ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF LUMEFANTRINE BY RP-HPLC

A Dissertation submitted to

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY,

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In partial fulfilment of the award of the degree of

MASTER OF PHARMACY

IN

Branch V – PHARMACEUTICAL ANALYSIS

Submitted by

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EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled, **ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF LUMEFANTRINE BY RP-HPLC,** submitted by student bearing **Reg. No. 261730208** to **"The Tamil Nadu Dr. M.G.R. Medical University"**, Chennai, for the partial fulfillment of the degree of **MASTER OF PHARMACY in Pharmaceutical Analysis,** was evaluated by us during the examination held on…….

Internal Examiner **External Examiner**

CERTIFICATE

This is to certify that the work embodied in the dissertation entitled, **ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF LUMEFANTRINE BY RP-HPLC,** submitted to "**The Tamil Nadu Dr. M.G.R. Medical University",** Chennai, was carried out by **NIDHIN GEORGE [Reg.No: 261730208],** for the partial fulfillment of the degree of **MASTER OF PHARMACY** in **PHARMACEUTICAL ANALYSIS**, under direct supervision of **Dr. V. SEKAR, M.Pharm., Ph.D.,** Professor and Head, Department of Pharmaceutical Analysis, J.K.K. Nattraja College of Pharmacy, Komarapalayam, during the academic year 2018-2019.

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DECLARATION

The work presented in this dissertation entitled, **ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF LUMEFANTRINE BY RP-HPLC,** was carried out by me, under the direct supervision of **Dr. V. SEKAR, M.Pharm., Ph.D.,** Professor and Head, Department of Pharmaceutical Analysis, J.K.K. Nattraja College of Pharmacy, Komarapalayam. I further declare that, this work is original and has not been submitted in part or full for the award of any other degree or diploma in any other university.

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Dedicated to

Almighty

My Beloved Parents,

&

My Family Members

CONTENTS

LIST OF ABBREVIATIONS USED

%	:	Percentage
%RSD	:	Percentage Relative Standard Deviation
λ	:	Lambda
α	:	Selectivity factor
°C	:	Degree Celsius
µ	:	Minute
µg/ml	:	Microgram per Millilitre
µl	:	Micro litre
A.R	:	Analytical Reagent
API′S	:	Active Pharmaceutical ingredients
As	:	Peak Asymmetry
AUC	:	Area under curve
B.P	:	British Pharmacopeia
CAS	:	Chemical Abstract service
CFR	:	Code Federal regulations
cm	:	centi meter
DMSO	:	Dimethyl sulfoxide
EC_{50}	:	Half maximal effective concentration
ELSD	:	Evaporate light scattering detector
GERD	:	Gastroesophageal reflux disease
Gms	:	Grams
I.D	:	Internal diameter
IC_{50}	:	Half maximal inhibitory concentration
ICH	:	International conference on Harmonization
IP	:	Indian pharmacopeia
IUPAC	:	International union of pure and applied chemistry
K′	:	Capacity factor
LOD	:	Limit of Detection
LOQ	:	Limit of Quantification
mg/tab	:	Milligram per tablet
min	:	Minute

ml	:	Millilitre
ml/min	:	Millilitre/Minute
mM	:	Milli Mole
MS	:	mass spectroscopy
N	:	column effecient
nm	:	Nanometre
NSAIDs	:	Non steroidal anti- inflammatory drugs
ODS	:	Octadecyl Silica (C_{18} Column)
OPA	:	Ortho Phosphoric Acid
PDA	:	photo diode array
pH	:	Negative Logarithm of Hydrogen Ion
PhoP	:	Transcriptional regulatory protein
Psi	:	pounds for square inch
RI	:	Refractive index
RP-HPLC	:	Reverse Phase-High Performance Liquid Chromatography
Rpm	:	Rotations per Minute
R_s	:	Resolution
RSD	:	Relative Standard Deviation
Rt or t_R	:	Retention time
S.D	:	Standard Deviation
S.E	:	Standard Error
USP	:	United States Pharmacopoeia
UV-VIS	:	Ultraviolet –Visible
v/v	:	Volume/Volume

INTRODUCTION

1.1 ANALYTICAL CHEMISTRY [1]

Everything is made of chemicals Analytical chemistry determine what and how much .In other words analytical chemistry is concerned with the separation, identification, and determination of the relative amounts of the components making up a sample of matter Analytical chemistry is concerned with the chemical characterization of matter and the answer to two important questions what it **(qualitative)** is and how much is it **(quantitative)**

A) Qualitative Qualitative Analysis refers to analyses in which substances are identified or classified on the basis of their chemical or physical properties, such as chemical reactivity, solubility, molecular weight, melting point, radioactive properties (emission, absorption), mass spectra, nuclear half-life, etc Chemical tests there are numerous qualitative chemical tests, for example, the acid test for gold and the Kastle-Meyer test for the presence of blood .

B) Quantitative. In quantitative chemical analysis, a sample is prepared and then analyzed to determine the concentration of one (or more) of its components, Gravimetric Methods – the mass of the analyte or some compound produced from the analyte was determined, Titrimetric Methods – the volume or mass of a standard reagent required to react completely with the analyte was measured. Titration involves the addition of a reactant to a solution being analyzed until some equivalence point is reached. Often the amount of material in the solution being analyzed may be determined.

Classification of Analytical Techniques: [2]

I) The type of analytical technique – classical or instrumental techniques;

II) The nature of the measurement data generated – single-channel or multi-channel

III) Quantitation method (by which the analyte concentration is calculated) – relative or absolute techniques

The type of analytical technique – classical or **instrumental techniques**

Classical methods in classical analysis, the signal depends on the chemical properties of the sample: a reagent reacts completely with the analyte, and the relationship between the measured signal and the analytical concentration is determined by chemical stoichioimetry Classical methods are most useful for accurate and precise measurements of analyte concentrations at the 0.1% level or higher Mass, volume, and charge are the most common signals for classical techniques, and the corresponding techniques are Coulometeric techniques. Classical methods of analysis are considered absolute techniques, because there is a direct and simple relationship between the signal (mass in gravimetry; endpoint volume in titrimetry) andthe analyte concentrations in the sample.

Precipitation, Extraction, Distillation

Instrumental methods [3] Analysis some physical property of the sample is measured, such as the electrical potential difference between two electrodes immersed in a solution of the sample, or the ability of the sample to absorb light. Some specialized instrumental techniques are capable of detecting individual atoms or molecules in a sample Analysis at the ppm (ug/mL) and even ppb (ng/mL) level is routine an instrumental method encompasses the use of more complicated

instrumentation based on analytical methods. The sampling, dissolution, change in oxidation state, removal of excess reagent, pH adjustment, addition of complexing agent, precipitation, concentration and the removal of interferences are the various chemical steps which are part of an instrumental method

Table. 1 Shows various Instrumental methods analysis

Spectrophotometry techniques	**Electrochemical techniques**	**Chromatographic techniques**	**Miscellaneous techniques**	**Hyphenated techniques**
Colorimetry UV-Visible Fluorescence Phosphorescence Atomic spectrometry IR spectrometry X-ray techniques NMR	Conductometry Potentiometry Coulometry Voltamery Electro gravimetry	TLC GC HPLC	Thermal analysis Mass-spectroscopy	LC- MS GC- MS LC- NMR LC-MS/MS

a) Spectrophotometry techniques

b) Electrochemical techniques

c) Chromatographic techniques

d) Miscellaneous techniques

Thermal analysis, Mass spectroscopy

e) Hyphenated techniques

LC MS, GC MS

a) Spectrophotometry techniques It measures the interaction of the molecules with Electromagnetic radiation. Spectrochemical methods of analysis, in which the analyte interacts with electromagnetic radiation. Most of the methods in this category are based on the measurement of the amount of light absorbed by a

sample; such absorption-based techniques include atomic absorption, molecular absorption, and NMRmethods. The rest of the methods are generally based on the measurement of light emitted or scattered by a sample; these emission-based techniques include atomic emission, molecular fluorescence, and Raman scatter methods Spectroscopy consists of many different applications such as atomic absorption spectroscopy, atomic emission spectroscopy, ultraviolet-visible spectroscopy, x-ray fluorescence spectroscopy, infraredspectroscopy, Ramanspectroscopy, Dualpolarization interferometer, nuclear magnetic resonance spectroscopy, photoemission spectroscopy, colorimetry, NMR. Scatter methods.

b) Electrochemical techniques Electro analytical methods measure the potential (volts) and/or current (amps) in an electrochemical cell containing the analyte. These methods can be categorized according to which aspects of the cell are controlled and which are measured. The three main categories are potentiometric (the difference in electrode potentials is measured), and voltammeter (the cell's current is measured while actively altering the cell's potential, conductometry. In voltammetric analysis, the analyte is part of an electrolytic cell. Current flows when voltage is applied to the cell due to the participation of the analyte in a redox reaction the conditions of the electrolytic cell are such that the magnitude of the current is directly proportional to the concentration of analyte in the sample solution.

c) Chromatographic technique Thin layer chromatography Separation processes are used to decrease the complexity of material mixtures. Chromatography, electrophoresis TLC, GC, HPLC.

d) Miscellaneous techniques

Mass spectrometry Anaccelerator mass spectrometer used for radiocarbon dating and other analysis. Mass spectrometry measures mass-to-charge ratio of molecules using electric and magnetic fields. There are several ionization methods: electron impact, chemical ionization, electro spray, fast atom bombardment, matrix assisted laser desorption ionization, and others. Also, mass spectrometry is categorized by approaches of mass analyzers: magnetic-sector, quadrupole mass analyzer, quadruple ion trap, time-of-flight. Mass spectroscopy is a powerful method for analysis in which the analyte isionized and subsequently detected. Although in common usage, the term “spectroscopy” is not really appropriate to describe this method, since electromagnetic radiation is not usually involved in mass spectroscopy.

Thermal analysis Calorimetry and Thermogravimetric analysis measure the interaction of a material and heat

e) Hyphenated techniques Combinations of the above techniques produce a "hybrid" or "hyphenated" technique several examples are in popular use today and new hybrid techniques are under development example gas chromatography mass spectrometry Liquid chromagraphy infrared spectroscopy and capillary electrophoresis- mass spectrometry. Hyphenated separation techniques refer to a combination of two (or more) techniques to detect and separate chemicals from solutions. Most often the other technique is some form of chromatography. Hyphenated techniques are widely used in chemistry and biochemistry. A slash is sometimes used instead of Hyphen, especially if the name of one of the methods contains a hyphen itself.

II) The nature of the measurement data generated single-channel or **multi-channel**

Although they do not actually generate a signal in and of themselves, some of the more sophisticated separation techniques are usually considered "instrumental methods." These Techniques include chromatography and electrophoresis. These techniques will separate a Chemical sample into its individual components, which are then typically detected by one of the methods listed above.

a) single-channel techniques will generate but a single number for each analysis of the sample. Examples include gravimetric and potentiometric analysis. In the former, the signal is a single mass measurement (e.g., mass of the precipitate) and in the latter method the signal is a single voltage value.

b) Multi-channel techniques will generate a series of numbers for a single analysis. Multi-channel techniques are characterized by the ability to obtain measurements while changing some independently controllable parameter. For example, in a molecular absorption method, an absorption spectrum may be generated, in which the absorbance of a sample is monitored as a function of the wavelength of the light transmitted through the sample. Measurement of the sample thus produces a series of absorbance values. It is the determination of the absolute or relative abundance (often expressed as a concentration) of one, several or all particular substance(s) present in a sample.

III) Quantitation method (by which the analyte concentration is calculated) – relative or absolute techniques

a) In absolute analytical techniques, the analyte concentration can be calculated directly from measurement of the sample. No additional measurements are required.

b) In relative analytical techniques, the measurement of the sample must be compared to measurements of additional samples that are prepared with the use of analyte standards Knowing the composition of a sample is very important and several ways have been developed to make it possible, like Gravimetric and volumetric analysis. Volumetric analysis can be simply a titration based in a neutralization reaction but it can also be a precipitation or a complex forming reaction as well as a titration based in a Redox reaction, Argentometry titration

Analytical Chemistry Applied to: [4]

In medicine, analytical chemistry is the basis for clinical laboratory tests which help Physician's diagnosis disease and chart progress in recovery.

In industry, analytical chemistry provides the means of testing raw materials and for assuring the quality of finished products whose chemical composition is critical. Many household products, fuels, paints, pharmaceuticals, etc. are analyzed by the Procedures developed by analytical chemists before being sold to the consumer.

Environmental quality is often evaluated by testing for suspected contaminants using the techniques of analytical chemistry.

Bioanalytical chemistry and analysis - detection and/or analysis of biological Components (i.e., proteins, DNA, RNA, carbohydrates, metabolites, etc.).

In pharmacy sciences. Pharmaceutical chemistry, Pharmaceutical industry (quality control). Analytical toxicology is concerned with the detection, identification and measurement of drugs and other foreign compounds (and their

metabolites in biological and related specimens, Natural products detection, isolation, and structural determination.

1.2 UV-VISIBLE SPECTROSCOPY

Ultraviolet–visible spectroscopy or ultraviolet-visible spectrophotometry (**UV-Vis**) [5] Refers to absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region. This means it uses light in the visible and adjacent (near-UV and near-infrared (NIR)) ranges. The absorption or reflectance in the visible range directly affects the perceived color of the chemicals involved. In this region of the electromagnetic spectrum, molecules undergo electronic transitions. This technique is complementary to fluorescence spectroscopy, in that fluorescence deals with transitions from the excited state to the ground state, while absorption measures transitions from the ground state to the excited state Colorimetric is the determination of the light absorbing capacity of a system. A quantitative determination is therefore, carried out by subjecting a colored solution to those wavelengths of visible energy which are absorbed by that solution. UV and visible absorption bands are due to electronic transitions in the region of 200nm nm to 780 nm.

Figure: 1. Shows Beckman DU640 UV/Vis spectrophotometer

Principle of ultraviolet-visible absorption- Molecules containing π-electrons or non-bonding electrons (n-electrons) can absorb the energy in form of

UV/Vis to excite these electrons higher anti-bonding molecular orbitals The more easily excited the electrons the longer the wavelength of light it can absorb. UV and visible absorption bands are due to electronic transitions in the region of 200 nm to 780 nm organic molecules, electronic transitions be s, p or n electron transitionfromthegroundstatetoanexcitedstate(s*,p*n*)

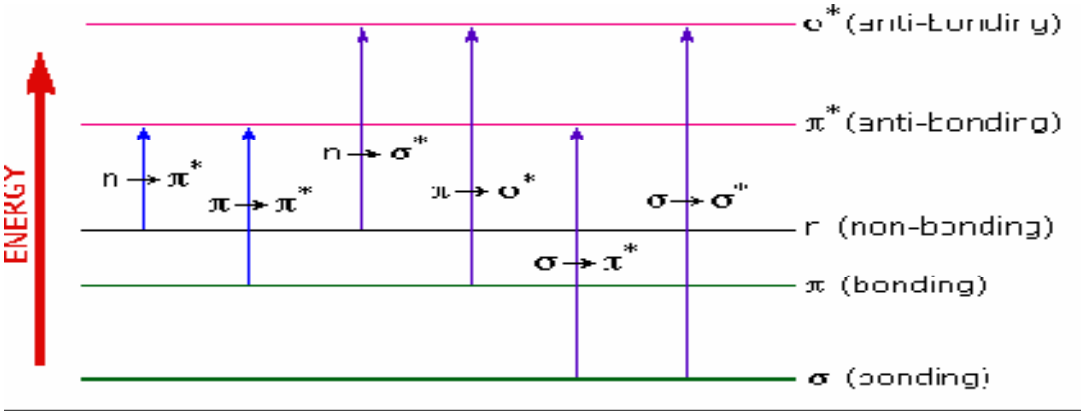


Figure: 2. Shows four types of absorption bands that occur due to the electronic transition of a molecule

Beer-Lambert's law

When light (monochromatic or heterogeneous) falls upon a homogeneous medium, a portion of the incident light is reflected, a portion is absorbed within the medium and the remainder is transmitted. If the intensity of the incident light is expressed by I, that of the absorbed light by I_a, that of the transmitted light by I_t, and that of the reflected light by I_r, then

$$I = I_a + I_t + I_r \text{..... (1)}$$

The change of absorption of light with the thickness of the medium is frequently given to Lambert, Beer later applied similar experiments to solutions of different concentrations and published his results, the two separate laws governing

absorption are usually known as Lambert's law and Beer's law. In the form they are referred to as the Beer-Lambert law mathematically, the radiation-concentration and radiation-path-length relation can be expressed by

$$\frac{dI}{dc} = -k_1 I \text{ and } \frac{dI}{dl} = -k_2 I \quad \text{..... (2)}$$

$$\text{Log } (I/I_O) = \hat{I} \times c \times l \text{..... (3)}$$

Where,

- I= intensity of the incident energy
- I_O=intensity of the emergent energy
- c= concentration(moles/L)
- l= thickness of the absorber (in cm)
- Î=molar absorptivity for concentration in moles/L

$E^{1\%}_{1cm}$, which is encountered less frequently in the literature, represents a concentration of 1% w/v and 1 cm cell thickness and is used primarily in the investigation of those substances of unknown or undetermined molecular weight. A typical UV absorption spectrum, shown in fig. 1, is the result of plotting wavelength v/s absorptivity, I_{max} is denoted by l_{max}.

