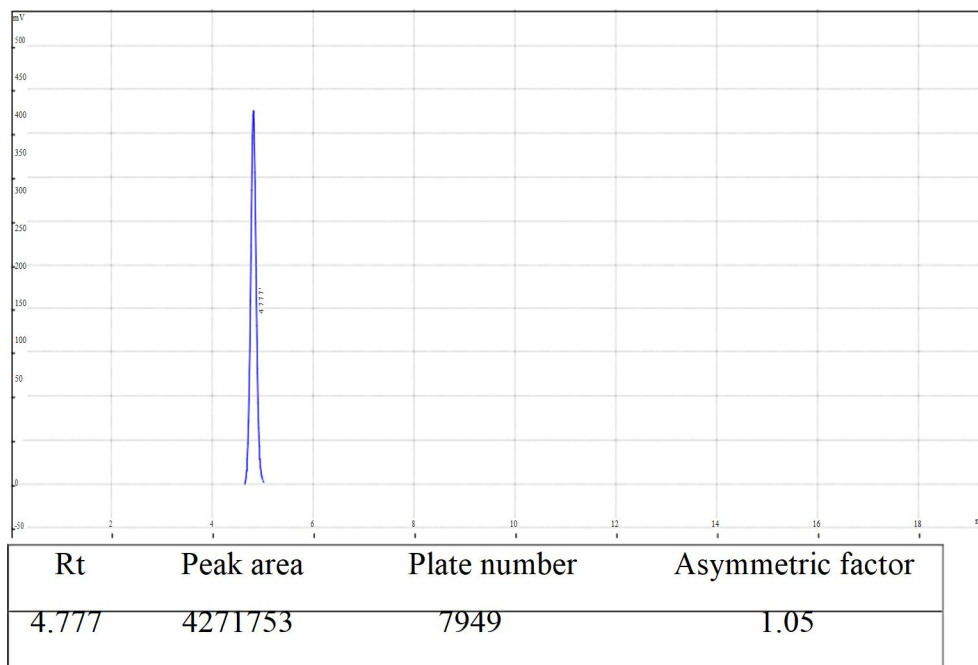


Fig 6.36 Chromatogram of Intraday precision morning (a)

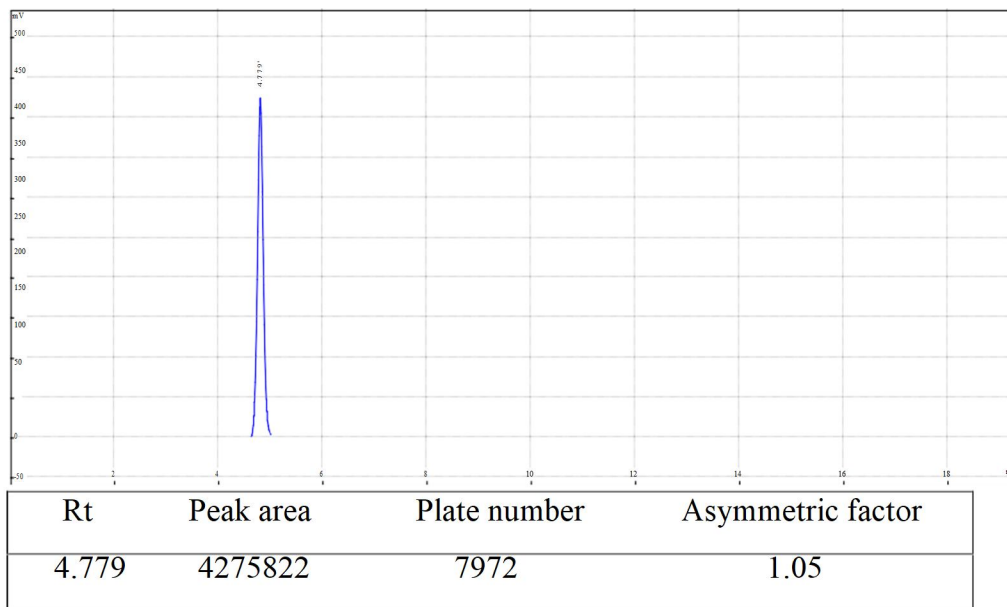


Fig 6.37: Chromatogram of Intraday precision morning (b)

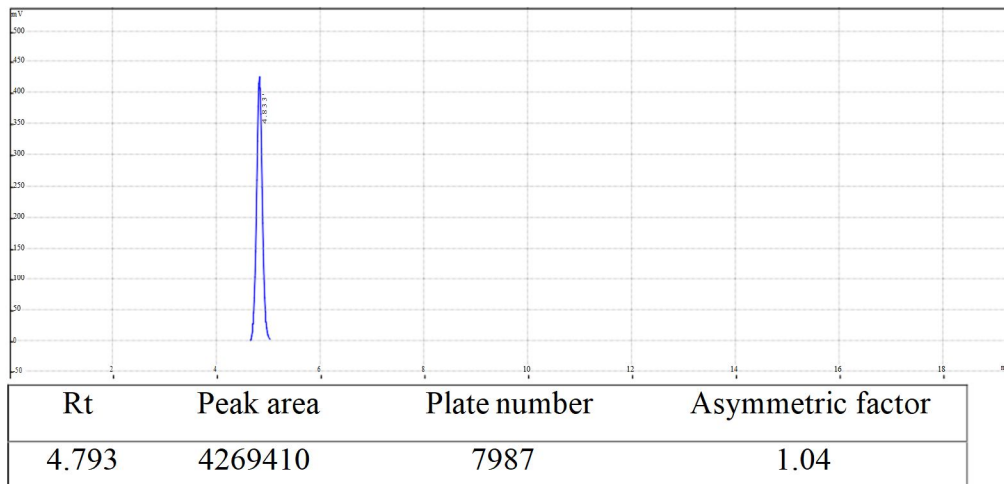


Fig 6.38 Chromatogram of Intraday precision morning (c)

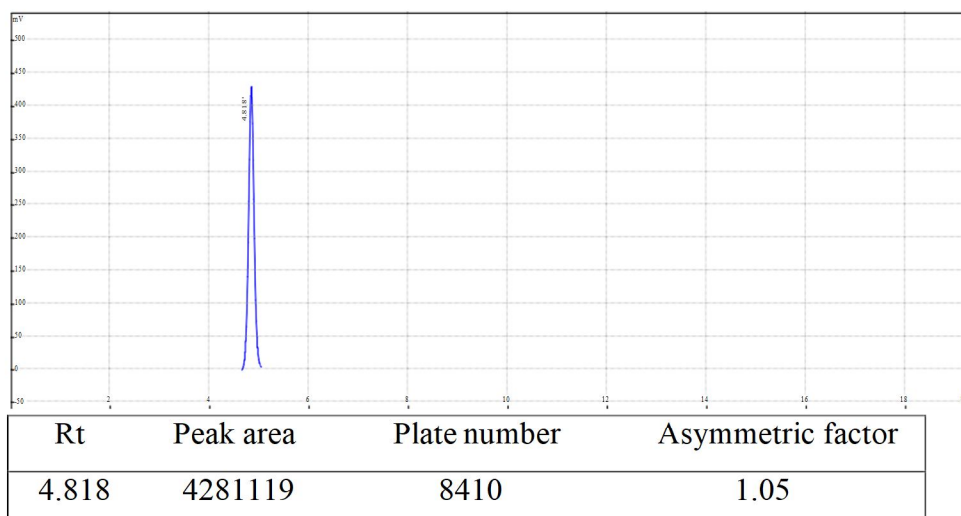


Fig 6.39: Chromatogram of Intraday precision evening (a)

