Development and Validation of Stability Indicating Liquid Chromatographic Methods for Simultaneous Determination of Antimalarial Drugs in Combination Dosage Forms.

THESIS

Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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CERTIFICATE

This is to certify that the thesis entitled **"Development and Validation of Stability Indicating Liquid Chromatographic Method for Simultaneous Determination of Antimalarial Drugs in Combination Dosage Form."** and submitted by Ms. Manisha U. Phadke, ID No. 2006PHXF001P for award of Ph.D. Degree of the institute, embodies original work done by her under my supervision.

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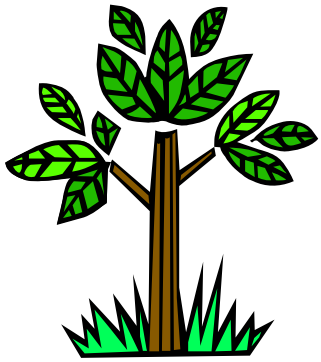
‖Shri Mhalasa Narayani Prasanna‖

DEDICATED TO

My Father Late Shri. Sadashiv Ramachandra Athalekar.

& My Mother Late Smt .Vidyadevi Sadashiv Athalekar.



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SUMMARY

Malaria is a protozoal parasite disease in humans with transmission in 103 countries affecting more than one billion people and causing around 3 million deaths each year, mostly children younger than 5 years.

Drug combinations, rather than monotherapy , are now seen to be the best solution for treating malaria . Artemisinin based drug combinations are highly effective, with cure rates similar to that of chloroquine 30 years ago . As per WHO, to improve efficacy and delay the onset of resistance, artemisinin drugs should always be used in combination with another effective antimalarial drug. Combination of one of these drugs with a longer half-life partner antimalarial drug allows a reduction in the duration of antimalarial treatment while at the same time enhancing efficacy and reducing the likelihood of development of resistance to the partner drug.

When such fixed dose combination formulations are developed, the development of the analytical method to evaluate the stability as well as the content of the drug substance in drug products becomes the attractive challenge in the analytical research. The development of a new drug in to its proper dosage forms involves series of stages where analytical methods play a vital role. These instrumental techniques are sensitive, precise and are developed by taking in to consideration their applications to the routine quality control analysis and to analyze drugs and drug products. Quality means the degree of excellence for the drug product of interest. In order to have the highest degree of excellence for the drug product, we need to control various factors during the course of which the product come in to existence. Thus, analytical technique plays a very important role in various studies carried out in pharmaceutical analysis. The major areas of analytical chemistry in pharmaceutical analysis are, assay of bulk drugs and formulations , quantification and detection of impurities, accelerated stability and in vitro dissolution study. Some of the antimalarial drug substances and the drug products like artemether, artesunate, arteether are official in International Pharmacopoeia but not all these are official in Indian Pharmacopoeia, British Pharmacopoeia or US Pharmacopoeia.

Literature survey shows various analytical methods for separation of artemisinin products, artesunate, artemether, amodiaquine and their metabolite (s) using high-performance liquid chromatography – mass spectrometry, gas chromatography – mass spectrometry, liquid chromatography with electrochemical detector.

All these methods are for determination of individual drug substance and its metabolite. To our knowledge, no methods are available in the reviewed literature for simultaneous determination of metabolite (s) or degradant (s) and drug substances (s) of fixed dose combination drug products. It is clear from the thorough literature review that some of the methods are reported for individual determination or single component determination but there is no work on simultaneous determination of artemether and lumefantrine or artesunate and amodiaquine and their degradants like dihydroartemisinin, artemisinin, glycan in fixed dose combination dosage form. The antimalarial drugs and drug products are receiving considerable attention because of their application in the treatment of life threatening diseases. Therefore fixed dose combinations of these drugs are selected for the present work. Various regulatory authorities like USFDA, WHO and other health agencies are emphasizing on the stability indicating assay and the identification of impurities in drug substance and products. A key component of the overall quality of a pharmaceutical is on control of impurities and content of drug (s) in the drug products through out the shelf life of the product, as presence of impurities, even in small amounts, may affect drug safety and efficacy. As per ICH Q3A(R) and ICH Q3B(R) guidelines, unknown impurities associated with bulk drug and dosage form, greater than the identification threshold should be identified. A need for analytical methods for consistent quality establishment through out the shelf life of the product arises. Therefore, the aim of the research work was to develop and validate specific, accurate and precise stability indicating methods for simultaneous determination of contents of antimalarial drug substances in fixed dose combination products. In view of the existing approaches for the development of degradation profile of fixed dose combination antimalarial drug products, the technique of Liquid Chromatography was developed into an invaluable laboratory tool for the separation and identification of components.