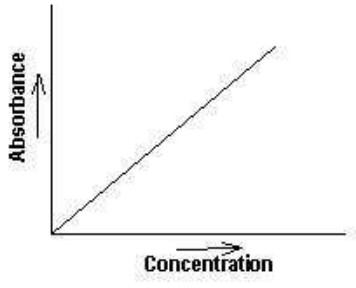


Figure: 3. Shows a representative Beer-Lambert law plot

Instrumentation of Ultraviolet–visible spectroscopy Instruments for measuring the absorption of U.V. or visible radiation are made up of the following components.

a) Sources (UV and visible)

b) Wavelength selector (monochromator)

c) Sample containers

d) Detector

e) Signal processor and readout

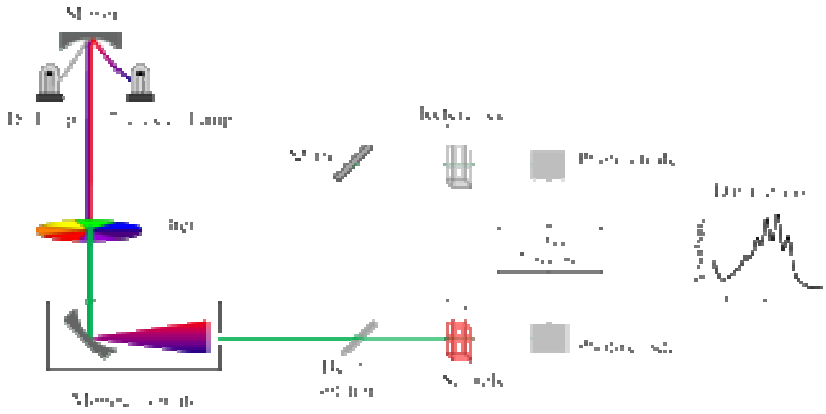


Figure: 4. Shows Schematic of UV- visible spectrophotometer [6]

a) Sources of UV radiation [7] The electrical excitation of deuterium or hydrogen at low pressure produces a continuous UV spectrum. The mechanism for this involves formation of an excited molecular species, which breaks up to give two atomic species and an ultraviolet photon, both deuterium and hydrogen lamps emit radiation in the range 160 - 375 nm. Quartz windows must be used in these lamps, and quartz Cuvettes must be used, because glass absorbs radiation of wavelengths less than 350 nm.

Sources of visible radiation The tungsten filament lamp is commonly employed as a source of visible light. This type of lamp is used in the wavelength range of 350 - 2500 nm. The energy emitted by a tungsten filament lamp is proportional to the fourth power of the operating voltage.

b) Wavelength selector (monochromator) All monochromator contain the following component parts; An entrance slit ,A collimating lens ,A dispersing device (usually a prism or a grating) , a focusing lens , an exit slit, Polychromatic radiation (radiation of more than one wavelength) enters the monochromator through the entrance slit. The beam is collimated, and then strikes the dispersing element at an angle. The beam is split into its component wavelengths by the grating or prism. By moving the dispersing element or the exit slit, radiation of only a particular wavelength leaves the monochromator through the exit slit

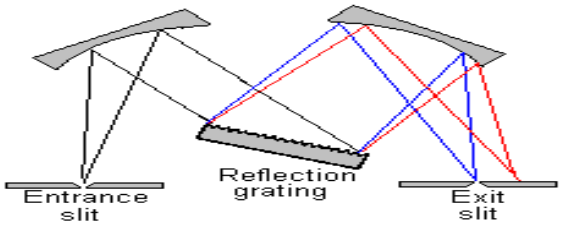


Figure: 5. Shows Czerney Turner grating monochromator

c) Cuvettes The containers for the sample and reference solution must be transparent to the radiation which will pass through them. Quartz or fused silica Cuvettes are required for spectroscopy in the UV region. These cells are also transparent in the visible region. Silicate glasses can be used for the manufacture of Cuvettes for use between 350 and 2000

d) Detectors [8]

The photomultiplier tube The photomultiplier tube is a commonly used detector in UV spectroscopy. It consists of a photo emissive cathode (a cathode which emits electrons when struck by photons of radiation), several dynodes (which emit several electrons for each electron striking them) and an anode. A photon of radiation entering the tube strikes the cathode, causing the emission of several electrons. These electrons are accelerated towards the first dynode (which is 90V more positive than the cathode). The electrons strike the first dynode, causing the emission of several electrons for each incident electron. These electrons are then accelerated towards the second dynode, to produce more electrons which are accelerated towards dynode three and so on. Eventually, the electrons are collected at the anode. By this time each original photon has produced 106 - 107 electrons. The resulting current is amplified and measured.

Photomultipliers are very sensitive to UV and visible radiation. They have fast response times. Intense light damages photomultipliers they are limited to measuring low power radiation.

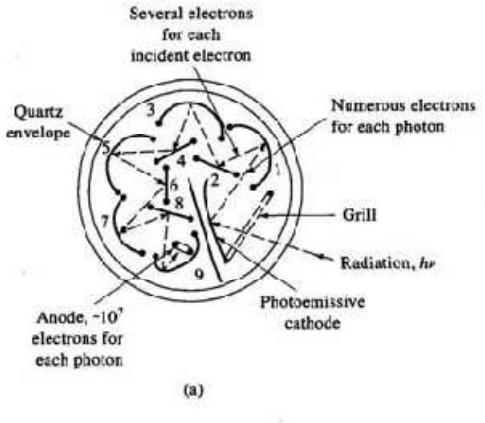


Figure: 6. Shows photomultiplier tube detector

b) Phototube A phototube consists of an evacuated glass bulb. There is light sensitive cathode inside it. The inner surface of cathode is coated with light sensitive layer such as potassium oxide and silver oxide. When radiation is incident upon a cathode, photoelectrons are emitted. These are collected by an anode. Then these are returned via external circuit. And by this process current is amplified and recorded

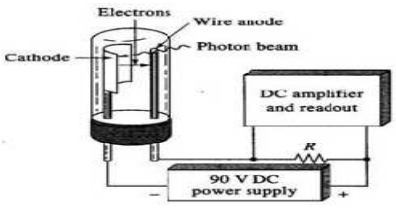


Figure 7-27 A phototube and accessory circuit. The photocurrent induced by the radiation causes a potential drop across *R*, which is then amplified to drive a meter or recorder.

Figure: 7. Shows Phototube detector

c) Photovoltaic cell is also known as barrier layer it consists of a metallic base plate like iron or aluminium which acts as one electrode. On its surface, a thin layer of a semiconductor metal like selenium is deposited. Then the surface of selenium is covered by a very thin layer of silver or gold which acts as a second collector tube. When the radiation is incident upon the surface of selenium, electrons are generated at the selenium- silver surface and the electrons are collected by the silver. This accumulation at the silver surface creates an electric voltage difference between the silver surface and the basis of the cell.

Applications [9] UV/Vis spectroscopy is routinely used in analytical chemistry for the quantitative determination of different analytes, such as transition metal ions, highly conjugated organic compounds, and biological macromolecules. Spectroscopic analysis is commonly carried out in solutions but solids and gases may also be studied. Solutions of transition metal ions can be colored (i.e., absorb visible light) because d electrons within the metal atoms can be excited from one

electronic state to another. The colour of metal ion solutions is strongly affected by the presence of other species, such as certain anions or ligands. For instance, the colour of a dilute solution of copper sulfate is a very light blue; adding ammonia intensifies the colour and changes the wavelength of maximum absorption (λ_{max}).

1.3 CHROMTOGRAPHY [10]

Chromatography is the ability to separat**e** molecules using partitioning characteristics of molecule to remain in a stationary phase versus a mobile phase. Once a molecule is separated from the mixture, it can be isolated and quantified.

Types of chromatographic techniques: Thin layer chromatography

- Paper chromatography
- Column chromatography
- Gas chromatography
- HPTLC
- HPLC

HPLC (High-performance liquid chromatography) [11]

HPLC is a technique in analytic chemistry used to separate the components in a mixture, to identify each component, and to quantify each component. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column. HPLC has been used for medical, legal, research and manufacturing.

Figure: 8. Shows High-performance liquid chromatography instruments

Classification of HPLC techniques:

Based on modes of chromatography

Based on principle of separation Based on elution technique

Table 2 shows Different modes of chromatography are as follows

Based on Modes of chromatography	**Based on Principle of separation**	**Based on Elution technique**
Normal phase Reversed Phase	Adsorption Ion Exchange Size Exclusion Chiral phase	Isocratic Gradient

I) Modes of Chromatography: Modes of chromatography are defined essentially according to the nature of the interactions between the solute and the stationary phase, which may arise from hydrogen bonding, Vander walls forces, electrostatic forces or hydrophobic forces or basing on the size of the particles (e.g. Size exclusion chromatography).

a) Normal phase-in a very non-polar environment, hydrophilic molecules will tend to associate with each other (like water drops on an oily surface). The hydrophilic molecules in the mobile phase will tend to adsorb to the surface on the inside and outside of a particle if that surface is also hydrophilic. Increasing the

polarity of the mobile phase will subsequently decrease the adsorption and ultimately cause the sample molecules to exit the column. This mechanism is called Normal Phase Chromatography.

b) Reversed Phase The opposite of normal phase, or Reversed Phase Chromatography, results from the adsorption of hydrophobic molecules onto a hydrophobic solid support in a polar mobile phase. Decreasing the mobile phase polarity by adding more organic solvent reduces the hydrophobic interaction between the solute and the solid support resulting in de-sorption. The more hydrophobic the molecule the more time it will spend on the solid support and the higher the concentration of organic solvent that is required to promote de-sorption.

II) Principle of seperation

a) Adsorption chromatography Chromatography in which separation is based mainly on differences between the adsorption affinities of the sample components for the surface of an active solid.

b) Partition chromatography Chromatography in which separation is based mainly on differences between the solubility of the sample components in the stationary phase (gas chromatography), or on differences between the solubilities of the components in the mobile and stationary phases (liquid chromatography).

c) Ion-exchange chromatography Chromatography in which separation is based mainly on differences in the ion-exchange affinities of the sample components. Anions like SO3- or cations like N (CH3)3+ are covalently attached to stationary phase, usually a resin.

d) Molecular exclusion chromatography A separation technique in which separation mainly according to the hydrodynamic volume of the molecules or particles takes place in a porous non-adsorbing material with pores of approximately the same size as the effective dimensions in solution of the molecules to be separated.

e) Affinity chromatography The particular variant of chromatography in which the unique biological specificity of the analyte and ligands interaction is utilized for the separation.

III) Based on elutation technique

Isocratic, gradient Isocratic versus Gradient Elution: Elution techniques are methods of pumping mobile phasethrough a column. In the isocratic method, the composition ofthe mobile phase remains constant, whereas in the gradient Method the composition changes during the separationprocess. The isocratic method is the simplest technique andshould be the first choice when developing a separation. Eluentgradients are usually generated by combining the pressurizedflows from two pumps and changing their individual flowrates with an electronic controller or data system while maintainingthe overall flow rate constant.

Instrumentation of HPLC

Components of HPLC

a) Mobile Phase Reservoirs and Pre-treatment system

b) Solvent Delivery System

c) Pump

d) Sample Injection System

e) Columns

f) Detectors

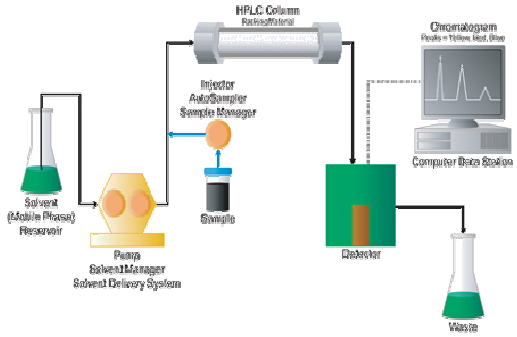


Figure: 9. Shows Schematic representation of an HPLC unit

a) Mobile Phase Reservoirs and Pre-treatment system a good HPLC unit should have 3-4 solvent reservoirs to release eluent into a mixing chamber at varying rate. Inert container for holding the solvent (mobile phase)

b) Solvent delivery systems Solvent delivery systems continuously provide eluent (solvent). Provide accurate mobile phase compositions. Includes solvent reservoirs, inlet filter, and degassing facilities which works in composition.

Inlet Filters: Inlet Filters Type of filter. Stainless Steel or glass with 10 micron porosity. Removes particulates from solvent.

Degassing System Degassing System Removed dissolved gases (such as oxygen and nitrogen). May consist of vacuum pump system, a distillation system, a heating and stirring device, or a system for spearing.

VacuumfiltrationIt can remove the air bubbles. But it is not always reliable and complete.

Heliumpurging Passage of helium through the solvent. This is very effective but Helium is expensive.

Ultrasonicator By using ultrasonicator, this converts ultra-high frequency to mechanical vibrations. This causes the removal of air bubbles.

c) Pumping System Pumping System C constant, reproducible, and pulse free supply of eluent to the HPLC column. Flow rate in between 0.1-10 cm 3 min -1 .Operating pressures from 3000 psi to 6000 psi.

Types of Pumping System Types of Pumping System Mainly three types Constant flow reciprocating pump Syringe (or displacement) type pump. Pneumatic (or constant pressure) pump.

Constant flow reciprocating pump Constant flow reciprocating pump the term "reciprocating" describes any continuously repeated backwards and forwards motion. Widely used (~90% in HPLC system) type of pump. It gives a pulsating delivery of the eluent. Pulse damper is used to make the flow pulse free. Deliver solvent(s) through reciprocating motion of a piston in a hydraulic chamber. Solvent is sucked during back stroke and gets deliver to the column in forward stroke. Flow rates of eluent can be set by adjusting piston displacement in each stroke.

Syringe (or displacement) type pump Syringe (or displacement) type pumps Consist of large syringe like chamber (capacity up to 500 Cm Plunger activated by screw-driven and hydraulic amplifier machine. Suitable for small bore column.

Pneumatic Pump Pneumatic Pump Gas is used to pressurize the mobile phase present in a collapsible solvent container Advantages: Not very costly. Provide pulse free flow.

d) Sample Injection system Sample Injection systems introduce required sample volume accurately into the HPLC system .Introduction of sample without depressurizing the system. Volume of sample must be very small (2 µL to 500 µL). Types of injection system: Manual injection (Rheodyne / Valco injectors) Automatic injection**.**

Manual Injector Manual Injector Also known as Rheodyne / Valcoinjectors. User manually loads sample into the injector using a syringe. Overloading of column causes band broadening hence volume used must be very small (2 µL to 500 µL). Sample should be introduced without depressing the system.

Automatic Injector System Automatic Injector System Also known as Autosampler. Programmed based sample delivery system. User loads vials filled with sample solution into the Autosampler tray (100 samples). Autosampler automatically 1.Measures the appropriate sample volume, 2.Injects the sample, 3. F lushes the injector to be ready for the next sample, etc., until all sample vials are processed. Also controls the sequence of samples for injection from vials.[12]

e) HPLC Columns Columns Material Stainless steel (highly polished surface) External diameter 6.35 mm Internal diameter: 4-5 mm (usual 4.6 mm) Length: 10-30 cm (usual 25 cm) Packing particles size (3 µm, 5µm, 10 µm) Stainless steel frits or mess discs (porosity <2 µm) retain packing material Efficiency or performance of a column may be measured by fallowing expression N = 16(V R /W B) 2 …(a) H = L/N …(b) V R = Retention volume of the solute W B = Volume occupied by a solute (For efficient column W B < V R) N = Plate number of the column (dimensionless) H = Height of the column (mm × µm) L = Length of the column (cm) For more efficient column 'N' should be larger and

correspondingly 'H' gets smaller. Factors affecting efficiency of column: Particle size Flow rate Thickness of stationary phase Mobile phase viscosity Diffusion of solute in mobile and stationary phases How well a column is packed For prolonged life of HPLC columns Guard column Scavenger column thermostats Guard column: Also know as pre-column. Placed in between injector and analytical column. Having same material as in column but with larger size particles ~ 30-40 µm. Scavenger column: Place between the pump and injection valve. Saturate the aqueous eluent (specially high or low pH buffers) with silica. Column thermostats: HPLC is performed at ambient temperature in number of cases. Controls temperature of the column for better resolutions (chromatograms). HPLC is performed at ambient temperature in many cases. On the basis of chromatographic objective HPLC column can be categorized as follows: 9/22/2013 28 Scale Chromatographic Objectives Analytical Information (compound identification and concentration) Semi-preparative Data and small amount of purified compound [<0.5 g] Preparative Large amount of purified compound [>0.5 g] Process (industrial) Manufacturing quantities (g to kg)

Figure: 10. Shows Picture of HPLC column

Precolumn/Guard Column A guard column is placed between the analytical column and sample injector port and is filled with the same packing material as that of the analytical column. It protects the analytical column from damage or loss of efficiency by strongly adsorbing the particulate matter or

impurities present in the sample or the solvent. These are made up of stainless steel that helps them withstand high pressure of about 5000psi.They are usually short in length ranging between 2-20 cm.Smaller inner diameter of Precolumn gives good results and helps to increase their working efficiency.