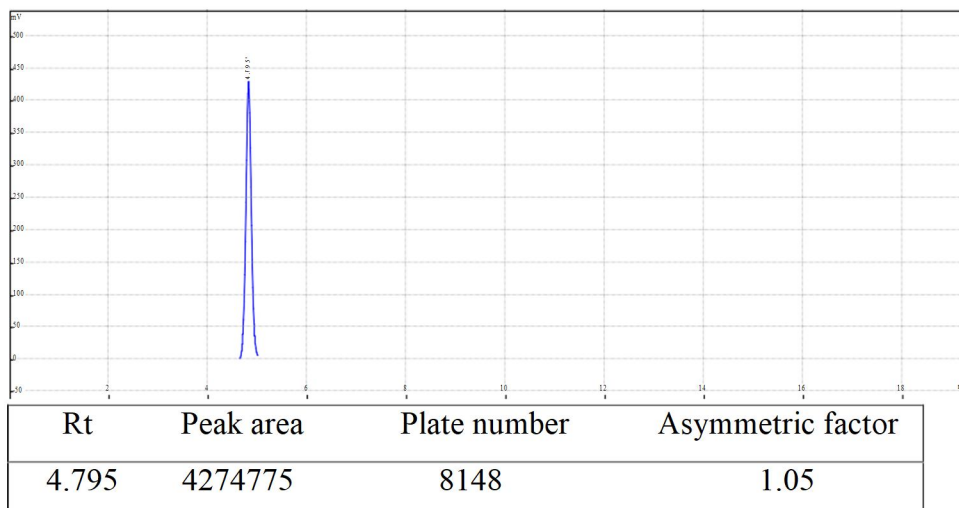
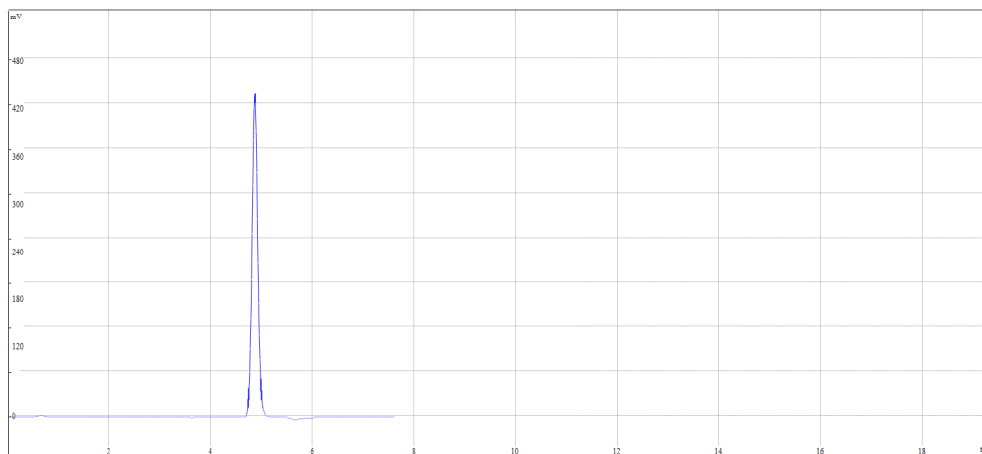


Fig 6.40: Chromatogram of Intraday precision evening (b)



Rt	Peak area	Plate number	Asymmetric factor
4.833	4272163	8457	1.05

Fig 6.41: Chromatogram of Intraday precision evening (c)

Table 6.19 Result of Intraday

	Concentration (µg/ml)	Peak area
Morning	30	4271753
	30	4275822
	30	4269410
Evening	30	4281119
	30	4274775
	30	4272163
	Mean	4274174
	%RSD	0.10

O. Specificity

For the specificity study, solutions of blank, sample and standard solution were used, the standard and sample solutions of 40 ppm were used and each solution were injected into the system and chromatograms were recorded. It was found that the no interference from impurity and excipients.

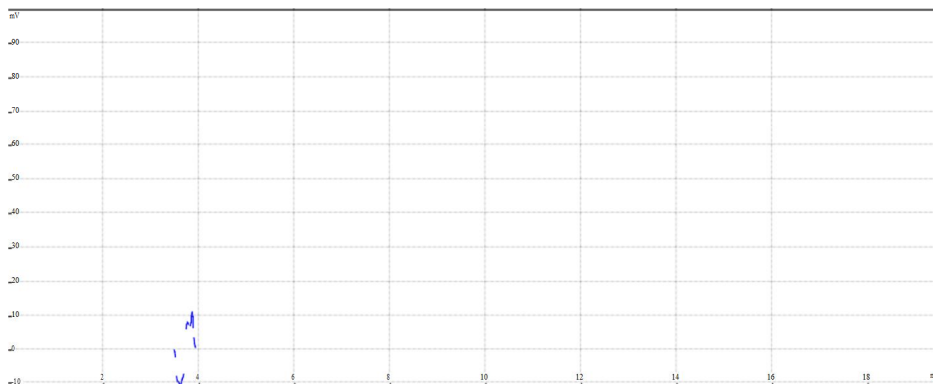


Fig 6.42: Chromatogram of blank preparation

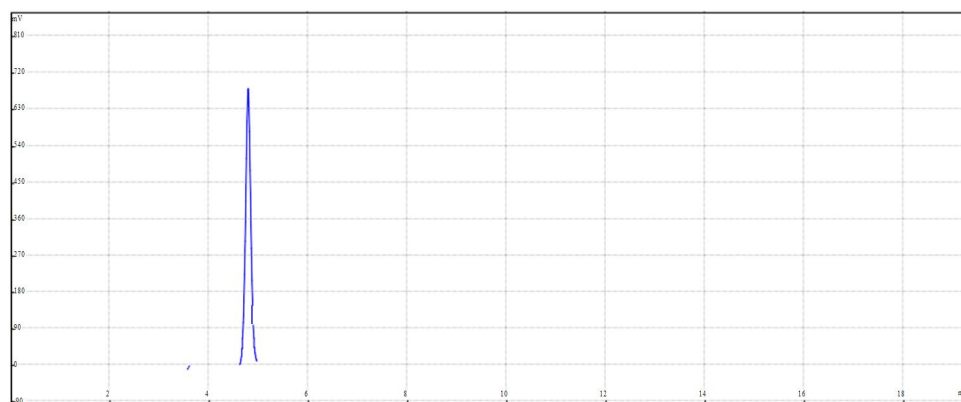


Fig 6.43: Chromatogram of standard preparation

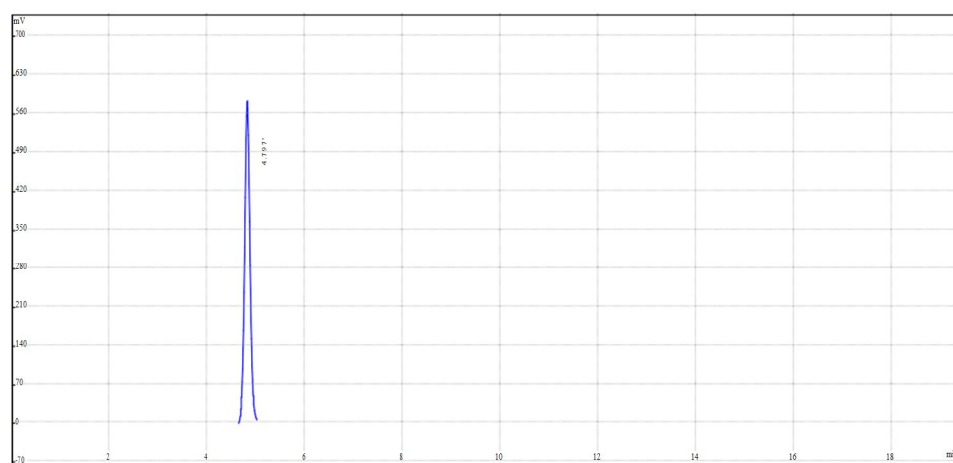
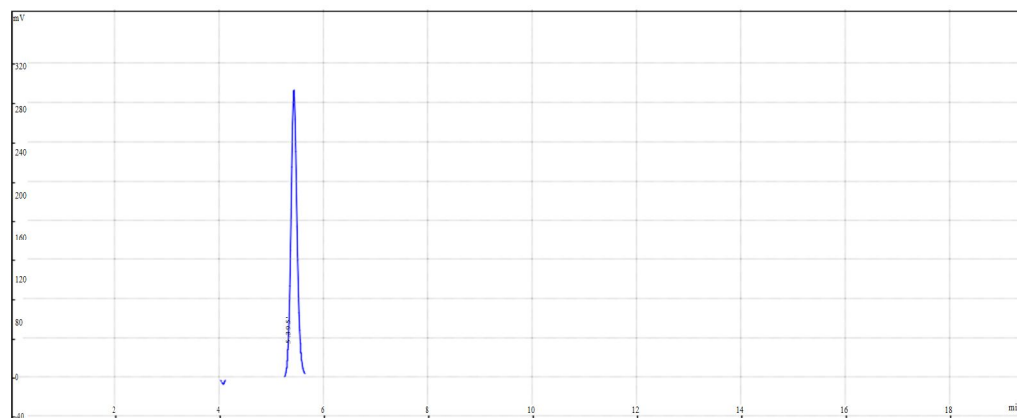