Stationary Phase (column packing) Stationary Phase (column packing) the stationary phase is the substance fixed in place for the chromatography procedure. The stationary phase can be a solid, a liquid, or a bonded phase. Bonded phase is a stationary phase that is covalently bonded to the support particles or to the inside wall of the column tubing. Chemically-modified silica's, unmodified silica or cross-linked co-polymers of styrene and divinyl benzene, commonly used as stationary phase. Silica particles as the basis of the support. Sizes 3 µm, 5 µm, and 10 µm (spherical and regular in shape). Pore size normally in the 60 – 100 Å range. Pore size of 300 Å or larger being used for larger biomolecules. Columns are packed using high-pressure to ensure that they are stable during use. Several types of particles are used in HPLC column packing. Micro porous (or diffusive) particle/Porous microsphere Perfusion particles Nonporous (or micropellicular) Chiral (bounded) stationary phase. Thorough pore Diffusive Pore Perfusion Particle 5 µm Microporous Particle Micropellicular particles Solid core Liquid or ion exchange film Microporous (or diffusive) Particles: Main surface area is within the pores to interact with the stationary phase. Small particles reduce the diffusion path length and thereby band broadening. Zorbax Rx (Sil) (Silica sol) is a porous microsphere silica particle with 50% porosity and a pore size of 100 Å.Perfusion Particles: The particle consists of both small (diffusive) and large (through) pores in them. Diffusive pore provide sorption power. Through pore permits the mobile phase to pass directly through the packing. Slightly larger than Microporous

particles (~ 12 µm).Nonporous particles: Made from either silica or resin. Smaller in size (1.5 - 2.5 µm) with thin porous layer.

Mobile Phase Mobile Phase Also known as eluent. Solvent used in HPLC must be of HPLC grade i.e. filtered using 0.2 µ m filter. Eluting power of the mobile phase is determined by its overall polarity, stationary phase polarity and the nature of the sample components. For 'normal-phase‘ separations eluting power increases with increasing polarity of the solvent, while for 'reverse-phase ' separations eluting power decreases with increasing solvent polarity.

f) Detectors detector refers to the instrument used for qualitative and quantitative detection of analytes after separation. Monitors the eluent as it emerges from column. Establishing both the identity and concentration of eluting components in the mobile phase stream. Characteristics of detectors: Adequate sensitivity (10 -8 to 10 -15 g solute sec -1). Desired stability and reproducibility Sort response time Minimal internal volume (minimize zone broadening). Detectors Continuous Sensitivity: E expressed as the noise equivalent concentration. The solute concentration, which produces a signal equal to the detector noise level. The lower the value of for a particular solute, the more sensitive is the detector for that solute. A linear response the linear range of a detector is the concentration range over which its response is directly proportional to the concentration of solute. Type of response: Detector is either universal or selective. Universal (sense all the constituents of the sample). Selective (respond to certain components).

Refractive Index Detector The response is dependent on changes in refractive index of eluting compounds in the mobile phase. The mobile phase itself should have refractive index different from the sample. The detector is less sensitive

than UV-VIS detector. Temperature control is necessary as it has high temperature sensitivity. Typical applications are in Size Exclusion Chromatography.

Light Scattering Detectors Light scattering detectors are useful for detection of high molecular weight molecules. After removal of mobile phase by passing through a heated zone the solute molecules are detected by light scattering depending on molecular sizes band moves fastest, eluting first from the column, it is the first peak drawn. A little while later, the red band reaches the flow cell. The signal rises up from the baseline as the red band first enters the cell, and the peak representing the red band begins to be drawn. The red band has not fully passed through the flow cell the red band and red peak would look like if we stopped the process at this moment. Since most of the red band has passed through the cell, most of the peak has been drawn, as shown by the solid line. If we could restart, the red band would completely pass through the flow cell and the red peak would be completed [dotted line]. The blue band, the most strongly retained, travels at the slowest rate and elutes after the red band. **[14]**

Conductivity detectors Conductivity detectors Conductivity measurement of effluent. Mainly measure inorganic ions and small organic substances, including organic acids and amines. Conductivity detector measures electronic resistance and measured value is directly proportional to the concentration of ions present in the solution .Employed as a detector in an ion chromatography.

Ultraviolet Detector Ultraviolet Detector based on the principle of absorption of UV visible light as the effluent from the column is passed through a small flow cell placed in the radiation beam. High sensitivity (detection limit of about 1x10 -9 g mL -1 for highly absorbing compounds). Detector cells are

generally 1 mm diameter tubes with a 10 mm optical path length. UV-VIS Detector is the most commonly used detector. Its response is specific to a particular compound or class of compounds depending on the presence of light absorbing functional groups of eluting molecules. Some compounds which do not have such light absorbing groups can give suitable response after post column derivatization to introduce light absorbing entities

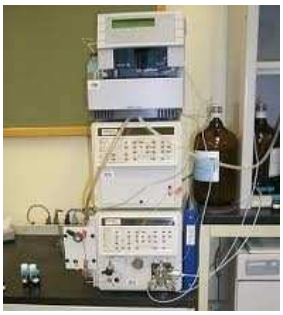


Figure: 11. Shows images of UV detectors of HPLC

Photodiode-array detectors a photodiode array (PDA) is a linear array of discrete photodiodes on an integrated circuit (IC) chip. A photodiode is a type of photo detector capable of converting light into either current or voltage, depending upon the mode of operation. Incorporation of large number of diodes which serve as detector elements makes possible simultaneous monitoring of more than one absorbing component at different wavelengths. This provides benefit of time saving and cost reduction on expensive solvent.

Fluorescence Detectors Fluorescence Detectors based on filter- fluorimeters or spectrofluorimeters. Flow cell has a capacity 10-25µL with a narrow depth (1.07 mm) and large surface area for excitation-emission collection. The fluorescent radiation emitted by the sample is usually measured at 90° to the incident beam. Simplest detector: mercury excitation source, and filters (one/more). Advanced

detector: xenon source and a grating monochromator to isolate emitted fluorescent radiation.

Electrochemical Detector Electrochemical Detector The term 'electrochemical detector' in HPLC normally refers to amperometric or coulometric detectors. Measure the current associated with the oxidation or reduction of solutes. Complete removal of oxygen is almost difficult, electrochemical detection is normally based upon the oxidation of the solute. Amperometric detector is presently considered to be the best electrochemical detector. Working electrode: Commonly made of glassy carbon, is the electrode at which the electro active solute species is monitored. Reference electrode: Usually a Ag- AgCl electrode, gives a stable, reproducible voltage to which the potential of the working electrode is referred. Auxiliary electrode: current-carrying electrode and usually made of stainless steel. Potentiostat from Column to waste Auxiliary Electrode Reference Electrode Working Electrode Electro Chemical Detector.

Mass Spectroscopic Detector Mass spectroscopy offers very high sensitivity and selectivity. Detection is based on fragmentation of molecules by electric fields and separation on basis of mass to charge ratios of fragmented molecules. LC –MS technique has opened up new application areas due to advantages of resolution and sensitivity.

Applications of HPLC Used for both qualitative and quantitative analyses of environmental, pharmaceutical, industrial, forensic, clinical, and consumer product samples. A few typical examples: I solation of natural pharmaceutically active compounds control on microbiological processes Assay of cephalosporin assay of frusemide Assay of theophylline Assay of corticosteroids assay of

dichlorphenamide Assay of human insulin.

1.4 ANALYTICAL METHOD DEVELOPMENT

Method development [12] : The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitorsIn general, methods for regulatory submission must include studies on specificity, linearity, accuracy, precision, range, detection limit, quantization limit, and robustness which ensure that the analytical methodology in question gives timely, accurate, reproducible and reliable data which are adequate for the intended purpose of useThe evolutionary process of HPLC and UPLC method development and utilization at each stage of the drug development process is to fulfil the scientific, regulatory, and business needs of a pharmaceutical company. [15]

Early Phase Method Development

The primary goal of early phase method development is to gain a fundamental knowledge of the chemistry of drug substances and drug products to facilitate optimization of synthetic schemes and drug product formulations. At the same time, methods are required for release and stability studies to support clinical trials and must assure that products are safe in vivo.

Late Phase Method Development

The late-phase methods are filed with regulatory authorities and are used for stability studies and for the release of the drug product (DP) or drug substance (DS) validation batches. For release testing of production batches, the methods are generally transferred to the operational quality control (QC) laboratories. In most cases, the development group tries to complete its task as well as possible with the talent and time available to them. The method is validated according to standard operating procedures and transferred to the application labs.

Method development is done

1) For new products
2) For existing products

Methods are developed for new products when no official methods are available. Alternate methods for existing (non-pharmacopoeial) products are developed to reduce the cost and time for better precision and ruggedness. Trial runs are conducted, method is optimized and validated. When alternate method proposed is intended to replace the existing procedure, comparative laboratory data including merit/demerits are made available.

Steps of Method Development

Documentation starts at the very beginning of the development process, a system for full documentation of the development studies must be established. All data relating to these studies must be recorded in laboratory notebook or an electronic database.

The analytical method development consists of following steps: [13]

- Analyte standard characterization
- Literature review
- Define the goal of method
- Choosing a method
- Instrumental setup and initial studies
- Optimization
- Method development
- Validation as per ICH guidelines
- Documentation of analytical figures of merit

Analyte standard characterization:

- All information about the standard analyte will be obtained
- The standard analyte (100% purity) is obtained and properly stored.
- When multiple components are to be analyzed in the sample matrix, the number of components is noted, data is assembled and the availability of standards for each one is determined.
- Only those methods (MS, GC, HPLC etc.,) that are compatible with sample stability are considered.

Literatures search and prior methodology:

- The information related to the analyte is surveyed for synthesis, physical and chemical properties, solubility and relevant analytical methods.
- Books, periodicals and USP/NF, and publications are reviewed.

Method requirements:

- The goals of the analytical method that need to be developed are considered.
- The detection limits, selectivity, linearity, range, accuracy and precision are defined**.**

Choosing a method:

- Using the information in the literatures, methodology is adapted.
- The methods are modified wherever necessary.
- If there are no prior methods for the literature, the compounds that are similar in structure and chemical properties are investigated and are worked out.

Instrumental setup and initial studies:

- The required instrumentation is setup Installation, operational and performance Qualifications of instrumentation verified by using laboratory SOP's.
- Always new solvents, filters are used, for example, method development is never started, on a HPLC column that has been used earlier.
- The analyte standard in a suitable injection/introduction solution and in known concentrations and solvents are prepared.
- Analysis is done using analytical conditions described in the existing literature.

Optimization:

- During optimization one parameter is changed at a time, and set of conditions are isolated, rather than using a trial and error approach.

- Work has been done from an organized methodical plan, and every step is documented in a lab notebook

Documentation of analytical figures of merit:

- The originally determined analytical figures of merit limit of quantitation (LOQ), limit of detection (LOD), linearity, time per analysis, cost, sample preparation etc., are documented.

Evaluation of method development with actual samples:

- The sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all other matrix components**.**

Determination of percent recovery of actual sample and demonstration of quantitative sample analysis:

- Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined.
- Reproducibility of recovery (average+/- standard deviation) from sample to sample and whether recovery has been optimized.
- It is not necessary to obtain 100% recovery as long as the results are reproducible and known with a high degree of certainty.
- The validity of analytical method can be verified only by laboratory studies.
- Therefore documentation of the successful completion of such studies is a basic requirement for determining whether a method is suitable for its intended applications

1.5 ANALYTICAL METHOD VALIDATION

Validation [14] : Validation may be defined as a systematic study which helps to prove that the systems and process perform their job adequately & consistent by as specified.

Objective of Validation The primary objective of validation is to form a basis for written procedures for production and process control which are designed to assure that the drug products have the identity, strength, quality and purity they purport or are represented to process. Quality, safety and efficacy must be designed and built into the products. Each step of the manufacturing process must be controlled to maximize the probability that the finished product meets all quality and design specifications. [18]

Steps followed for validation procedures

- Proposed protocols or parameters for validations are established.
- Experimental studies are conducted.
- Analytical results are evaluated
- Statistical evaluation is carried out.
- Report is prepared documenting all the results.

According to ICH, typical analytical performance characteristics that should be considered in the validation of the types of methods are

a) Accuracy

b) Precision

c) Linearity Range

d) Limit of Detection and limit of Quantitation

e) Selectivity and Specificity

f) Ruggedness

g) Robustness

h) System suitability

(a) Accuracy: -The accuracy of an analytical method may be defined as the closeness of the test results obtained by the method to the true value. It is the measure of the exactness of the analytical method developed.

(b) Precision: -The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of homogenous samples. This is usually expressed as the standard deviation or the relative standard deviation (coefficient of variation). Precision is a measure of the degree of reproducibility or of the repeatability of the analytical method under normal operating circumstances.

Determination of Repeatability- Repeatability can be defined as the precision of the procedure when repeated by same analyst under the same operating conditions (same reagents, equipment, settings and laboratory) over a short interval of time.

Determination of reproducibility- Reproducibility means the precision of the procedure when it is carried out under different conditions-usually in different laboratories-on separate, putatively identical samples taken from the same homogenous batch of material. Comparisions of results obtained by different analysts, by the use of different equipments, or by carrying out the analysis at different times can also provide valuable information.

(c) Linearity and range:-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 analyte concentration in samples within a given range. The range of an analytical method is the interval between the upper and lower levels of the analyte (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written.

(d) Limit of Detection and limit of Quantitation:-

Limit of detection- The limit of detection is the parameter of limit tests. It is the lowest level of analyte that can be detected, but not necessarily determined in a quantitative fashion, using a specific method under the required experimental conditions.

Limit of quantitation- Limit of quantitation is a parameter of quantitative assays for low levels of compounds in sample matrices such as impurities in bulk drugs and degradation products in finished pharmaceuticals. The limit of quantitation is the lowest concentration of analyte in a Sample that may be determined with acceptable accuracy and precision when the required procedure is applied.

(e) Selectivity and Specificity:-The selectivity of an analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of components that may be expected to be present in the sample matrix. If an analytical procedure is able to separate and resolve the various components of a mixture and detect the analyte qualitatively the method is called selective. On the other hand, if the method determines or measures quantitatively the component of interest in the sample matrix without separation, it is said to be specific

(f)Ruggedness:- The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a

variety of normal test conditions such as different laboratories, different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay

(g) Robustness:-The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. The determination of robustness requires that methods characteristic are assessed when one or more operating parameter varied.

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

The ICH documents give guidance on the necessity for revalidation in the Following circumstances

- Changes in the synthesis of the drug substances.
- Changes in the composition of the drug product, and
- Changes in the analytical procedures.

DRUG PROFILE OF LUMEFANTRINE

Name of Drug **:** Lumefantrine [15, 16]

Affected organisms: caused by plasmodium falciparum

Chemical Structure:

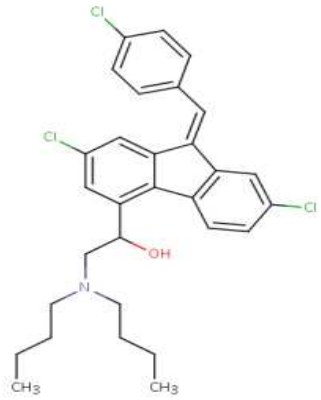


Figure: 12. Shows structure of Lumefantrine

Brand names	:	marketed by Falcidoc
Company Name	**:**	Dwarkesh pharmaceuticals pvt.ltd
Category:	:	Anti malarial
Molecular formula	:	$C_{30}H_{32}Cl_3NO$
Molecular weight	:	528.94
IUPAC Name	**:**	2-Dibutylamino-1-[2, 7-dichloro-9-(4-chlorobenzylidene)-9H-fluoren-4-yl]-ethanol
Physical properties	**:**	Yellow crystalline powder
Ph:	**:**	7.5
Pka value:	:	9.78
Protein binding	:	99.7% bound
Half life	**:**	4.5days

Solubility: practically soluble in water, free soluble in ethyl acetate, soluble in Dichloromethane, slightly soluble in ethanol.

Mechanism action of drug: Involves an interaction with ferrous ions, in the acidic parasite food vacuole, which results in the generation of cytotoxic radical species. Mechanism of action of peroxide anti malarials involves interaction of the peroxide-containing drug with heme, a hemoglobin degradation byproduct, derived from proteolysis of hemoglobin. This interaction is believed to result in the formation of a range of potentially toxic oxygen and carbon-centered radicals.

Dosage Forms and strength: 120 mg

Adult dose 4 tablets as single initial dose followed by 4 tablets after 8 hours and then 4 tablets twice a day (morning& evening) for the following 2 days total course 24 tablets.

Pediatric dose 1 tablet as single initial dose followed 1 tablet after 8 hours and then 1 tablet twice a day (morning&evening) for the following 2 days total course 6 tablets.

Storage: Lumefantrine stored in well closed container at room temperature away from heat moisture.

FDA Approval of drug: The Food and Drug Administration approved Lumefantrine in December 3, 2009 for the treatment of malaria.

Uses: Lumefantrine is an anti malarial drug, used in treatment of malaria. It also acts by killing the parasites.

Side effects: In adults: headache, vomiting, anorexia

Children: cough, vomiting

LITERATURE REVIEW

1. Rajasekarann et al, (2014) reported method development and validation for the determination Lumefantrine in sold dosage form by Rp-hplc. [17]

The mobile phase consisting of acetonitrile and methanol in ratio the of (50:50 % v/v) was delivered at the flow rate of 2.0 ml/min and diode array detection was carried out at 235 nm. C18 reverse-phase column (250 X 4.6mmI.D, particle size 5μm). The method was linear over the concentration range of 50 – 150 μg/ml for LumefantrineThe analytical recovery obtained was 99.76% the method was validated for precision, accuracy, linearity and robustness.

2. Foram D. Naik et al, (2014) reported Development and Validation of UV Spectrophotometric Methods for Simultaneous Estimation of Artemether and Lumefantrine in Synthetic Mixture and Tablet Dosage form. [18]

Simultaneous Equation method, Absorption correction method and first order derivative Spectrophotometric methods were developed and validated. Artemether linearity over the range of 3-5 μg/mL Lumefantrine showed linearity over the range of 18–30 μg/ml with r2 greater than 0.9985.The wavelengths Artemether 254nm, 254 nm and 236 nm and for Lumefantrine were 236 nm, 338 nm and 220.55 nm for The percentage recoveries of Artemether and Lumefantrine for all three methods were found to be in the range of 98.28-101.71%and 98.08-102.00% Validation of the proposed methods was carried out for its accuracy precision, linearity and robustness.

3. Mannur Vinodh et al, (2013) reported analytical method development and validation for simultaneous estimation of artemether and Lumefantrine in pure and pharmaceutical dosage form using Rp-hplc method. **[19]**

BDS Hypersil C18 (150 × 4.6 mm i.d. 3 μm particle size) column, with mobile phase comprising of 0.01M tetra butyl ammonium hydrogen sulphate and acetonitrile in the ratio of 20: 80 (v/v) The flow rate was 1.0ml/min and the detection was carried out using UV-visible detector at 222 nm Limit of detection (LOD) and limit of quantification (LOQ) The retention time were found to be 4.19 and 5.22 min for AT and LU Correlation coefficient (r2) of 0.999 for both over concentration range of 3.2-19.2μg/ml and 16-96μg/ml for AT and LU The percentage recovery for AT and LU were ranged between 99.18-100.19 and 99.96-100.07 The LOD for AT and LU were found to be 0.201 and 2.99 μg/ml and the LOQ were 0.609 and 9.086 μg/ml respectively. Method was found to be reproducible with relative standard deviation (RSD) for intra and inter day precision less than 2%. The method was validated for precision, accuracy, linearity and robustness

4. Gupta n.k. et al, (2013) reported simultaneous estimation of artemether and Lumefantrine by Rp-hplc method development in pharmaceutical tablet dosage form. **[20]**

Hypersil BDS C18, 250 × 4.6 mm, 5 μ particle size with mobile phase consisting of buffer and acetonitrile in the ratio of 50:50v/v, orthophosphoric acid used as buffer (pH 3.0 + 0.6), at a flow rate of 1.5 ml/min and eluents monitored at 215nm.. The retention times of artemether and Lumefantrine were 2.464 and 6.236 min, respectively. The calibration curves of peak area versus concentration, which was linear from 4-24μg/ml for artemether and 24-144μg/ml for Lumefantrine, had

regression coefficient (r2) greater than 0.999The method was validated for precision, accuracy, linearity and robustness.

5. Naveen S Kotur et al, (2012) reported Analytical Method Development and Validation for Estimation of Lumefantrine in Pharmaceutical Dosage Forms by HPLC. [21]

Waters Symmetry C18 (250 X 4.5 mm) analytical column with maintained the column oven temperature 35 °C and isocratic pump mode. The mobile phase compressing of water, acetonitrile and glacial acetic acid in the ratio of 48: 52: 1, v/v/with delivered the flow rate of 1.2mL /min and the detected the Lumefantrine at 276 nm from PDA detector. The retention time of Lumefantrine was 4.65 minutes. The method is linear from 10µgmL-1 to100µgmL-1 and linear correlation coefficient (R2) was more than 0.9990. The accuracy of the method by recovery was found between99.44 and 100.14 % Mean inter and intraday assay relative standard deviation (RSD) were less than 1.0%The proposed method provided an accurate and precise analysis of Lumefantrine in its Pharmaceutical dosage form

6. T.M. Kalyankar et al, (2011) reported reversed-phase liquid chromatographic method for simultaneous determination of artemether and Lumefantrine in pharmaceutical preparation.[22] Hypersil ODS C18 (250mm~4.6mm~5µ particle Size) analytical column, a mobile phase of methanol: 0.05 % trifluroacetic acid with triethylamine buffer pH 2.8 adjusted with Orthophosporic acid in ratio (80:20 v/v) flow rate of 1.5 ml/min and PDA detector wavelength at 210 nm The retention times for Artemether and Lumefantrine are 6.15 min and 11.31min, respectively The linearity range for Artemether and Lumefantrine are 20-120 & 120-720 µg/mlrespectively. The Percentage recovery for Artemether and Lumefantrine are

ranged between 99.50–101.16 and99.78–101.21. The correlation coefficients of Artemether and Lumefantrine are 0.999, and 0.999The method was validated for precision, accuracy, linearity and robustness

7. J.sunil et al, (2010) reported Hplc method development and validation for simultaneous estimation of Artemether an Lumefantrine in pharmaceutical dosage forms [23]

C18, 250 x 4.6 mm, i.d, 5µm particle size in isocratic mode, with mobile phase compressing of buffer and acetonitrile in the ratio of 40:60 (v/v), pH 3 ± 0.5. The flow rate was 1.5 ml/min and the detection was carried out by UV detector duali.e, 210 and 303 nm. The retention times were 13.887 and 7.218 mins for Artemether and Lumefantrine The percentage recovery was found to be 98.87 and 99.78 % for Artemether and Lumefantrine the method was validated for precision, accuracy, linearity and robustness.

8. B. Sridhar et al, (2010) reported a validated reverse phase hplc method for the simultaneous estimation of artemether and Lumefantrine in pharmaceutical dosage forms. [24]

C18, 250 x 4.6 mm, i.d, 5µm the mobile phase consisted of Acetonitrile: buffer (0.1% v/v ortho phosphoric acid, PH – 3) in the ratio of 60:40 v/ flow rate of 1.5 ml / min and wavelength of detection at 303 nm. The retention times of Artemether and Lumefantrine were 13.888 min and 7.207 min the proposed method can be used for determination of these drugs in combined dosage forms and the method was validated for precision, accuracy, linearity and robustness.

OBJECTIVE AND PLAN OF WORK

Aim of the study:

- To develop analytical method for Lumefantrine by RP – HPLC and to validate the developed method as per ICH & USFDA guidelines.

Objective of the work:

- ✓ Analytical methods are required to characterize drug substances and drug products composition during all phases of pharmaceutical development.
- ✓ In view of the need for a suitable method for routine analysis of Lumefantrine Bulk Drug, attempts are being made to develop simple, precise and accurate analytical methods for estimation of Lumefantrine in pharmaceutical Bulk Drug.

PLAN OF WORK

To obtain thorough knowledge in practical HPLC method development. A step-by-step procedure of method development to be implemented and initial chromatographic conditions for assay of Lumefantrine is to be established. For the initial chromatographic conditions and trails the methods to be optimized.

Plan of work was designed into following section,

- Name of category of drug
- Selection of specific category of drug
- Study about drug profile

- Review of literature
- Selection of analytical method
- Solubility study of the drug
- Selection of wavelength
- Method development

METERIALS AND METHODS

5.1. Chemicals and reagents

Table: 3. Shows chemicals used for experimental work

CHEMICAL/SOLVENT	GRADE	MANUFACTURER
Lumefantrine		Hetero Pharma
Methanol	HPLC grade	FINER chemical LTD
Acetonitrile	HPLC grade	Rankem chemicals
Purified water	HPLC grade	Rankem chemicals

5.2. Instruments

Table: 4. Shows instruments used for experimental work

S. No.	Instrument	Make& model
1	HPLC	Analytical technologies
2	UV	Elico SL-196
3	Detector	UV detector, Analytical technologies
4	Software	Analchrome, Clarity
5	Column	Hypersil ODS C18, (150 *4.6 mm , 5µ
6	Analytical balance	Digital Analytical balance Shimadzu
7	Sonicator	Analytical technologies
8	Filter	Nylon 0.45 µm

EXPERIMENTAL WORK

6.1. Determination of absorption maxima by UV/Vis Spectrophotometry

Accurately weigh 100 mg of drug in to 100 ml volumetric flask. To this add 90 ml and 10 ml of diluents (acetonitrile 90: 10 methanol) and sonicate it and further make up the volume with diluent. From this take 1 ml and make up to 10ml. The solutions were scanned in the range of 200-400 nm in 1cm cell against blank.

Figure: 13. Shows UV spectrum of Lumefantrine

6.2. Method development

Method optimization

Preparation of mobile phase: Accurately measured 90 ml of Acetonitrile and10 ml of methanol (90:10) HPLC grade were degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ nylon filter under vacuum filtration.

Diluent: Mobile phase is used as diluents

Standard preparation: Accurately weigh 50 mg of Lumefantrine and transfer in to 50ml volumetric flask. Add about 10ml of solvent mixture sonicate to dissolve. Cool the solution to room temperature and dilute to volume with solvent

mixture. Transfer 1ml of above solution in to a 10ml volumetric flask and make up the volume with diluent.

Optimized chromatographic conditions

Column - Hypersil ODS C18 (150x4.6mm, 5 µ)

Flow rate - 1. 6ml/min

Wavelength - 235nm

Column temperature - 35□c

Injection volume - 10 µl

Run time - 5 min

Percentage Assay

Inject 10 µL of the standard, sample into the chromatographic system and measure the area of Lumefantrine peaks and calculate the %Assay by using the formulae.

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. Wt}}{\text{Label Claim}} \times 100$$

Where:

AT = average area counts of sample preparation.

AS = average area counts of standard preparation.

WS = Weight of working standard taken in mg.

P = Percentage purity of working standard

LC = Label claim of tablet mg/ml.

6.3. Validation of developed RP-HPLC method:

Validation: Establishing documentation evidence, which provides a high degree of assurance that specific process, will consistently produce a product meeting its predetermined specification and quality attributes.

The following parameters were considered for the analytical method validation of Lumefantrine in bulk Dosage form.

(1) Accuracy

(2) Precision

(3) Linearity

(4) Robustness.

(5) Ruggedness

(6) Limit of detection.

(7) Limit of quantification.

1) Accuracy: For accuracy determination, three different concentrations were prepared separately i.e. 50%, 100% and 150% for the analyte and chromatograms are recorded for the same.

Preparation of 50% solution: About 25mg of Lumefantrine was weighed and transferred to 50ml volumetric flask. Add 50ml of mobile phase, Sonicate for 10min & filter through 0.45µ nylon filter & make up to the mark with same solvent. Further 3ml of above solution was diluted to 10ml with the diluents to get 50%.

Preparation of 100% solution: About 50mg of Lumefantrine was weighed and transferred to 50ml volumetric flask. Add 50ml of mobile phase, Sonicate for 10min & filter through 0.45µ nylon filter & make up to the mark with same solvent. Further 3ml of above solution was diluted to 10ml with the diluents to get 100%.

Preparation of 150% solution: About 75mg of Lumefantrine was weighed and transferred to 50ml volumetric flask. Add 50ml of mobile phase, Sonicate for 10min & filter through 0.45µ nylon filter & make up to the mark with same solvent. Further 3ml of above solution was diluted to 10ml with the diluents to get 150%.

Acceptance criteria: The method is considered accurate if the average recovery is not less than 98% and not more than 102%.

2) Precision: Accurately weigh and transfer 50 mg of Lumefantrine working standard into a 50 ml clean dry volumetric flask add diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 3 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Then the standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Acceptance criteria: RSD of six replicate injections should be NMT 2%.

3) Linearity and range: Appropriate aliquot of standard Lumefantrine stock solution was taken in 50ml volumetric flask and resultant solution was diluted up to the mark with diuent to obtain final concentration of Lumefantrine. This solution was injected into chromatographic system. The chromatograms were obtained and

peak area was determined for each concentration of drug solution. Calibration curve was constructed by plotting peak area against applied concentrations. The slope, intercept and correlation coefficient (r^2) were also determined.

Linearity of the analytical method for assay by injecting the linearity solutions prepared in the range of 100µg to 500 µg of test concentration, into the chromatograph, covering minimum 5 different concentrations. Draw a plot between the concentration vs peak response of Artemether. Report the slope, intercept and regression coefficient from the plot.

Standard Preparation:

Accurately weigh 50 mg of Lumefantrine and transfer in to 50ml volumetric flask. Add about 10 ml of solvent mixture sonicate to dissolve. Cool the solution to room temperature and dilute to volume with solvent mixture (Stock solution).

100 µg Linearity standard solution Preparation: Transfer 1ml of Linearity standard stock solution in to 10ml volumetric flask and make up to the volume with diluent and inject into the HPLC.

200µg Linearity standard solution Preparation: Transfer 2ml of Linearity standard stock solution in to 10ml volumetric flask and make up to the volume with diluent and inject into the HPLC.

300µg Linearity standard solution Preparation: Transfer 3ml of Linearity standard stock solution in to 10ml volumetric flask and make up to the volume with diluent and inject into the HPLC.

400µg Linearity standard solution Preparation: Transfer 4ml of Linearity standard stock solution in to 10ml volumetric flask and make up to the volume with diluent and inject into the HPLC.

500µg Linearity standard solution Preparation: : Transfer 5ml of Linearity standard stock solution in to 10ml volumetric flask and make up to the volume with diluent and inject into the HPLC.

Acceptance criteria: The relationship between the concentration and response of Lumefantrine should be linear in the specified range and Regression Coefficient should not be less than 0.999.

4) Robustness: As part of the Robustness, deliberate change in the temperature Variation was made to evaluate the impact on the method.

Robustness of assay method is demonstrated by changing the flow rate for 1.4 ml / min and 1.8 ml / min instead of 1.6 ml / min by injecting the 6 replicate injections of standard in 1.4 ml / min and 1.8 ml / min flow rate and found that system suitability parameters are passed.

By changing the column temperature for 30 □c and 40 □c instead of 35 □c by injecting the 6 replicate injections of standard in 30 □c and 40 □c temperature and found that system suitability parameters are passed.

Solution stability: standard solution should be tested over 24-48hrs period under normal laboratory and potency of solution should be determined by comparison to freshly prepared standards.

Acceptance criteria: The solutions are considered stable if the difference in %RSD results from initial to 24hrs and 48 hrs is NMT 2%.

Selectivity:

Blank preparation: Dilute 5 ml of diluents in to 50 ml volumetric flask and dilute to volume with mobile phase.

Placebo Preparation: Weigh accurately about 50 mg of placebo powder in a 50ml volumetric flask add 50ml diluent and sonicate for 20 minutes and cool, after cooling make up to the volume. Further dilute 0.5 ml of this solution to 10ml with diluent and inject into the chromatogram.

Standard Preparation: Accurately weigh 25 mg of Artemether and transfer in to 25 ml volumetric flask. Add about 10 ml of solvent mixture sonicate to dissolve. Cool the solution to room temperature and dilute to volume with solvent mixture. Transfer 1ml of above solution in to a 10 ml volumetric flask and dilute to volume with diluent.

Acceptance Criteria: No peak should be observed due to blank and placebo at retention time of Lumefantrine peak.