Fig 6.44: Chromatogram of sample preparation

P. Robustness

The robustness of an analytical method was carried out to confirm the analytical method remains unaffected by small variations in the optimized method parameters, the 30 ppm of standard solution were injected for a each varied conditions like change in flow rate ± 2 mL/min, change in wavelength ± 2 nm and change in composition of mobile phase ± 5 mL of methanol and the chromatograms wererecorded, then results were obtained by calculating the %RSD of a peak area for each varied conditions.



Rt	Peak area	Plate number	Asymmetric factor
5.395	2891426	8077	1.10

Fig 6.45: Chromatogram of robustness (0.6 mL/min)

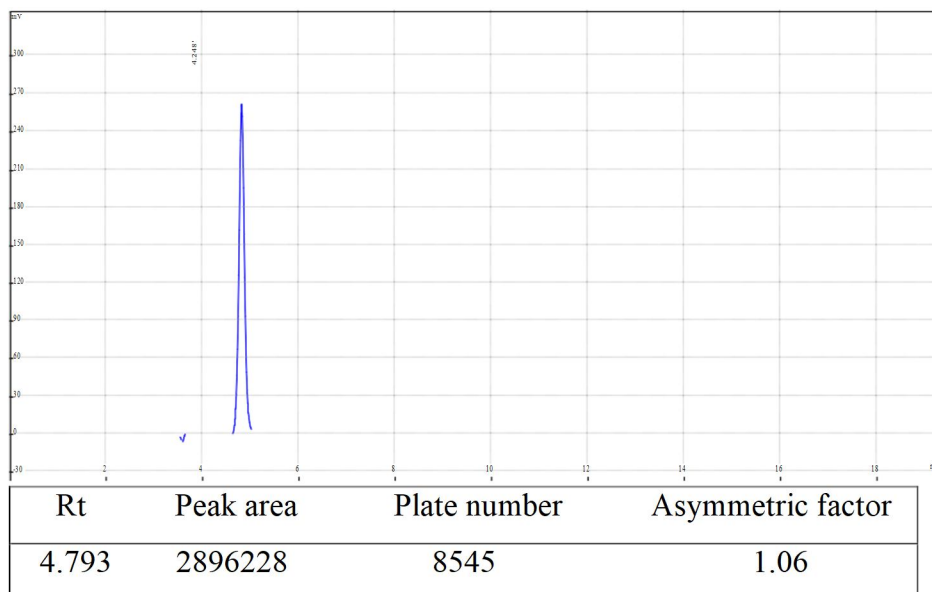


Fig 6.46: Chromatogram of robustness (0.8 mL/min)

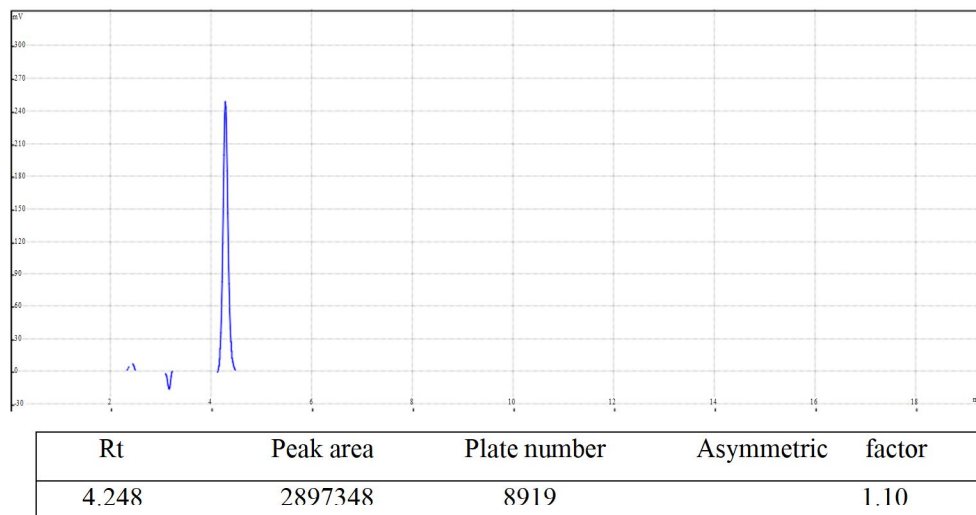
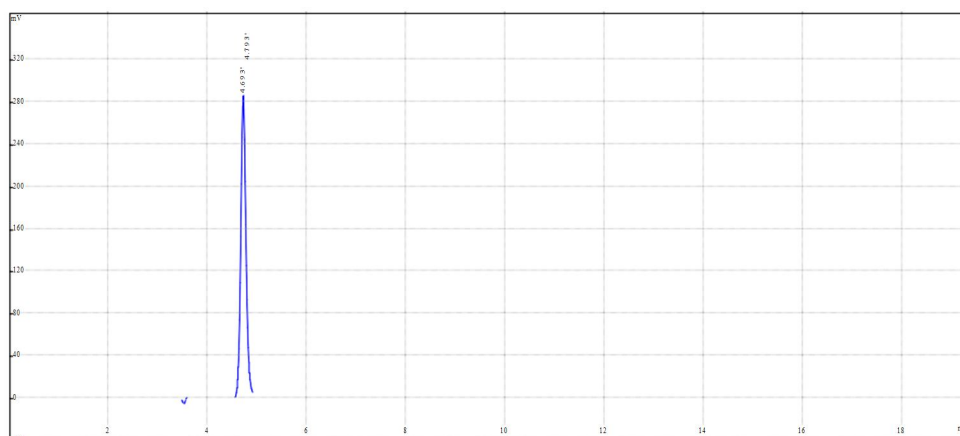


Fig 6.47: Chromatogram of robustness (1.0 mL/min)

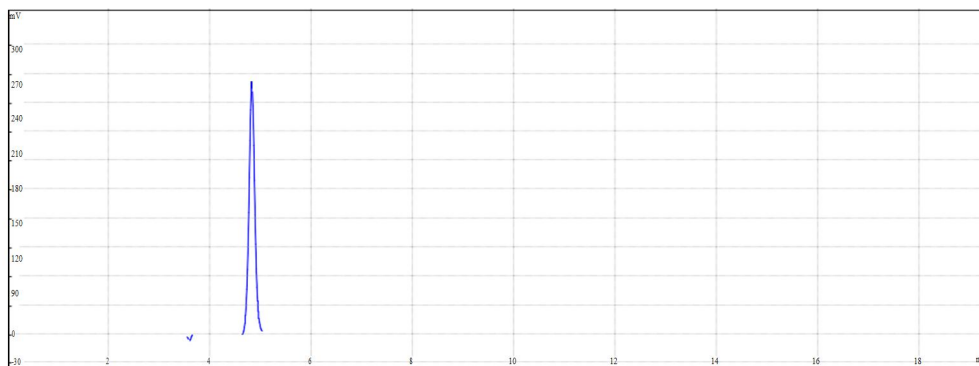
Table 6.20: Result of robustness (change in flow rate)