5) Ruggedness (Intermediate Precision): Intermediate Precision expresses with in laboratory variation as on different days or with different analysts or equipment with in the same laboratory. Establish the ruggedness of the analytical method by using the assay of 6 different sample preparations of same batch by a different analyst using a different HPLC System. Calculate assay for all 6 sample preparations and report the %RSD for the same.

Acceptance criteria: The RSD for the assay values of 6 sample preparations of same batch should not be more than2.0%.

6) Limit of Detection (LOD) The detection limit is determined by the analysis of samples with known concentration of analyte and by establishing that minimum level at which the analyte can reliably detected.

The LOD are calculated from the calibration curve by using the formulas

$$LOD = 3.3 \times SD / b$$

Where, SD- the estimate is the standard deviation of the peak area of the drugs. b -is slope of the corresponding calibration curve.

7) Limit of Quantification: The quantification limit is generally determined by the analysis of sample with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

The LOQ are calculated from the calibration curve by using the formulas

$$LOQ = 10 \times SD / b$$

Where, SD- the estimate is the standard deviation of the peak area of the drugs. b -is slope of the corresponding calibration curve.

RESULTS AND DISCUSSION

7.1. Method development and optimization of chromatographic parameters:

Selection of mobile phase

TRIAL 1

Mobile phase : Methanol

Flow rate : 1ml/min

Column : Hypersil ODS C_{18} (150×4.6mm, 5µ)

Detector wavelength : 266nm

Injection volume : 10µl

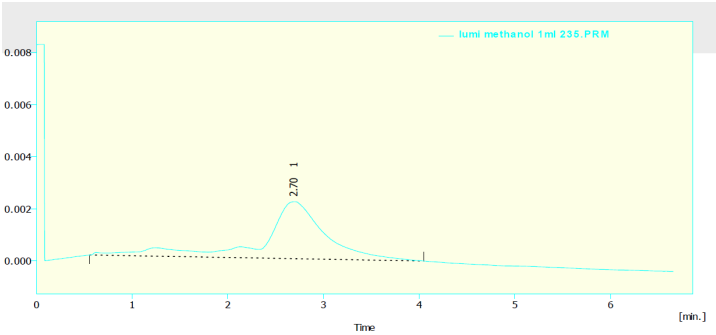


Figure: 14. Shows trail-1 chromatogram of Lumefantrine

TRIAL 2

Mobile phase	**:**	Acetonitrile
Flow rate	:	1ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm, 5µ)
Detector wavelength	:	250nm
Injection volume	:	10µl

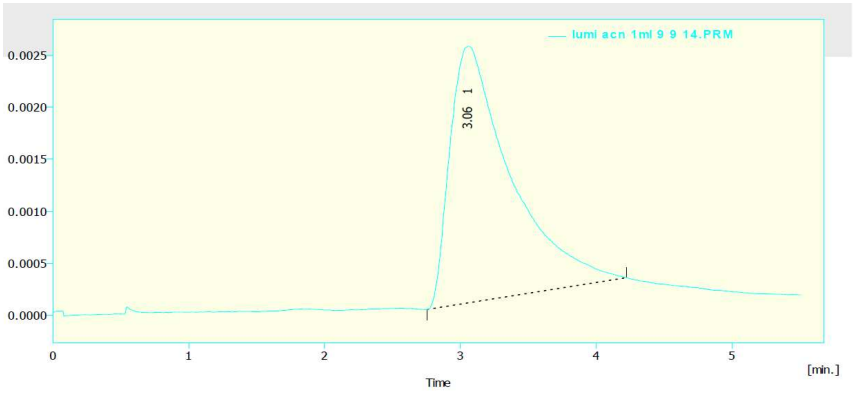


Figure: 15. Shows trail-2 chromatogram of Lumefantrine

TRIAL 3

Mobile phase	**:**	Acetonitrile
Flow rate	:	1. 2ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm,5µ)
Detector wavelength	:	250nm
Injection volume	:	10µl

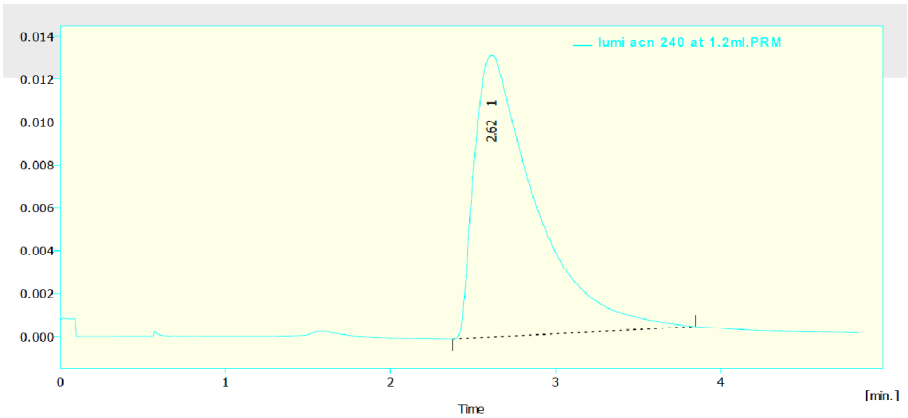


Figure: 16. Shows trail-3 chromatogram of Lumefantrine

TRIAL 4

Mobile phase	**:**	Acetonitrile
Flow rate	:	1. 4ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm,5µ)
Detector wavelength	:	250nm
Injection volume	:	10µl

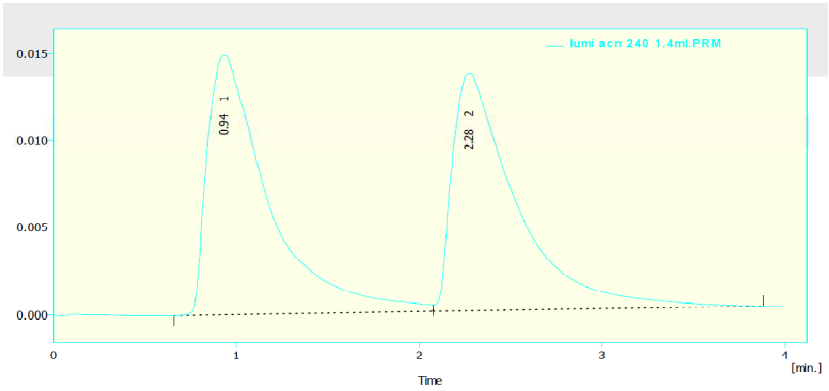


Figure: 17. Shows trail-4 chromatogram of Lumefantrine

TRIAL 5

Mobile phase	**:**	Acetonitrile
Flow rate	:	1. 6ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm,5µ)
Detector wavelength	:	250nm
Injection volume	:	10µl

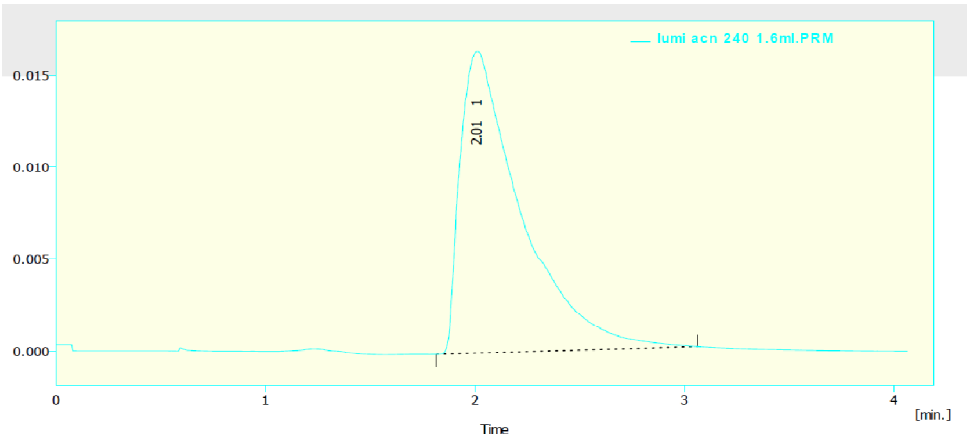


Figure: 18. Shows trail-5 chromatogram of Lumefantrine

TRIAL 6

Mobile phase	**:**	Acetonitrile, Water
Flow rate	:	1. 6ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm,5µ)
Detector wavelength	:	276nm
Injection volume	:	10µl

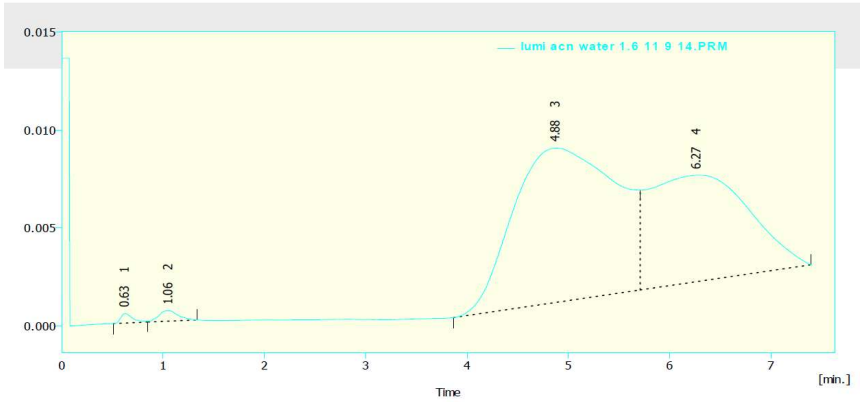


Figure: 19. Shows trail-6 chromatogram of Lumefantrine

TRIAL 7

Mobile phase	**:**	Acetonitrile, Water
Flow rate	:	1. 8ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm,5µ)
Detector wavelength	:	276nm
Injection volume	:	10µl

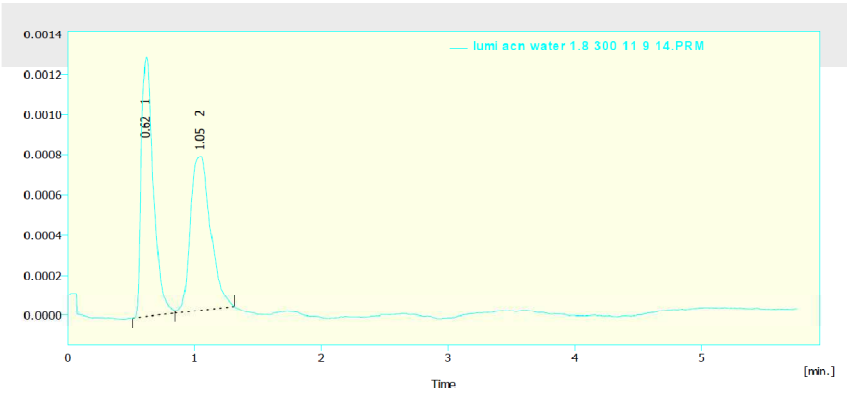


Figure: 20. Shows trail-7 chromatogram of Lumefantrine

TRIAL 8

Mobile phase	**:**	Acetonitrile, methanol, Triethylamine
Flow rate	:	1.6ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm, 5µ)
Detector wavelength	:	235nm
Injection volume	:	10µl

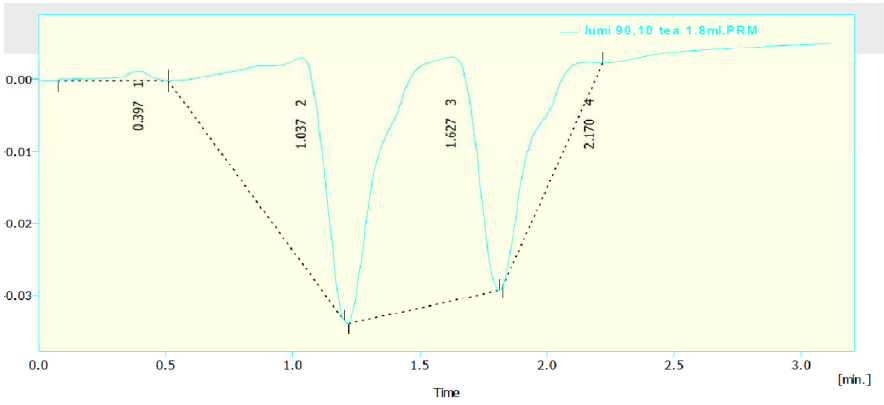


Figure: 21. Shows trail-8 chromatogram of Lumefantrine

TRIAL 9

Mobile phase	**:**	Acetonitrile, methanol (60:40)
Flow rate	:	1.2ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm, 5µ)
Detector wavelength	:	235nm
Injection volume	:	10µl

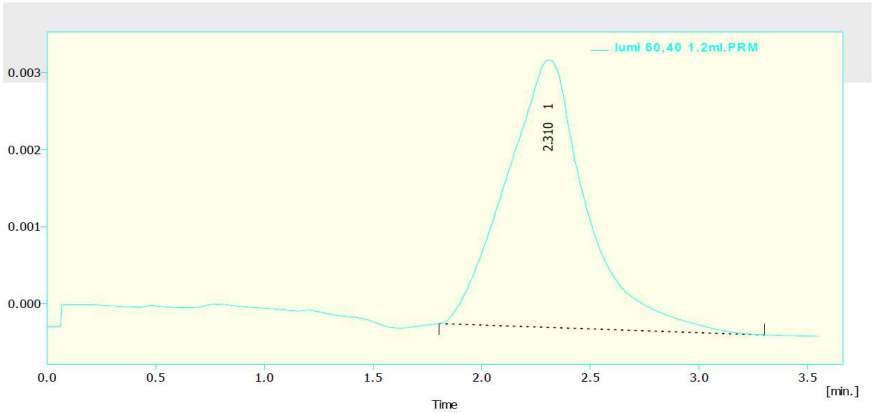


Figure: 22. Shows trail-9 chromatogram of Lumefantrine

TRIAL 10

Mobile phase	**:**	Acetonitrile, methanol (60:40)
Flow rate	:	1.4ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm,5µ)
Detector wavelength	:	235nm
Injection volume	:	10µl

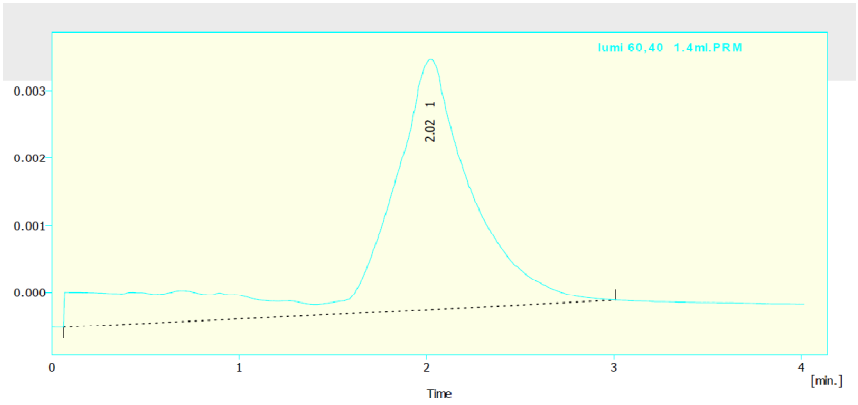


Figure: 23. Shows trail-10 chromatogram of Lumefantrine

TRIAL 11

Mobile phase	**:**	Acetonitrile, methanol (60:40)
Flow rate	:	1.6ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm,5µ)
Detector wavelength	:	235nm
Injection volume	:	10µl

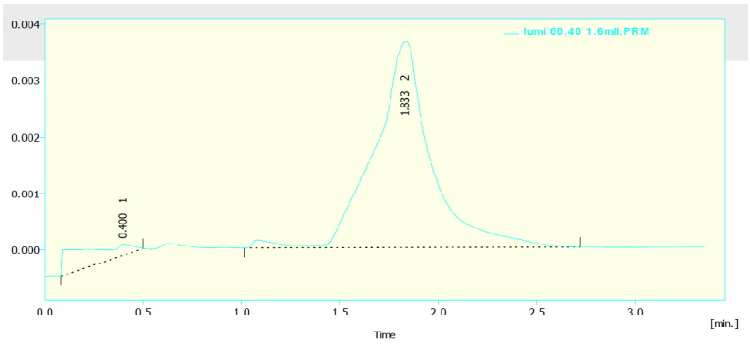


Figure: 24. Shows trail-12 chromatogram of Lumefantrine

TRIAL 12

Mobile phase	**:**	Acetonitrile, methanol (70:30)
Flow rate	:	1.2ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm,5µ)
Detector wavelength	:	235nm
Injection volume	:	10µl

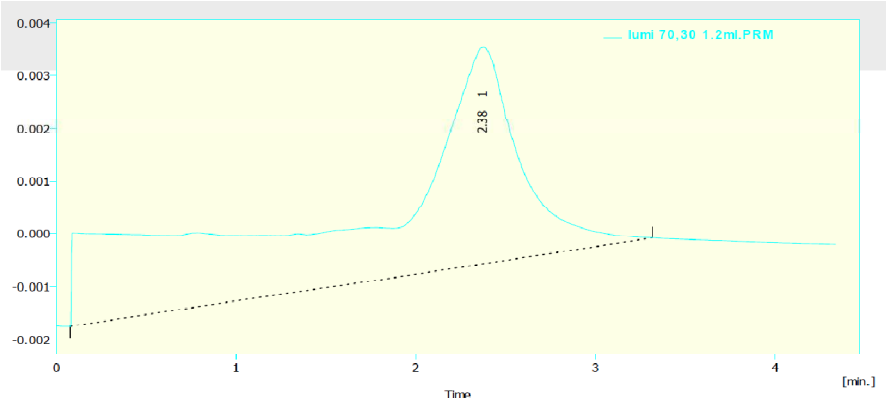


Figure: 25. Shows trail-12 chromatogram of Lumefantrine

TRIAL 13

Mobile phase	**:**	Acetonitrile, methanol (70:30)
Flow rate	:	1.4ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm,5µ)
Detector wavelength	:	235nm
Injection volume	:	10µl

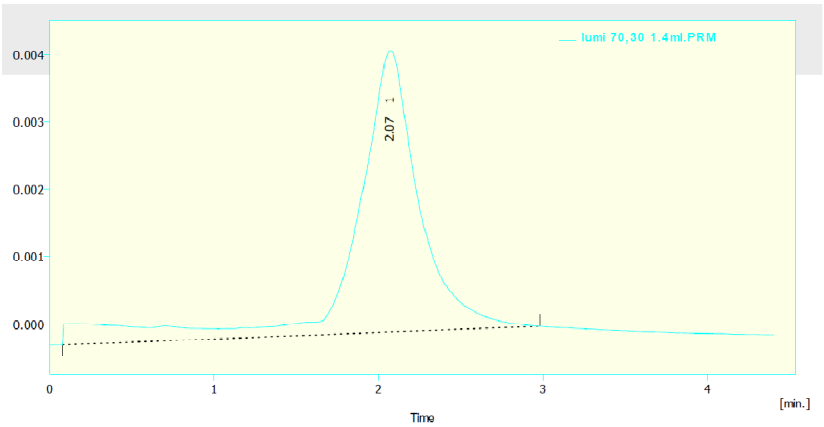
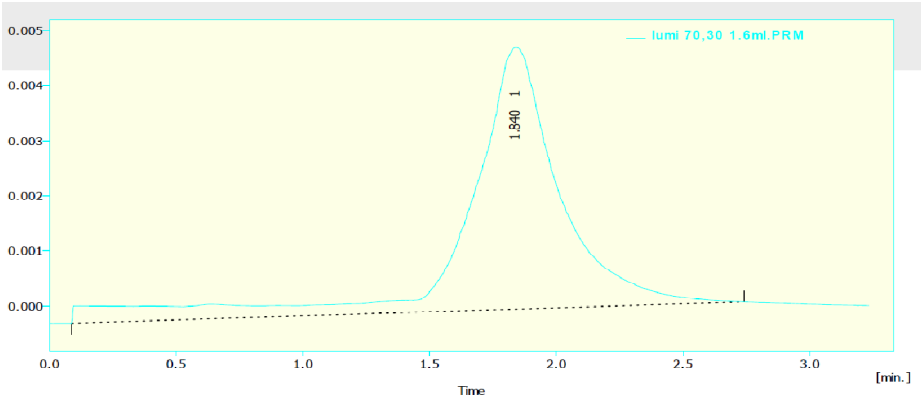


Figure: 26. Shows trail-13 chromatogram of Lumefantrine

TRIAL 14

Mobile phase	**:**	Acetonitrile, methanol (70:30)
Flow rate	:	1.6ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm,5µ)
Detector wavelength	:	235nm
Injection volume	:	10µl

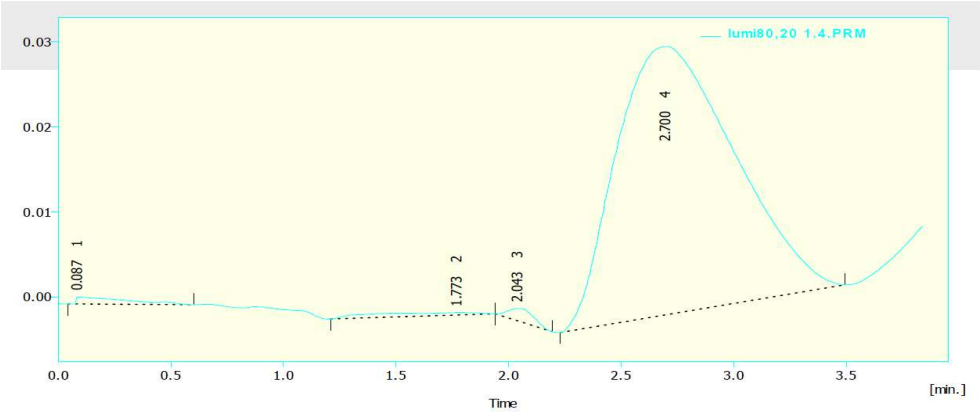


Observation: peak is eluted at 1.840 and sharp peak was not observed

Figure: 27. Shows trail-14 chromatogram of Lumefantrine

TRIAL 15

Mobile phase	**:**	Acetonitrile, methanol (80:20)
Flow rate	:	1.4ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm,5µ)
Detector wavelength	:	235nm
Injection volume	:	10µl

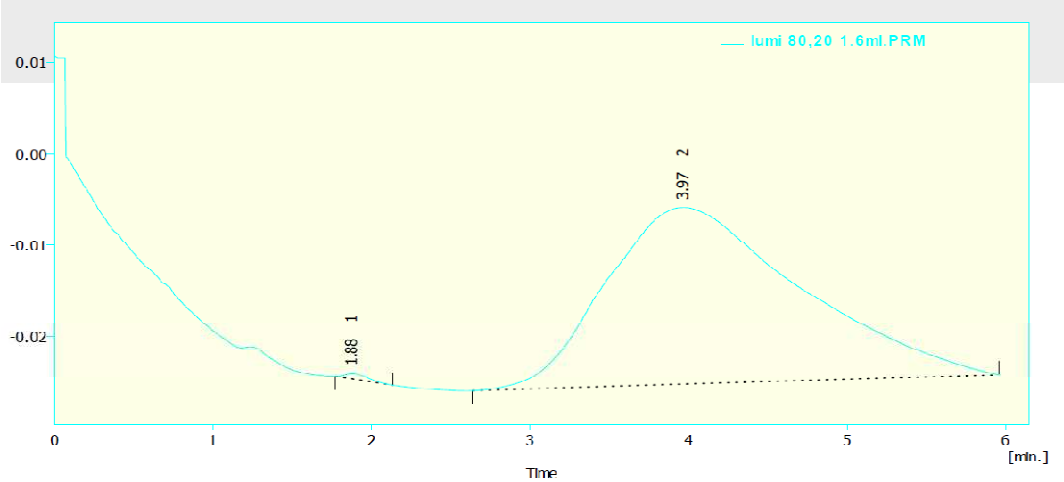


Observation: peak is eluted at 2.700 and Broad peak was not observed.

Figure: 28. Shows trail-15 chromatogram of Lumefantrine

TRIAL 16

Mobile phase	**:**	Acetonitrile, methanol (80:20)
Flow rate	:	1.6ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm,5µ)
Detector wavelength	:	235nm
Injection volume	:	10µl

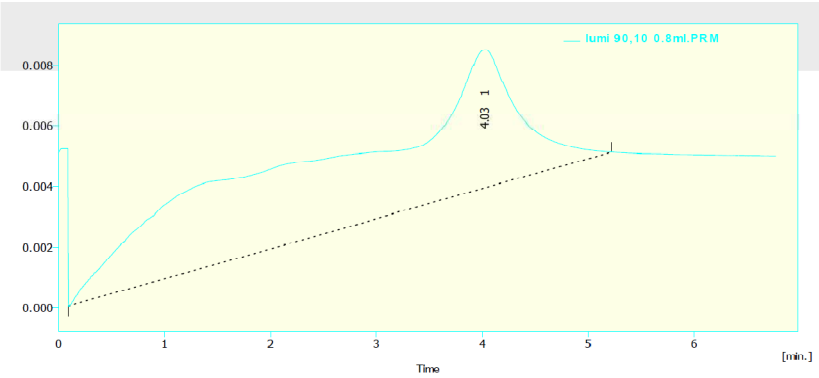


Observation: peak is eluted at 3.97 and Broad peak was not observed

Figure: 29. Shows trail-16 chromatogram of Lumefantrine

TRIAL 17

Mobile phase	**:**	Acetonitrile, methanol (90:10)
Flow rate	:	0.8ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm,5µ)
Detector wavelength	:	235nm
Injection volume	:	10µ

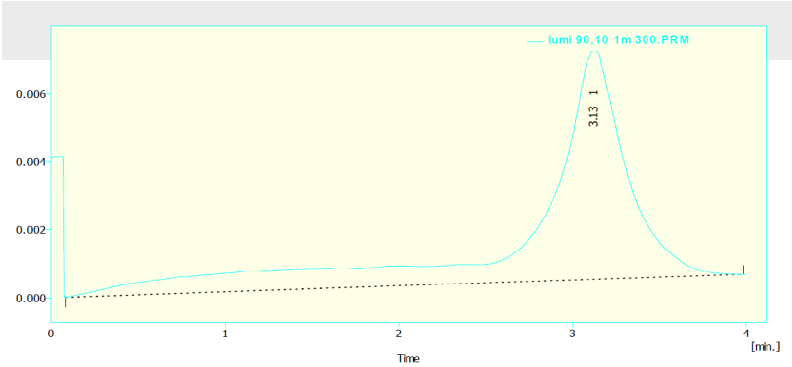


Observation: peak is eluted at 4.03 and Broad peak was not observed

Figure: 30. Shows trail-17 chromatogram of Lumefantrine

TRIAL18

Mobile phase	**:**	Acetonitrile, methanol (90:10)
Flow rate	:	1ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm,5µ)
Detector wavelength	:	235nm
Injection volume	:	10µ

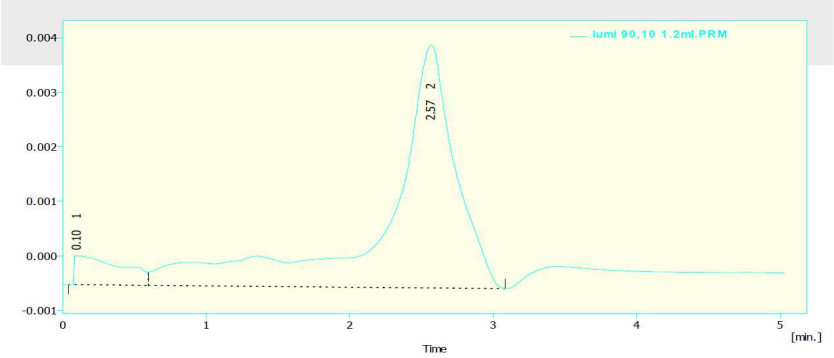


Observation: peak was eluted at 3.13 and sharp peak was not obtained.

Figure: 31. Shows trail-18 chromatogram of Lumefantrine

TRIAL 19

Mobile phase	**:**	Acetonitrile, methanol (90:10)
Flow rate	:	1.2ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm,5µ)
Detector wavelength	:	235nm
Injection volume	:	10µ

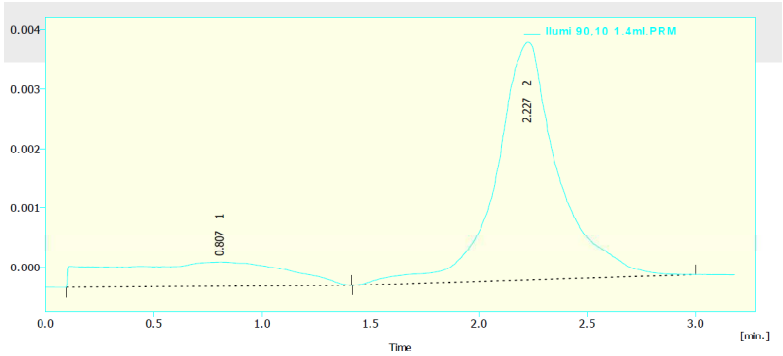


Observation: peak is eluted at 2.57 and sharp peak was not observed

Figure: 32. Shows trail-19 chromatogram of Lumefantrine

TRIAL 20

Mobile phase	**:**	Acetonitrile, methanol (90:10)
Flow rate	:	1.4ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm,5µ)
Detector wavelength	:	235nm
Injection volume	:	10µ

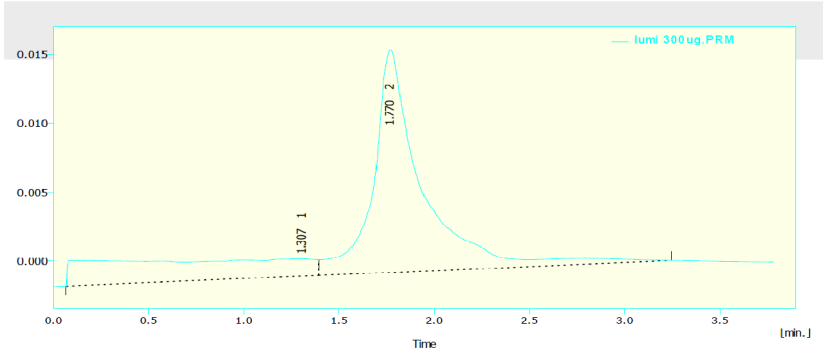


Observation : From the above peak it was observed that the obtained peak has Fronting

Figure: 33. Shows trail-20 chromatogram of Lumefantrine

TRIAL 21

Mobile phase	**:**	Acetonitrile, methanol (90:10)
Flow rate	:	1.6ml/min
Column	:	Hypersil ODS C_{18} (150×4.6mm, 5µ)
Detector wavelength	:	235nm
Injection volume	:	10µ



Observation: peak was eluted at 1.770 and sharp peak was obtained and no tailing was Observe

Figure: 33. Shows trail-20 chromatogram of Lumefantrine

7.2. Validation results

1) Accuracy

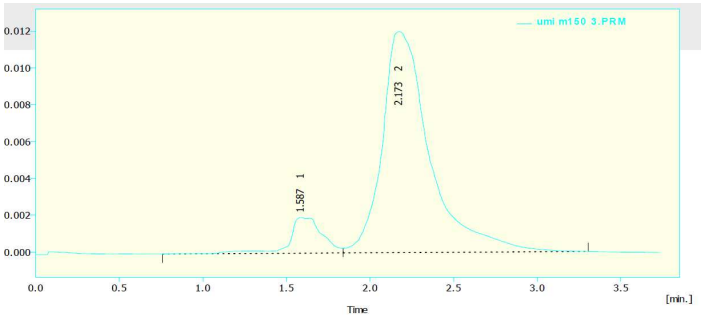


Figure: 34. Shows Chromatogram for Accuracy at 50% level

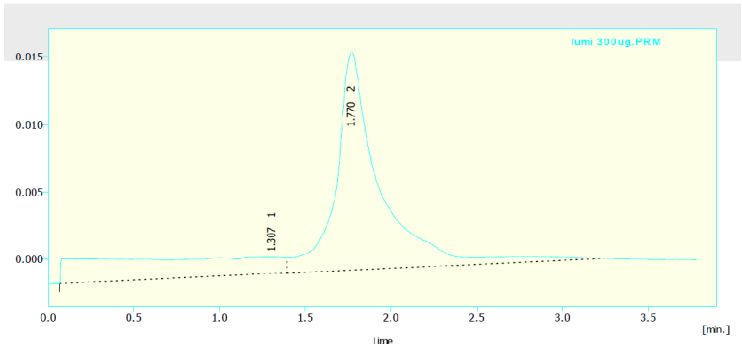


Figure: 35. Shows Chromatogram for Accuracy at 100% level

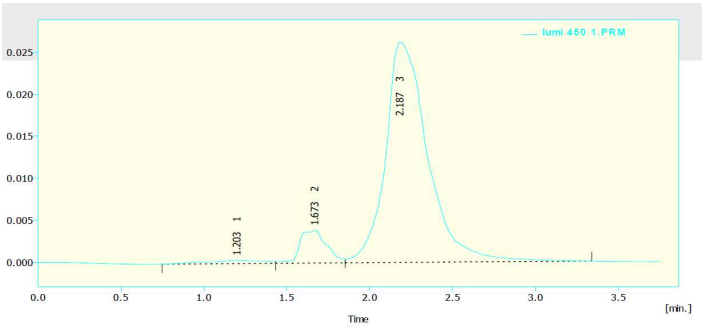


Figure: 36.Shows Chromatogram for Accuracy at 150% level

Table: 5 .Shows Accuracy results of Lumefantrine

Concentration level	Amount added (mg)	Amount found(mg)	%recovery	Average % recovery
50%	25 mg 25 mg 25 mg	25.5 mg 25.4 mg 24.3 mg	102% 101.6% 97.2%	100.2%
100%	50 mg 50 mg 50 mg	50.5 mg 50.45 mg 50.40 mg	101% 100.9% 100.8%	100.9%
150%	75 mg 75 mg 75 mg	75.5 mg 75.40 mg 74.65 mg	100.6% 100.5% 99.53%	100.2%