Changed condition	Peak area
0.6 ml/min	2891426
0.8 ml/min	2896228
1.0 ml/min	2897348
Mean	2895001
%RSD	0.10



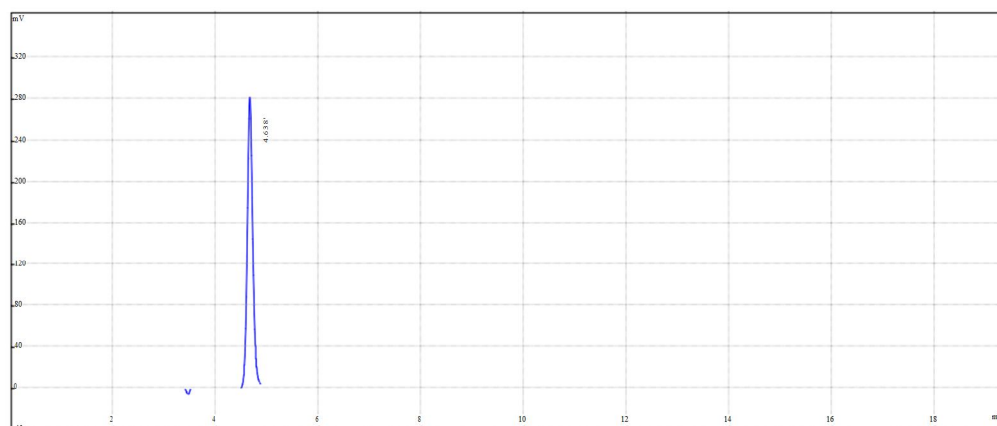
Rt	Peak area	Plate number	Asymmetric factor
4.693	2908376	7780	1.13

Fig 6.48: Chromatogram of robustness (216 nm)



Rt	Peak area	Plate number	Asymmetric factor
4.793	2896228	8545	1.06

Fig 6.49: Chromatogram of robustness (218 nm)



Rt	Peak area	Plate number	Asymmetric factor
4.638	2892805	8390	1.13

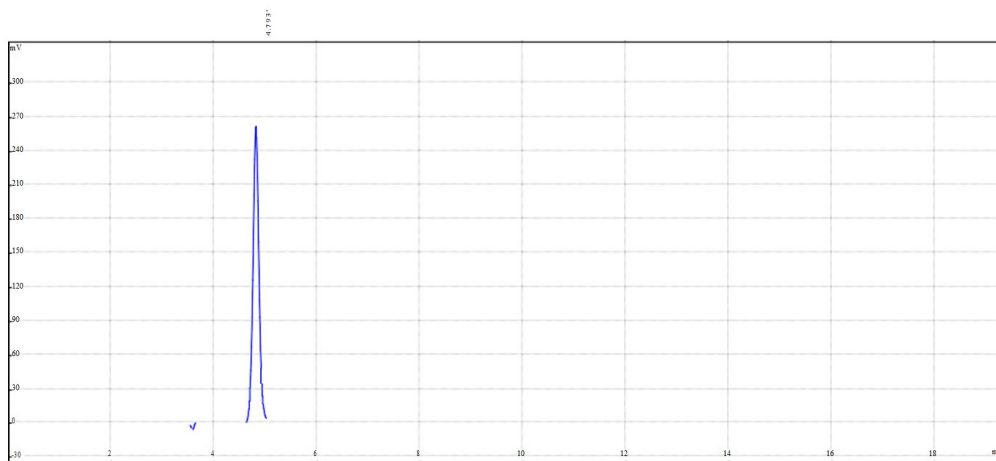
Fig 6.50: Chromatogram of robustness (220 nm) Table 6.21: Result of robustness (change in wavelength)

Changed condition	Peak area
216 nm	2908376
218 nm	2896228
220 nm	2892805
Mean	2899136
%RSD	0.28



Rt	Peak area	Plate number	Asymmetric factor
5.052	2900892	8092	1.06

Fig 6.51: Chromatogram of robustness (75:25 v/v)



Rt	Peak area	Plate number	Asymmetric factor
4.793	2896228	8545	1.06

Fig 6.52: Chromatogram of robustness (80:20 v/v)

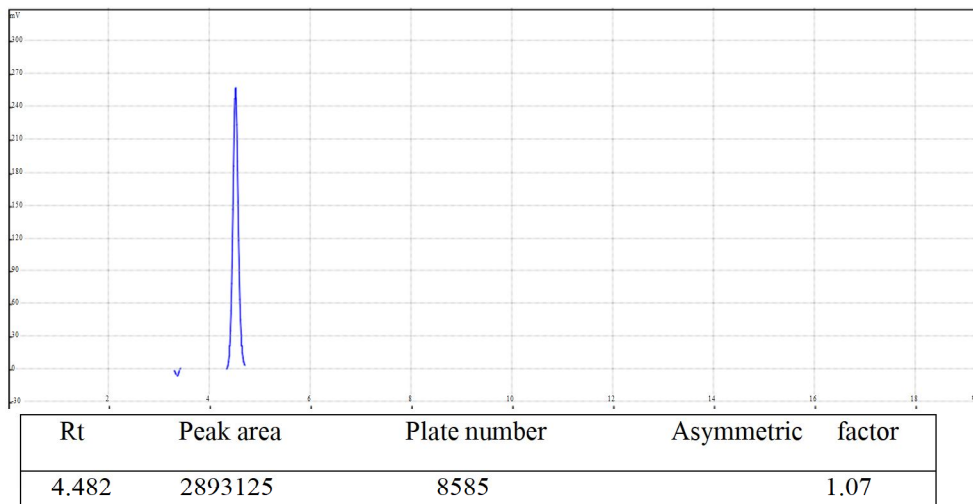


Fig 6.53: Chromatogram of robustness (85:15 v/v)

Table 6.22: Result of robustness (Change in mobile phase composition)

Composition	Peak area
75:25	2900892
80:20	2896228
85:15	2893125
Mean	2896748
%RSD	0.13

Q. Ruggedness

The ruggedness or Intermediate precision of a method was assessed to check the effect of change in analyst on analysis of Methoxsalen, the linearity as a method validation parameter was repeated by second/other analyst to verify the ruggedness, and % RSD was calculated.

The % RSD was found to be 0.070 which is under accepted criteria; hence the developed method was rugged.

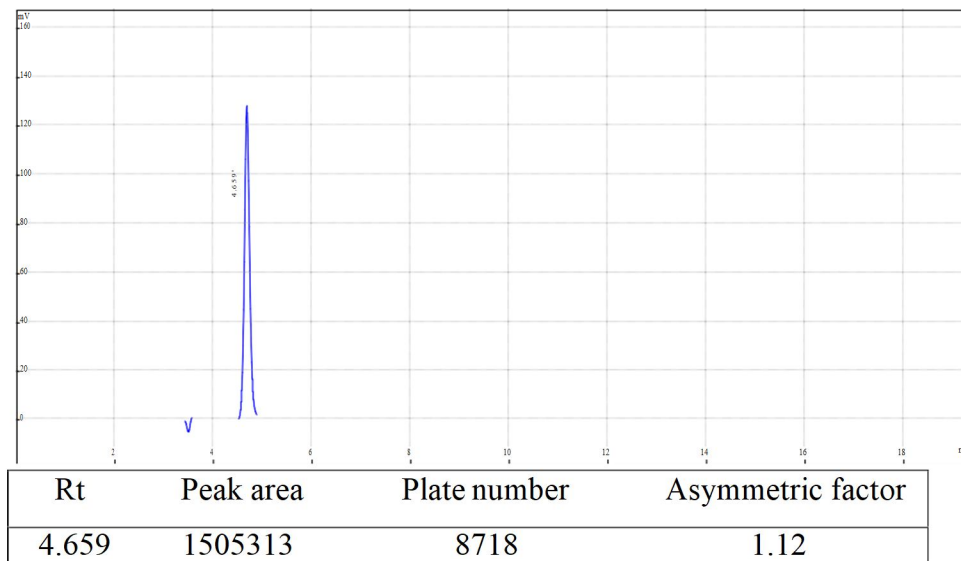
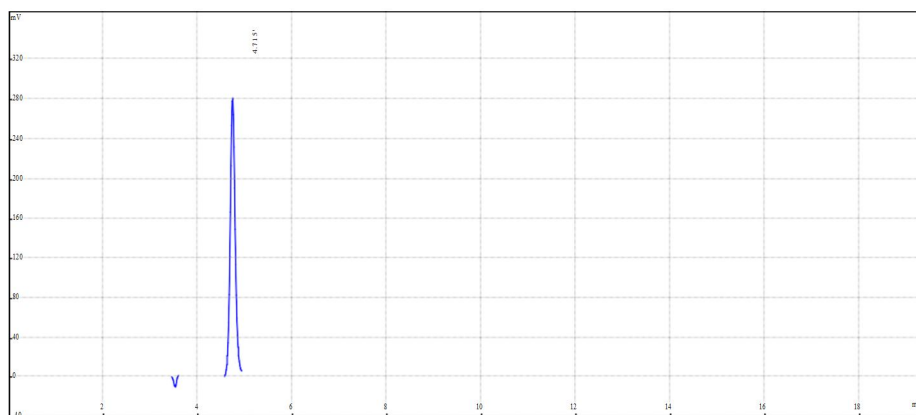
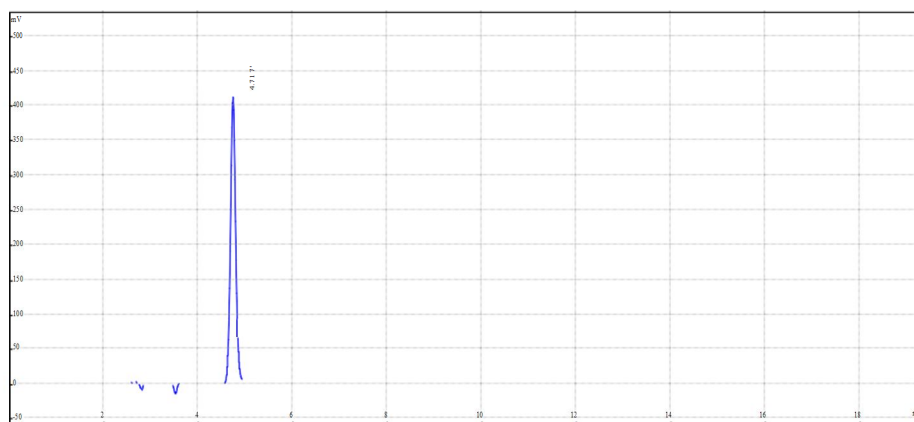


Fig 6.54: Chromatogram of analyst-2 linearity (10 µg/mL)



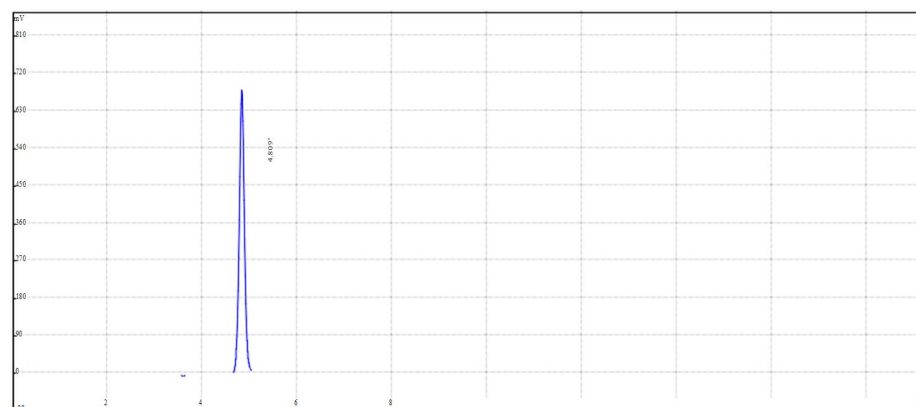
Rt	Peak area	Plate number	Asymmetric factor
4.715	2703320	8008	1.12

Fig 6.55: Chromatogram of analyst-2 linearity (20 µg/mL)



Rt	Peak area	Plate number	Asymmetric factor
4.717	4258983	8200	1.06

Fig 6.56: Chromatogram of analyst-2 linearity (30 µg/mL)



Rt	Peak area	Plate number	Asymmetric factor
4.809	5660353	8673	1.04

Fig 6.57: Chromatogram of analyst-2 linearity (40 µg/mL)

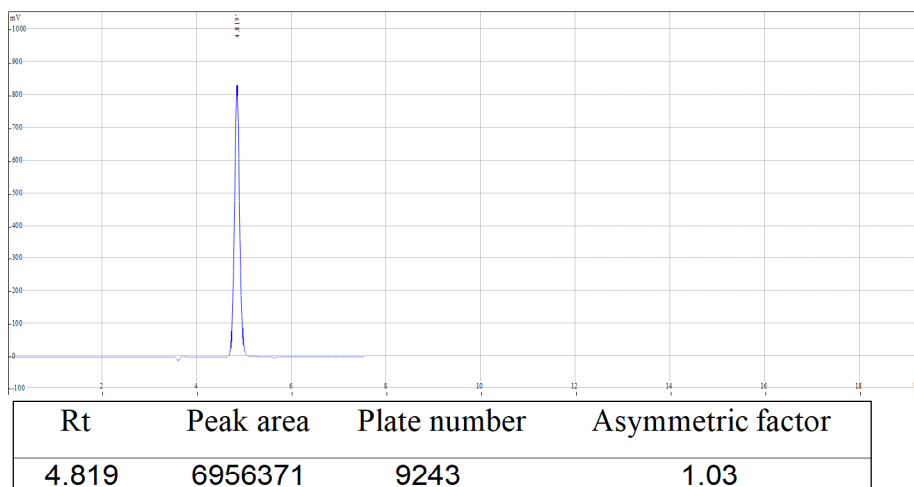


Fig 6.58: Chromatogram of analyst-2 linearity (50 µg/mL)

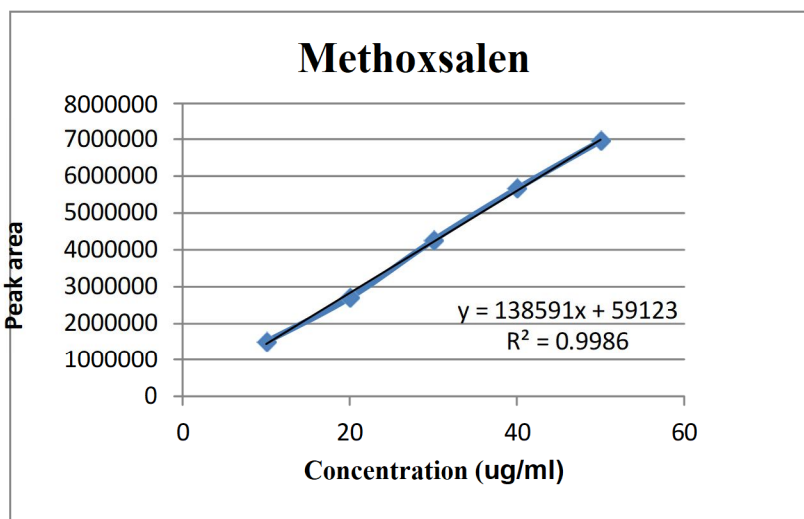


Fig 6.59: Standard calibration curve by analyst 2 Table 6.23: Result of ruggedness

Analyst-1		Analyst-2	
Concentration (µg/mL)	Peak area	Concentration (µg/mL)	Peak area
10	1509903	10	1505313
20	2898763	20	2703320
30	4275906	30	4258983
40	5659586	40	5660353
50	6954494	20	6956371
R ²	0.999	R ²	0.998
	%RSD	0.070	

R. LOD and LOQ

The limit of detection and limit of quantitation were calculated on the basis of standard deviation of the accuracy response and slope of the linearity calibration curve.