Result: The accuracy for the average of triplicate in each concentration samples are within the limit

Table: 6. Shows % Recovery of Lumefantrine

Amount added (mg)	Amount found(mg)	Average % recovery
50 mg	50.2 mg	100.4%

3) Precision

Intraday precision

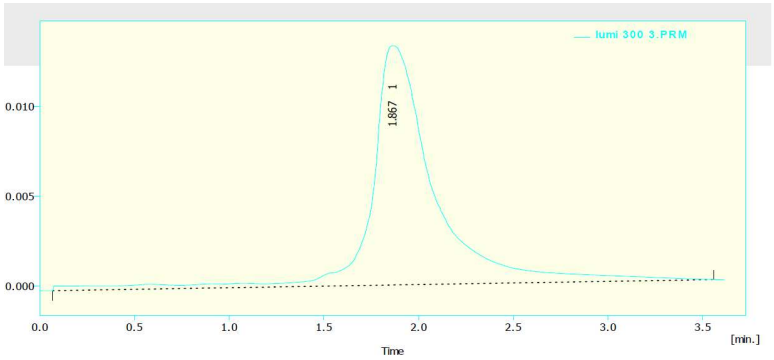


Figure: 37. Shows Chromatogram for Intraday precision (1st injection)

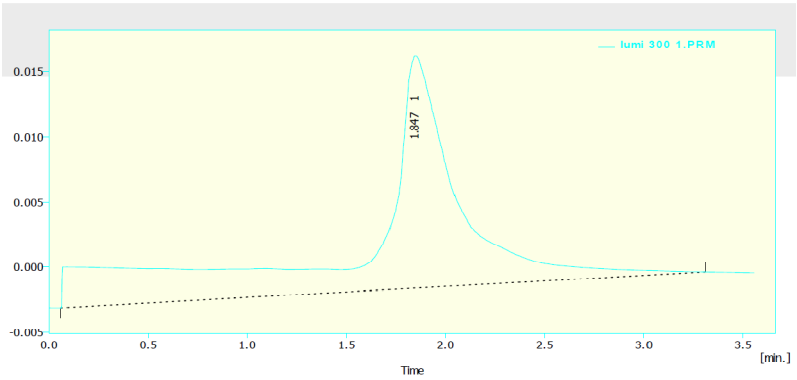


Figure: 38. Shows Chromatogram for Intraday precision (2nd injection)

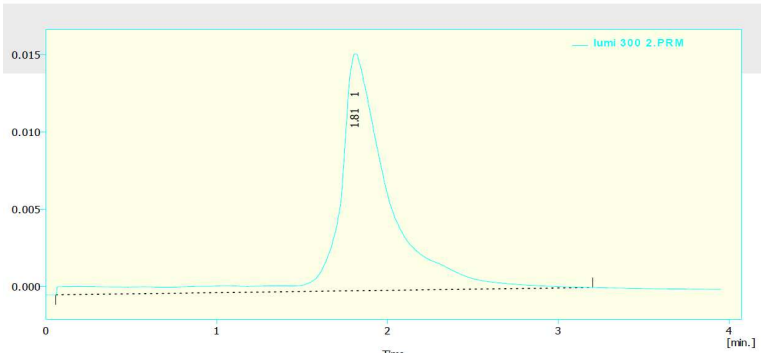


Figure: 39.Shows Chromatogram for Intraday precision (3rd injection)

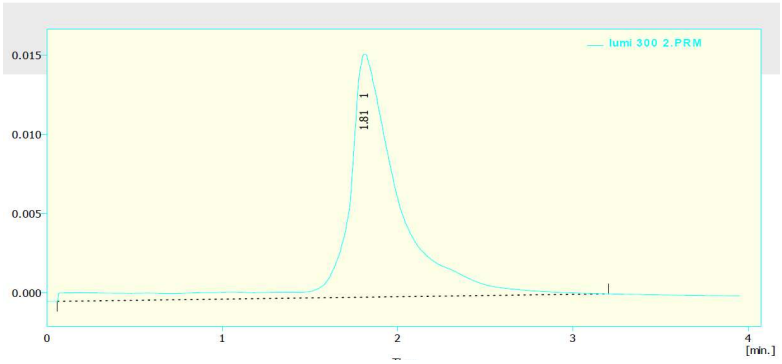


Figure: 40. Shows Chromatogram for Intraday precision (4th injection)

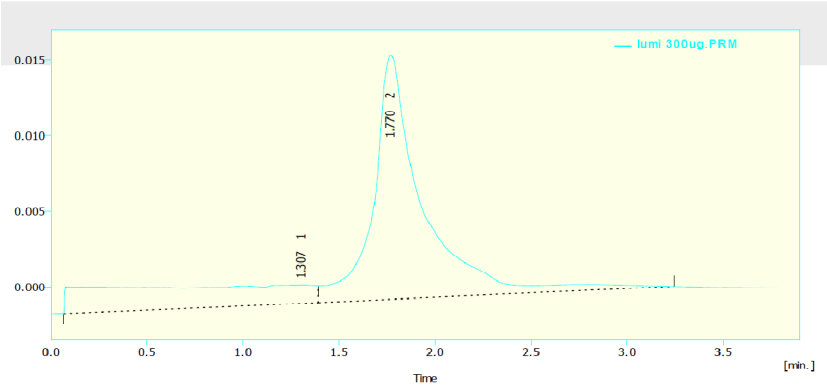


Figure: 41. Shows Chromatogram for Intraday precision (5th injection)

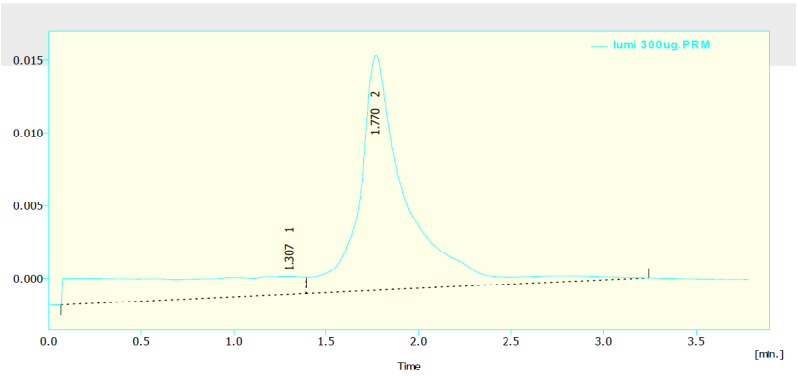


Figure: 42.Shows Chromatogram for Intraday precision (6th injection)

Table: 7 Shows precision results of Lumefantrine

Sample. No	Peak area of Lumefantrine
Injection 1	284.421
Injection 2	287.884
Injection 3	291.462
Injection 4	291.462
Injection 5	291.514
Injection 6	291.514
Mean	**289.7095**
Standard deviation	**2.964983491**
%RSD	**1.02**

Result: The precision values are found within the limits.

4) Specificity

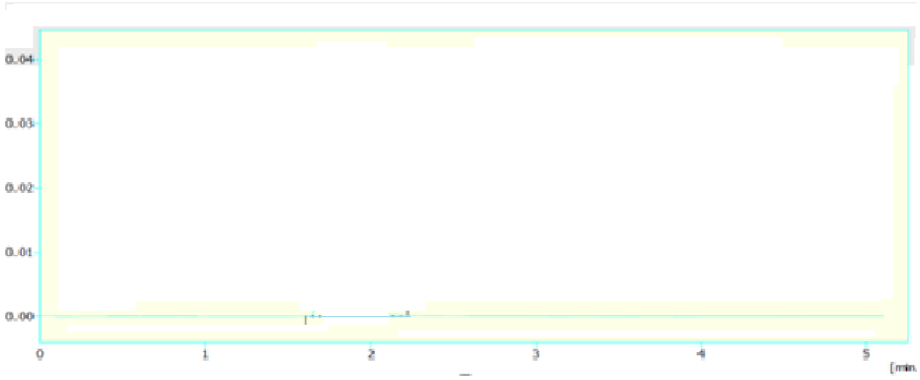


Figure: 43. Shows Chromatogram of blank

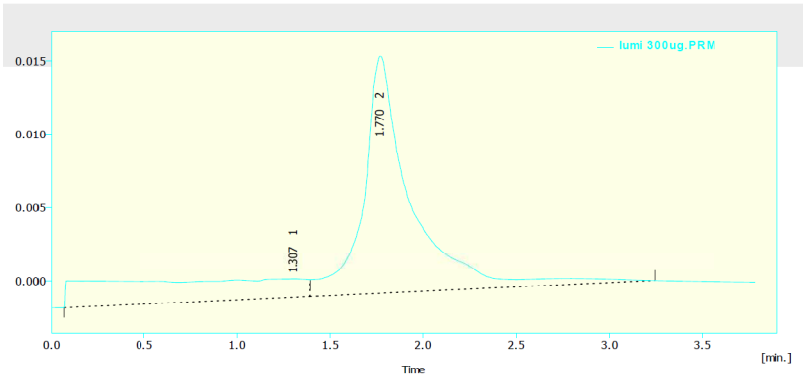


Figure: 44. Shows Chromatogram of standard

Result: There is no interference between the blank and standard, so the results were met with the acceptance criteria.

6) Linearity and range

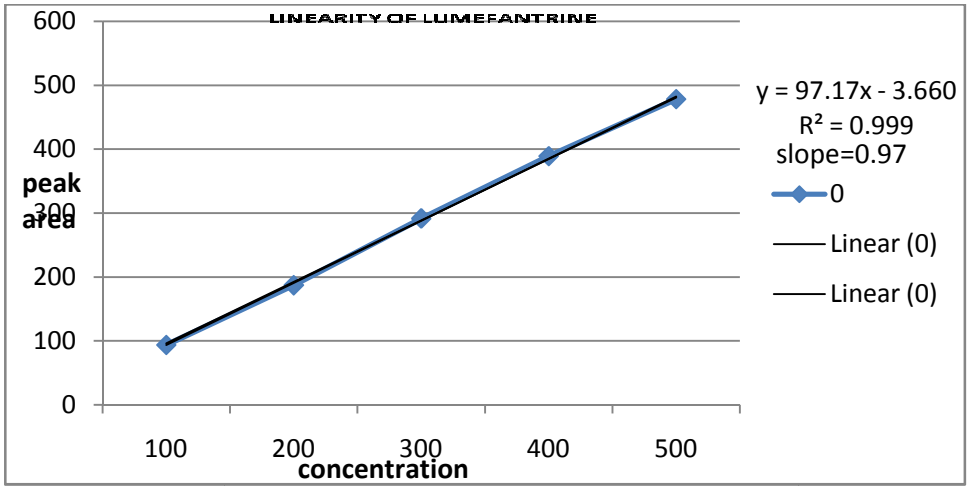


Figure: 45. Shows Calibration graph of Lumefantrine

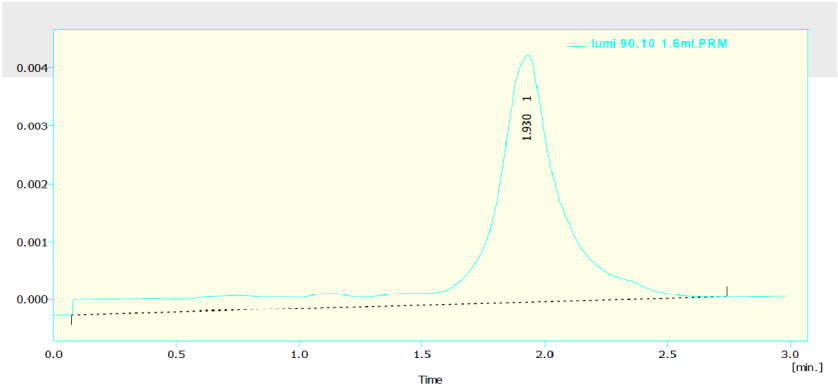


Figure: 46.Shows Chromatogram for Linearity at 100 µg/ml

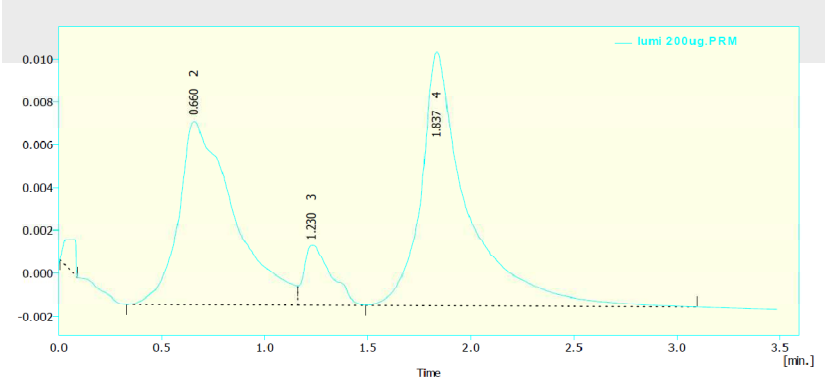


Figure: 47. Shows Chromatogram for Linearity at 200 µg/ml

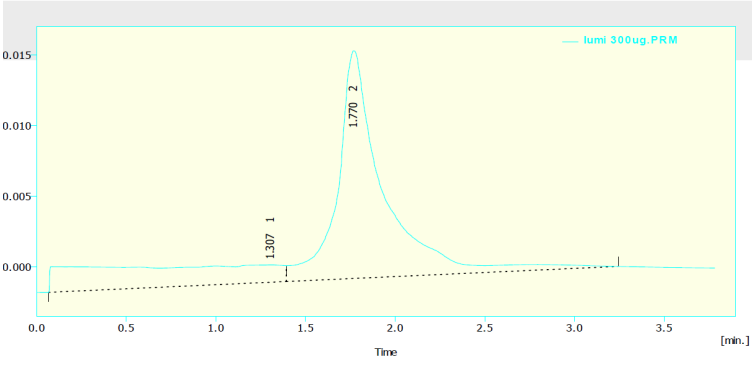


Figure: 48. Shows Chromatogram for Linearity at 300 µg/ml

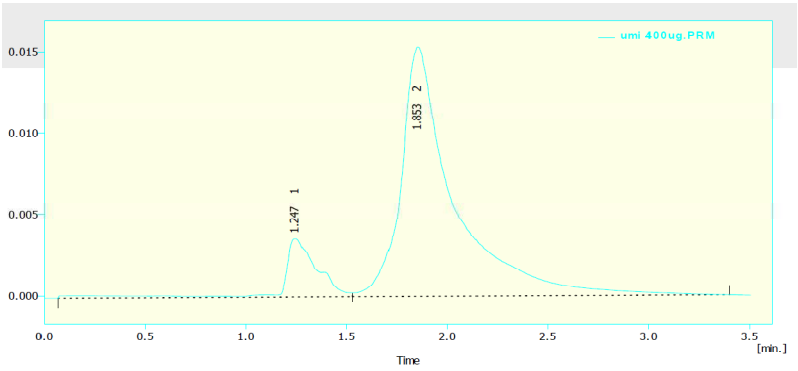


Figure: 49. Shows Chromatogram for Linearity at 400 µg/ml

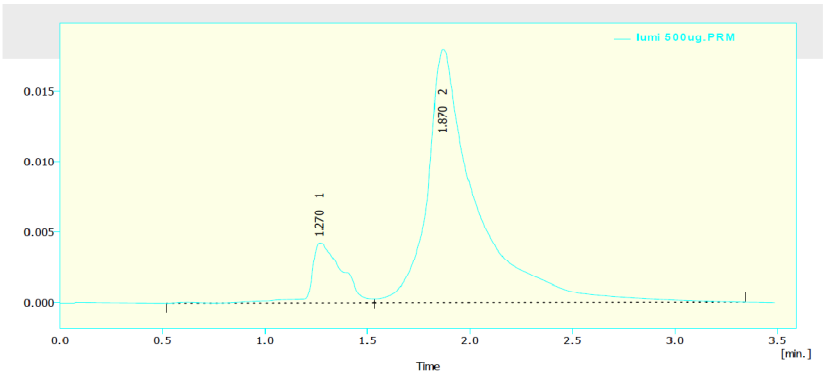


Figure: 50.Shows Chromatogram for Linearity at 500 µg/ml

Table: 8. Shows linearity results of Lumefantrine

%level	**Concentration(µg/ml)**	**Peak area**
33	100	93.48
66	200	186.96
100	300	291.514
133	400	389.15
166	500	478.28
Y Intercept		**96.18**
Correlation co-efficient (r^2)		**0.999**
Slope		**0.97**
Linearity range	**100-500**	