Formulas;

Table 6.24: Result of LOD and LOQ

Drug	LOD	LOQ
Methoxsalen	0.14	0.45

S. Forced degradation study

In order to establish the forced degradation study, the standard API was subjected in to the various stress conditions as follows:

T. Alkaline degradation

An accurately weighed quantity of 10 mg Methoxsalen was transferred to the round bottom flask, then 10 mL of 0.1N NaOH was added into it, afterwards; refluxed at 60°C for 1hr by using reflux assembly, after 1hr the flask was removed to cool the solution, 500 µL (0.5 mL) solution was withdrawn and transferred into 10 mL of volumetric flask and the final volume was made up to mark with selected mobile phase, the resultant sample was filtered through 0.45 µ membrane syringe filter. Finally, the sample was injected into the system to record the chromatogram. The percent degradation of drug in alkaline condition was found to be 6.21.

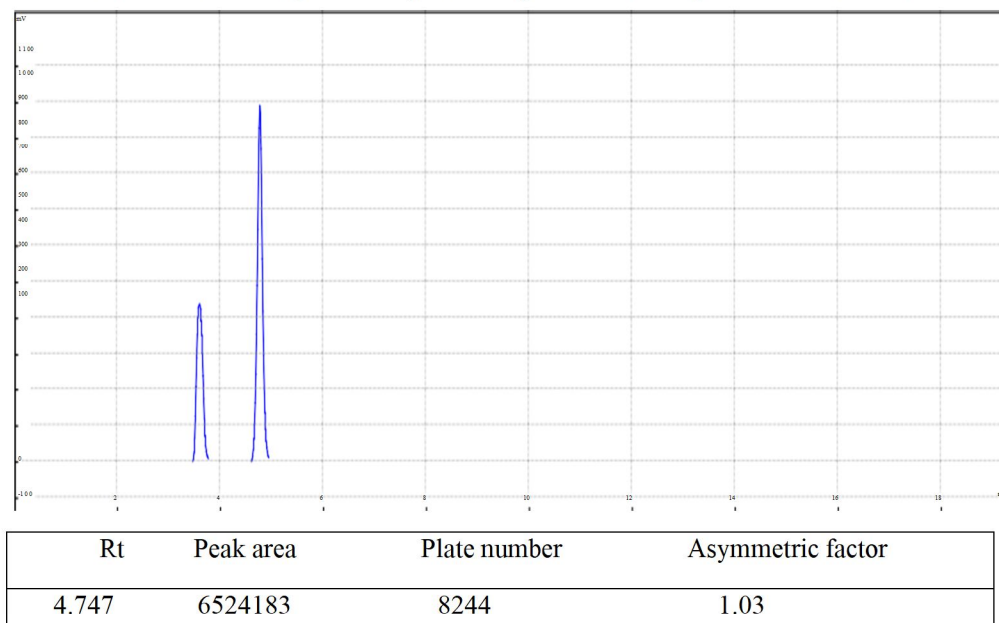


Fig 6.60: Chromatogram of base degradation sample

U. Acid degradation

An accurately weighed quantity of 10 mg Methoxsalen was transferred to the round bottom flask, then 10 mL of 0.1N HCl was added into it, afterwards; refluxed at 60°C for 1hr by using reflux assembly, after 1hr the flask was removed to cool the solution, 500 µL (0.5 mL) solution was withdrawn and transferred into 10 mL of volumetric flask and the final volume was made up to mark with selected mobile phase, the resultant sample was filtered through 0.45µ membrane syringe filter. Finally, the sample was injected into the system to record the chromatogram. **The degradation of drug in acidic condition was found to be 1.17%.**

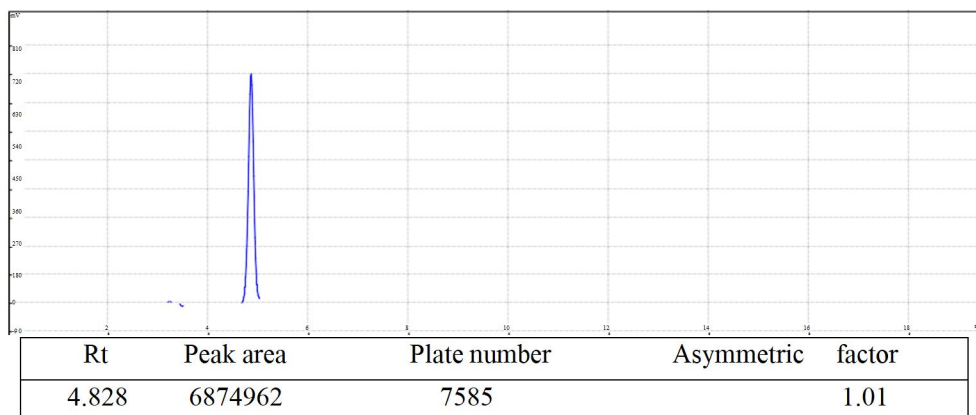


Fig 6.61: Chromatogram of acid degradation sample

V. Peroxide Degradation

An accurately weighed quantity of 10 mg Methoxsalen was transferred to the round bottom flask, 10 mL of 3% H₂O₂ was added, afterwards; refluxed at 60°C for 1hr. after 1hr flask was removed to cool the solution, 500 μL (0.5 mL) of solution was withdraw and transferred to a 10 mL volumetric flask and the final volume was adjusted up to mark with mobile phase, the resultant sample was filtered through 0.45μ membrane syringe filter, and inject it. **The degradation of drug in oxidative condition was found to be 3.18%.**

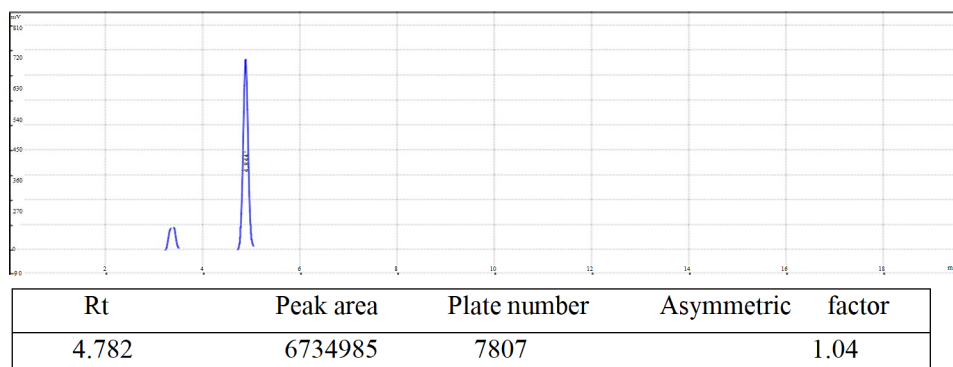


Fig 6.62: Chromatogram of oxidative degradation sample

W. Thermal degradation

The sample was heated in LOD OVEN at 60°C in Petri plate for 24 Hour. An accurately weighed quantity of 10 mg sample was transferred into 10 mL of volumetric flask and 5 mL of mobile phase was added to it, the resultant solution was sonicated for 25 min with intermittent shaking and diluted up to the mark with mobile phase, then it was allowed to settle for 15 min, the 100 μL of supernatant solution was diluted up to 10 mL by mobile phase, then filtered through 0.45μ membrane syringe filter-media and injected into the system to obtain the chromatogram. **The degradation of drug in alkali condition was found to be 3.54%.**

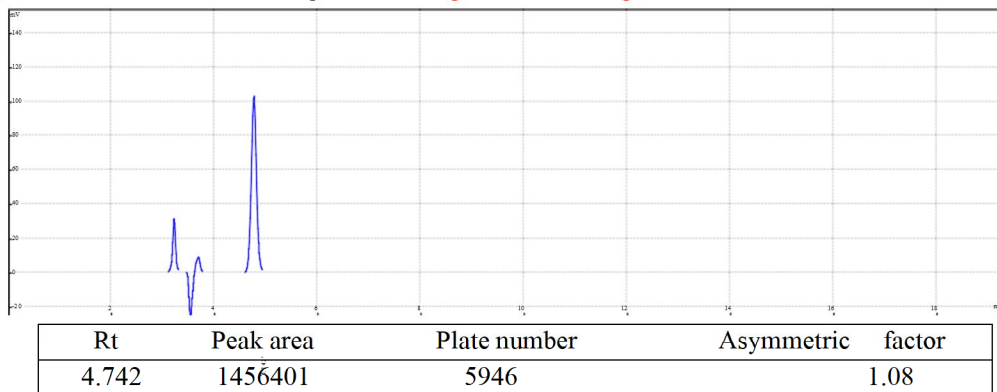


Fig 6.63: Chromatogram of thermal degradation sample

The result of forced degradation study is shown in Table 6.26.

Table 6.25: Result of forced degradation study

Sr. No.	Stress Condition	% degradation
1	Alkaline condition	6.21%
2	Acidic condition	1.17%
3	Oxidative condition	3.18%
4	Thermal condition	3.54%

VIII. DISCUSSION

The present study included development of stability indicating RP-HPLC method for estimation of Methoxsalen and the results obtained were found to be satisfactory. The work was started with solubility testing; the Methoxsalen was soluble in water, methanol and acetonitrile. Then the wavelength was selected by UV spectrophotometric method in methanol : water (80:20 v/v), the Methoxsalen was shown λ_{max} at 218 nm, and this 218 nm was selected as the detector wavelength in a HPLC.

For the selection of mobile phase the different trials were taken, the trail -1 was not selected due to the broad peak observed with 100 % methanol in the recorded chromatogram. By reducing the methanol: water ratio in (90:10 v/v) in trail-2, the recorded chromatogram was shown the solvent peak too close to the principal peak of Methoxsalen, In trial-3 the methanol : water (80:20 v/v) was selected as mobile phase due to sharp peak obtained in chromatogram, which fulfilled the acceptance criteria, therefore trial-3 chromatographic condition was optimized for the analysis of Methoxsalen.

For estimation of drug in marketed formulation, drug solution equivalent to -----

The validation of the developed method was carried out as per ICH guidelines and the results were found to be complying with the acceptable limit. The compiled validation results are shown in Table 7.1

Table 7.1: Results of validation

Sr. No.	Parameter	Result	Acceptance criteria
1	Linearity	10-50 μ g/mL, $R^2 = 0.999$	$R^2 \geq 0.999$
2	System suitability test	T.plates=8089, A. factor=	T.Plates ≥ 2000 , A. factor < 1.75
3	Accuracy	99.74	98-102%
4	Interday precision	0.15%	%RSD $< 2\%$
5	Intraday precision	0.10%	%RSD $< 2\%$
6	Specificity	No Interference	No Interference
7	Robustness	Change in wavelength:0.28%, Change in flow rate:0.10%, Change in composition: 0.13%	%RSD $< 2\%$
8	Ruggedness	0.070	%RSD $< 2\%$
9	LOD and LOQ	0.14 and 0.45	LOQ is three times of LOD

The forced degradation study was carried out in acid, base, peroxide and thermal stress condition. In alkaline condition 6.21% drug was degraded may be due to the breakdown of coumarin ring. In acidic condition 1.17% drug was degraded may be due to the double bond break in furocoumarin ring and in oxidative drug was 3.18% degraded may be due to the oxidation of lactone ring. In thermal degradation, 3.54% of drug was degraded at 60°C.

IX. CONCLUSION

The present research work based on development of stability indicating RP-HPLC method for estimation of Methoxsalen, was successfully completed. The parent drug and degradation products were well resolved under optimized chromatographic condition indicating the selectiveness of developed method. Based on the test results, it was concluded that the method is simple, accurate, sensitive, precise, rapid, and free from any kind of interference of the excipients from the formulation; therefore the proposed method can be used for routine analysis of estimation of Methoxsalen in its tablet formulation.

X. FUTURE SCOPE

There may be several scopes in future for further studies are given as follows:

- 1) The developed RP-HPLC method can be further optimized for the estimation of Methoxsalen in biological fluids.
- 2) Stability indicating UPLC and UHPLC methods can be developed for much faster results.
- 3) Degradation pathway of the drug can be established by identifying the possible structure of degraded product through hyphenated techniques like LC-MS, LC-NMR.

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List Of Abbreviations

Sr. No	CHEMICALS	
1	H ₂ O ₂	Hydrogen Peroxide
2	HCl	Hydrochloric acid
3	H ₂ O	Water
4	C ₃ H ₆ O	Acetone
5	MeOH	Methanol
6	ACN	Acetonitrile
7	NaOH	Sodium hydroxide
SYMBOLS		
8	%	Percentage
9	λ Max	Wavelength of maximum absorption
10	<	Less than
11	>	Greater than
12	µg	Microgram (S)
13	µL	Micro liter (S)
15	Cm	Centimeter (S)
16	i.d.	Internal diameter
19	Mg	Milligram (S)
20	Min	Minute (S)
21	mL	Milliliter (S)
25	r ²	Correlation coefficient
26	Sec	Second (S)
27	Temp	Temperature
28	°C	Degree centigrade
29	No.	Number

30	Sr. No.	Serial number
32	v/v	Volume by volume
34	Conc.	Concentration
35	ppm	Parts per million
OTHERS		
36	RSD	Relative standard deviation
37	SD	Standard deviation
38	UV	Ultraviolet
39	Rt	Retention Time
42	API	Active Pharmaceutical Ingredient
43	RP-HPLC	Reversed Phase HighPerformance Liquid Chromatograph
44	ICH	International Conference onHarmonization
46	LOD	Limit of detection
47	LOQ	Limit of Quantitation
50	SIAM	Stability indicating assay method

List Of Figures

Sr. No	Fig No.	Figure Name	Page No.
01	1.1	Adsorption of solutes over stationary phase	3
02	1.2	Ion Exchange chromatography	4
03	1.3	Size Exclusion chromatography	5
04	1.4	Schematic diagram of HPLC system	7
05	1.5	Fundamental parameters of Chromatography	13
06	1.6	Negative peaks	17
07	1.7	Peak Fronting	18
08	1.8	Peak Tailing	18
09	1.9	Broad Peak	18
10	1.10	Absorption and Intensity shifts	20