Result: From the above graph, it can be concluded that, the relationship between the concentration and peak response of Lumefantrine peak is linear in the range examined as all the points lie in a straight line with the regression coefficient of 0.999 for Lumefantrine which is within the limits. Hence the developed method is linear in the specified range for estimation of Lumefantrine

5) Robustness

i) **Change in flowrate**

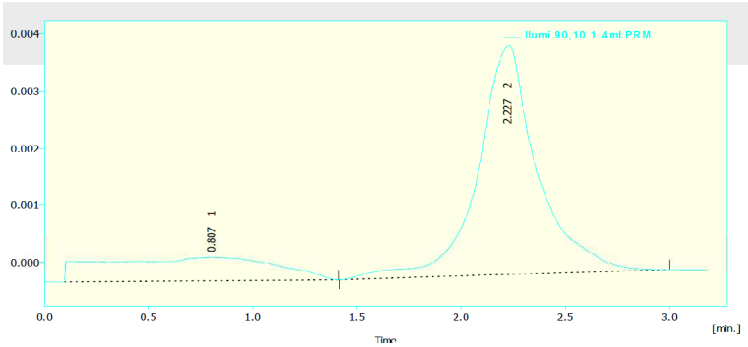


Figure: 51. Shows Chromatogram for flow rate at 1.4ml/min

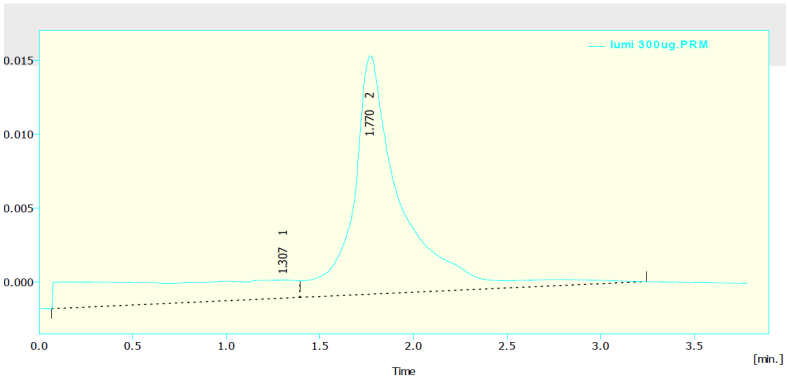


Figure: 52. Shows Chromatogram for flow rate at 1.6ml/min

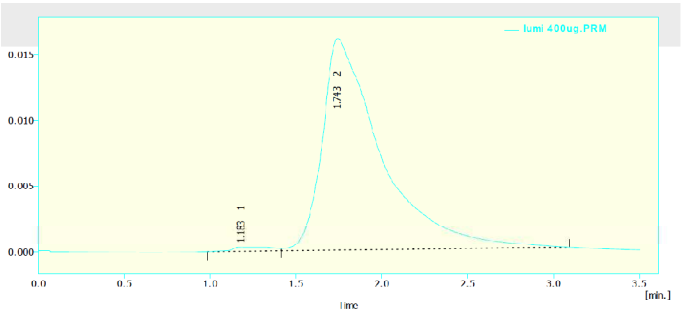


Figure: 53.Shows Chromatogram for flow rate at 1.8ml/min

Table: 9. Shows robustness results of Lumefantrine

S.NO	flow rate	Peak area of Lumefantrine		Average	SD	%RSD
1	1,4ml/min	79.921	78.910	79.4155	0.7	0.88
2	1.6ml/min	291.514	289.512	290.513	1.41	0.48
3	1.8ml/min	389.150	387.45	388.3	1.20	0.30

Result: From the above values it was found that %RSD values are within the limit A;

ii) Change in Column temperature

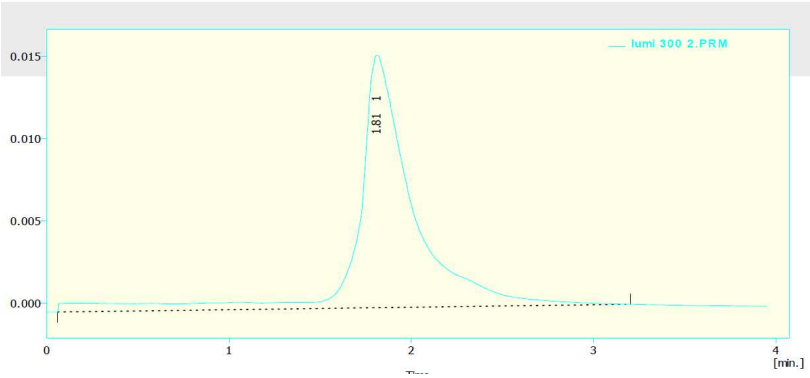


Figure: 54.Shows Chromatogram for temperature at 30 □c

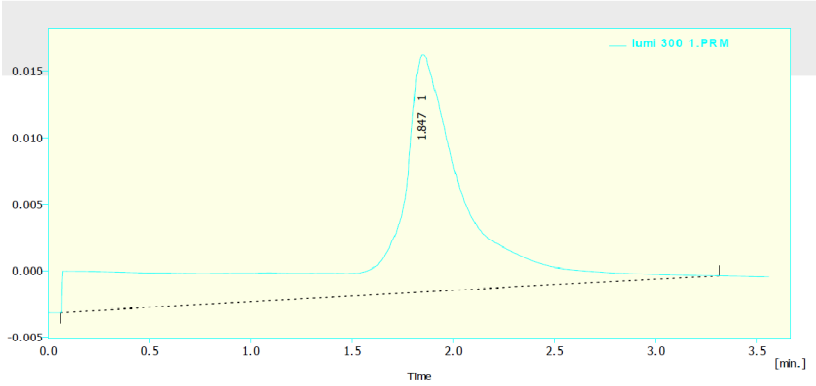


Figure: 55. Shows Chromatogram for temperature at 35 □c

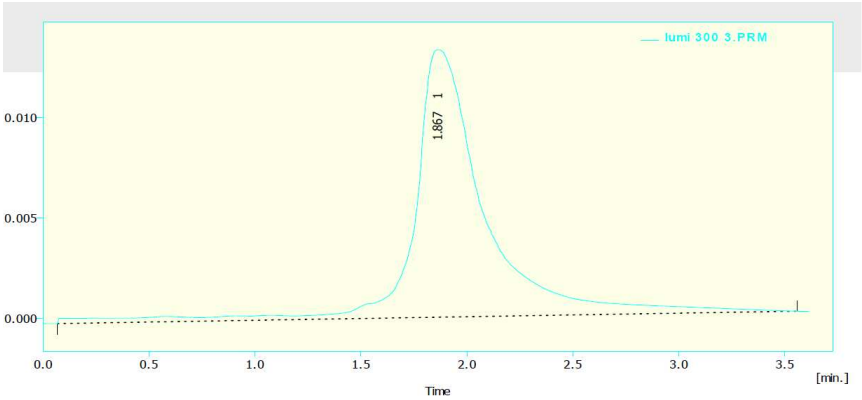


Figure: 56. Shows Chromatogram for temperature at 40 □c

Table: 10. Shows robustness results of Lumefantrine

S.NO	Temperature	Peak area of Lumefantrine		Average	SD	%RSD
1	30 □c	291.462	285.452.	288.457	1.41	0.48
2	35 □c	287.884	285.882	286.883	1.415	0.49
3	40 □c	284.421	282.420	283.420	1.414	O.498

Result: From the above values it was found that %RSD values are within the limits

7) Ruggedness (Intermediate precision)

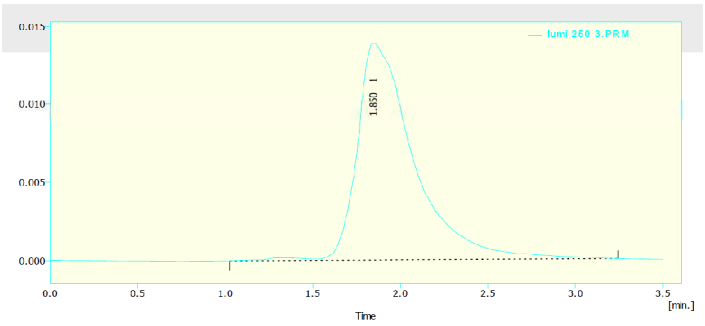


Figure: 57. Shows Chromatogram for Interday precision (1st injection

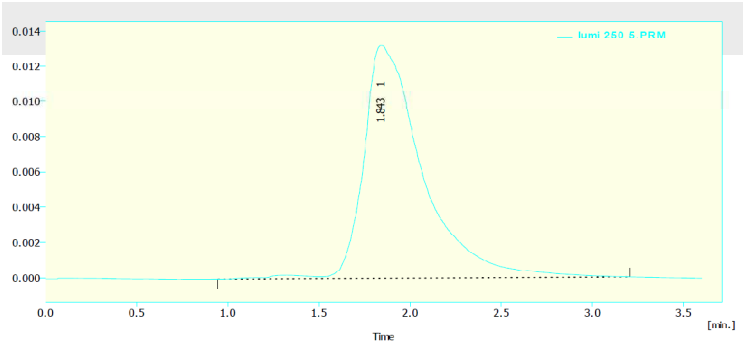


Figure: 58.Shows Chromatogram for Interday precision (2nd injection)

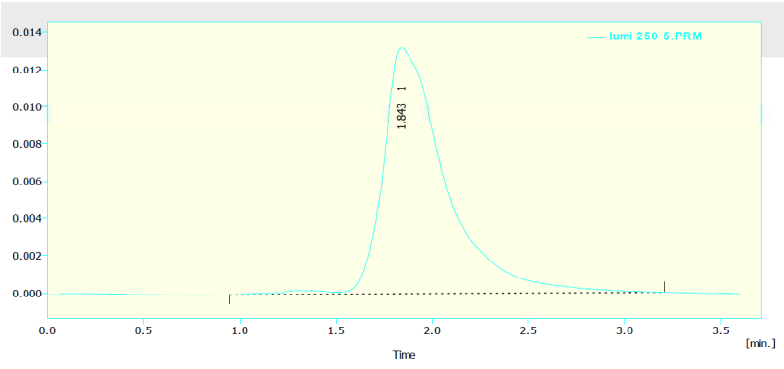


Figure: 59. Shows Chromatogram for Interday precision (3rd injection)

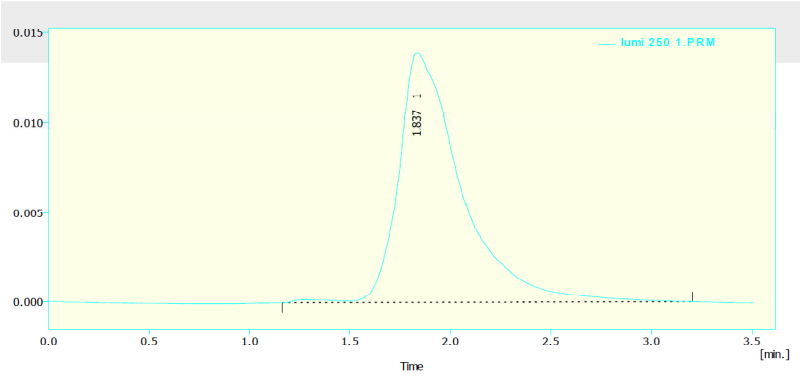


Figure: 60. Shows Chromatogram for Interday precision (4th injection)

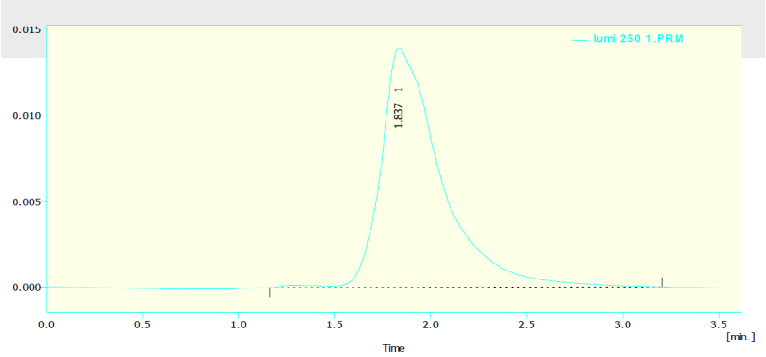


Figure: 61. Shows Chromatogram for Interday precision (5th injection)

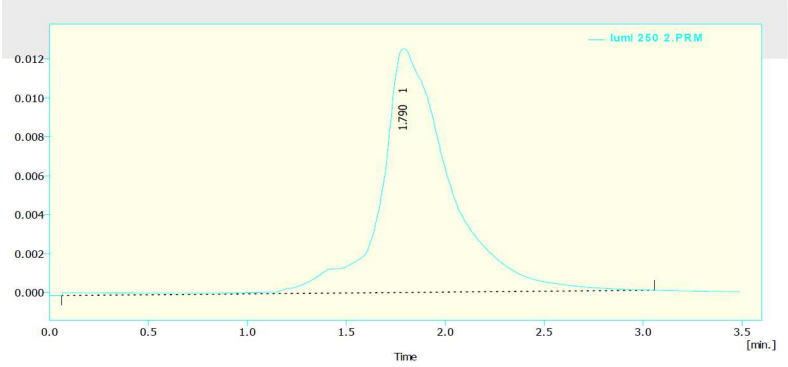


Figure: 62. Shows Chromatogram for Interday precision (6th injection)

Table: 11. Shows Ruggedness results of Lumefantrine

Name	Peak area of Lumefantrine
Ruggedness-(Day-1)-1	295.230
Ruggedness-(Day-1)-2	297.238
Ruggedness-(Day-1)-3	297.238
Ruggedness-(Day-1)-4	305.319
Ruggedness-(Day-1)-5	305.319
Ruggedness-(Day-1)-6	308.437
Average	**301.4635**
SD	**5.530383088**
% RSD	**1.83**

Result: The % RSD values were within 2 and the method was found to be precise

8) LOD&LOQ

Table: 12. Shows LOD & LOQ results of Lumefantrine

Parameters	Lumefantrine
LOD	10.0 µg/ml
LOQ	30.5 µg/ ml

Table: 13. Shows summary of validation parameter Results

S.NO	Parameter	Acceptance criteria	HPLC
1	%recovery	98-102%	100.4
2	Linearity range((µg/ml)	-	100-500(µg/ml)
3	Correlation coefficient	NLT 0.999	0.999
4	No .of Theoretical plates	NLT 2500	10877
5	Method precision	%RSD (NMT 2%)	1.02
6	System precision	%RSD (NMT 2%)	0.48
7	Intermediate precision	%RSD (NMT 2%)	1.83
8	LOD	-	10.0 (µg/ml)
9	LOQ	-	30.5 (µg/ml)

CONCLUSION

Method development & validation of Lumefantrine was done by RP-HPLC method. The estimation was done by using Hypersil C_{18} (4.6 x 150mm, 5µm, Make: Analytical technologies) mobile phase as Acetonitrile, methanol (90:10) at a flow rate 1.6ml/min.

Accuracy parameter is considered accurate if the average recovery is not less than 98% and not more than 102%. Precision parameter RSD of six replicate injections should be NMT 2%.

The linearity range of Lumefantrine was found to be 100-500 µg/ml in HPLC. Linear regression was not more than 0.999.the values of %RSD was <2

Robustness of assay method is demonstrated by changing the flow rate for 1.4ml/min and 1.8ml/min instead of 1.6ml/min by injecting the 6 replicate injections of standard in 1.4ml/min and 1.8ml/min flow rate and found that system suitability parameters are passed.

By changing the column temperature for 30 □c and 40 □c instead of 35 □c by injecting the 6 replicate injections of standard in 30 □c and 40 □c temperature and found that system suitability parameters are passed

Ruggedness parameter RSD for the assay values of 6 sample preparations of same batch should not be more than2.0%.

The LOD are calculated from the calibration curve by using the formulas LOD = 3 x SD/ bWhere, SD- the estimate is the standard deviation of the peak area of the drugs. b -is slope of the corresponding calibration curvepassed.The LOQ are calculated from the calibration curve by using the formulas LOQ = 10 x SD/ where,

SD- the estimate is the standard deviation of the peak area of the drugs. b -is slope of the corresponding calibration curve the %recovery varies in the range of 97-102%.LOD & LOQ values are found within the limits.

These results shows the method is accurate, precise, sensitive, economic & rugged. The HPLC method is more rapid. The proposed method is successfully applied to the bulk dosage form. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

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