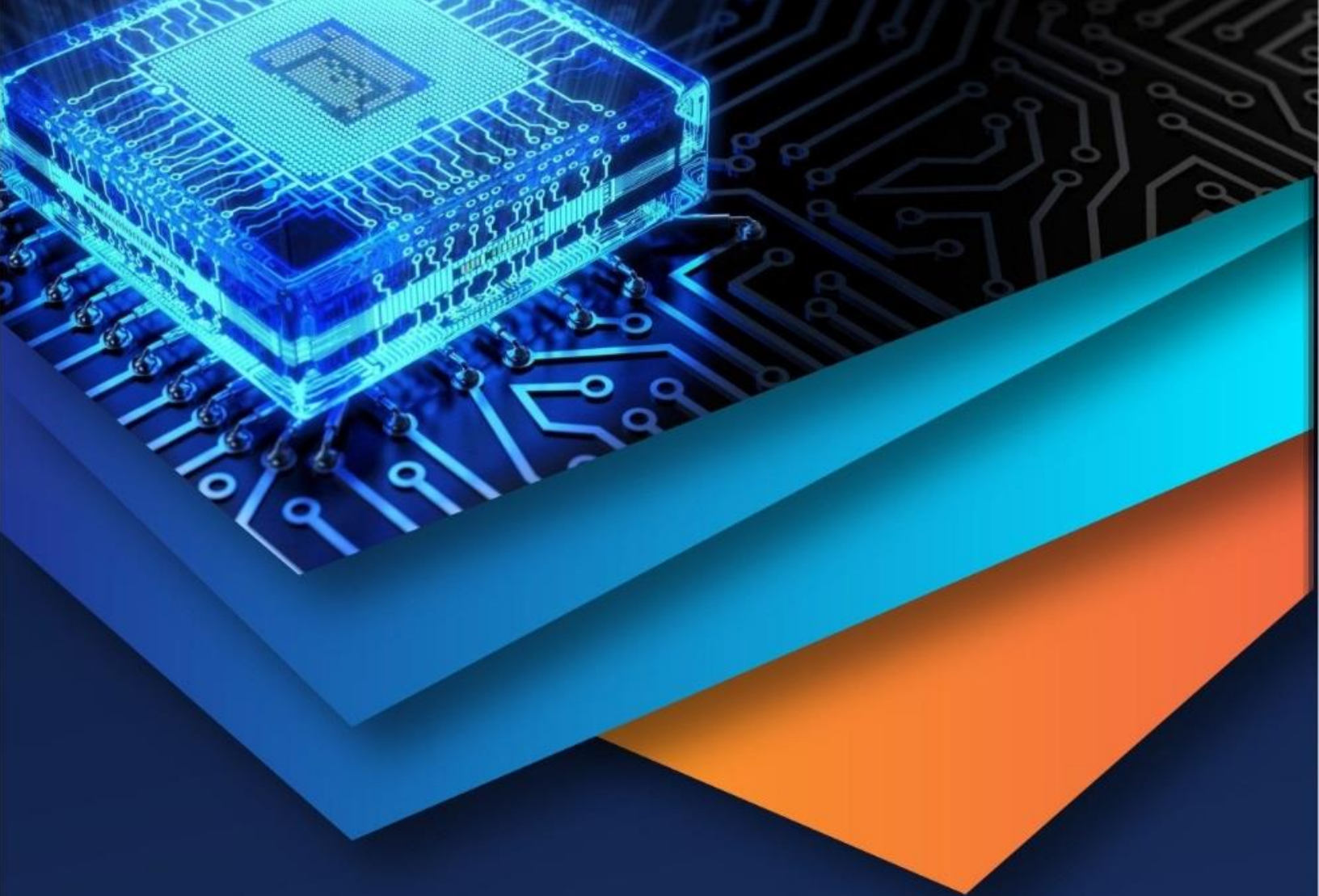
11	1.11	Schematic diagram of UV-Visible spectrophotometer	21
12	1.12	Prerequisites for Method development and Validation	23
13	6.1	UV spectra of Methoxsalen	
14	6.2	Typical chromatogram of Trial-1	
15	6.3	Typical chromatogram of Trial-2	
16	6.4	Typical chromatogram of Trial-3	
17	6.5	Typical chromatogram of Trial-4	
18	6.6	Standard calibration curve of Methoxsalen	
19	6.7	Linearity Chromatogram (10µg/mL)	
20	6.8	Linearity Chromatogram (20µg/mL)	
21	6.9	Linearity Chromatogram (30µg/mL)	
22	6.10	Linearity Chromatogram (40µg/mL)	
23	6.11	Linearity Chromatogram (50µg/mL)	
24	6.12	Typical chromatogram System Suitability-1	
25	6.13	Typical chromatogram System Suitability-2	
26	6.14	Typical chromatogram System Suitability-3	
27	6.15	Typical chromatogram System Suitability-4	
28	6.16	Typical chromatogram System Suitability-5	
29	6.17	Chromatogram of standard solution of Methoxsalen	
30	6.18	Chromatogram of sample solution of Methoxsalen	
31	6.19	Chromatogram of sample solution of Methoxsalen	
32	6.20	Chromatogram of sample solution of Methoxsalen	
33	6.21	Chromatogram of Accuracy 80% Level 1	
34	6.22	Chromatogram of Accuracy 80% Level 2	
35	6.23	Chromatogram of Accuracy 80% Level 3	
36	6.24	Chromatogram of Accuracy 100% Level 1	
37	6.25	Chromatogram of Accuracy 100% Level 2	
38	6.26	Chromatogram of Accuracy 100% Level 3	

39	6.27	Chromatogram of Accuracy 120% Level 1	
40	6.28	Chromatogram of Accuracy 120% Level 2	
41	6.29	Chromatogram of Accuracy 120% Level 3	
42	6.30	Chromatogram of Interday precision Day 1	
43	6.31	Chromatogram of Interday precision Day 1	
44	6.32	Chromatogram of Interday precision Day 1	
45	6.33	Chromatogram of Interday precision Day 2	
46	6.34	Chromatogram of Interday precision Day 2	
47	6.35	Chromatogram of Interday precision Day 2	
48	6.36	Chromatogram of Intraday precision morning (a)	
49	6.37	Chromatogram of Intraday precision morning (b)	
50	6.38	Chromatogram of Intraday precision morning (c)	
51	6.39	Chromatogram of Intraday precision evening (a)	
52	6.40	Chromatogram of Intraday precision evening (b)	
53	6.41	Chromatogram of Intraday precision evening (c)	
54	6.42	Chromatogram of blank preparation	
55	6.43	Chromatogram of standard preparation	
56	6.44	Chromatogram of sample preparation	
57	6.45	Chromatogram of robustness (0.6 mL/min)	
58	6.46	Chromatogram of robustness (0.8 mL/min)	
59	6.47	Chromatogram of robustness (1.0 mL/min)	
60	6.48	Chromatogram of robustness (216 nm)	
61	6.49	Chromatogram of robustness (218 nm)	
62	6.50	Chromatogram of robustness (220 nm)	
63	6.51	Chromatogram of robustness (75:25 v/v)	
64	6.52	Chromatogram of robustness (80:20 v/v)	
65	6.53	Chromatogram of robustness (85:15 v/v)	
66	6.54	Chromatogram of analyst-2 linearity (10 µg/mL)	
67	6.55	Chromatogram of analyst-2 linearity (20 µg/mL)	
68	6.56	Chromatogram of analyst-2 linearity (30 µg/mL)	
69	6.57	Chromatogram of analyst-2 linearity (40 µg/mL)	

70	6.58	Chromatogram of analyst-2 linearity (50 µg/mL)	
71	6.59	Standard calibration curve by analyst-2	
72	6.60	Chromatogram of base degradation sample	
73	6.61	Chromatogram of acid degradation sample	
74	6.62	Chromatogram of oxidative degradation sample	
75	6.63	Chromatogram of thermal degradation sample	

List Of Tables

Sr. No	Table No.	Name of Table	Page No.
1	1.1	Modes of Chromatography	6
2	1.2	Pump's advantages and Disadvantages	10
3	1.3	Commonly used Columns and their manufacturer	12
4	1.4	Characteristics to be Validated in HPLC	29
5	6.1	Details of Reference Standard Used	36
6	6.2	Details of Marketed Formulation Used	36
7	6.3	Details of Instrument Used	37
8	6.4	Details of Chemicals and Reagents used	37
9	6.5	Trial-1 Chromatographic condition	39
10	6.6	Trail-2 Chromatographic condition	40
11	6.7	Trail-3 Chromatographic condition	41
12	6.8	Trail-4 Chromatographic condition	41
13	6.9	Optimization of mobile phase strength	43
14	6.10	Optimized Chromatographic Conditions	43
15	6.11	Preparation of linearity samples	
16	6.12	Results of linearity	
19	6.13	Result of system suitability	
20	6.14	Result of assay	
21	6.15	Preparation of % recovery samples	
22	6.16	Result of accuracy (% recovery)	
	6.17	Statistical result of accuracy	
23	6.18	Result of Interday precision	
24	6.19	Result of Intraday precision	
25	6.20	Result of robustness (change in flow rate)	
26	6.21	Result of robustness (change in wavelength)	
27	6.22	Result of robustness (change in composition)	
28	6.23	Result of ruggedness	
29	6.24	Result of LOD and LOQ	
30	6.25	Result of forced degradation study	



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