Method development was based on several considerations such as chemical structure, molecular weight, pKa values, UV Spectra of the components, concentration range of components in samples of interest and lastly sample solubility pattern.

The present work includes:

(i) The selection of chromatographic methods for simultaneous separation and detection of impurities in the drug products.

(ii) Optimization of the chromatographic methods (stationary phase, mobile phase composition, Buffer/pH, wavelength etc)

(iii) Extraction of drug substance(s) from the combination dosage form

(iv) Identification of unknown impurity (> 0.1%)

(v) Validation of developed method(s).

To support the research work, the degradation profile of the selected combination drug products were studied. Based on the experimental trials the best results were obtained by using dual wavelength and C_{18} column and mixture of acidic buffer and acetonitrile, as a mobile phase. Analytical methods for estimation of antimalarial drug products containing artesunate + amodiaquine, and artemether + lumefantrine in fixed dose combination were finalized separately based on number of experimental trials, their evaluation and their application for stability studies. The methods were validated for parameters like specificity (Placebo Interference and Force Degradation), Linearity & (LOQ for Related impurities, accuracy (Recovery), method precision, intermediate precision and robustness. All the parameters were validated and values obtained for each parameter was found within the regulatory requirements. These methods were used for the monitoring of the consistent quality of the drug products through out the shelf life of the drug products.

Further scope of research is that these developed methods can be applied to forensic laboratories, in bioanalytical studies. Concept of two extreme concentration formulations to be analysed by dual wavelength can be applied to other formulations with other antimalarial fixed dose combinations like artesunate and lumefantrine , dihydroartemisinin and piperaquine phosphate and of other therapeutic categories like anti-hypertensive, anti diabetic , anti-inflammatory etc.

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LIST OF ABBREVIATIONS / SYMBOLS

Abbreviation / Symbol	Meaning
ACN	Acetonitrile
Approx.	Approximately
ANDA	Abbreviated New Drug Application
ANOVA	Analysis of Variance
AUC	Area Under the Curve
B.P.	British Pharmacopoeia
C_{max}	Concentration Maximum of Drug
D.R	Drug Release
E.P.	European Pharmacopoeia
f_2	Similarity Factor
FL	Fluorescence
GCP	Good Clinical Practices
GLP	Good Laboratory Practices
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin layer Chromatography
INP	International Pharmacopoea
ICH	International Conference on Harmonization
ID	Internal Diameter
I.P.	Indian Pharmacopoeia
I.P.A	Iso Propyl Alcohol
IR	Infra Red
K’	Capacity factor
LC	Liquid Chromatography
LOD	Limit Of Detection
LOQ	Limit Of Quantification
NDA	New Drug Application

Abbreviation / Symbol	Meaning
NAMP	National Association of Malarial Programgramme
NIR	Near Infra Red
NP	Normal Phase
NMT	Not More Than
N	Theoretical plate/ plate count
PDA	Photodiode Array Detector
R	Correlation coefficient
R^2	Residual sum of square
RH	Relative Humidity
RPM	Revolutions Per Minute
RP	Reversed Phase
RSD	Relative Standard Deviation
RRT	Relative Retention Time
Rs	Resolution
RT	Retention Time
SD	Standard Deviation
T_{max}	Time at which maximum drug concentration has reached
THF	Tetrahydrofuran
TLC	Thin layer chromatography
USFDA	United States Food and Drug Administration
USP	United states Pharmacopoeia
UV	Ultraviolet
WHO	World Health Organization
w.r.t.	with respect to

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1.1 Malaria

Malaria, the most important parasitic disease of humans, remains a major health and economic burden in most tropical regions [1]. It exacts a heavy toll of illness and death - especially among children and pregnant women. It also poses a risk to travelers and immigrants, with imported cases increasing in non-endemic areas. Treatment and control have become more difficult with the spread of drug-resistant strains of parasites and insecticide-resistant strains of mosquitoes that carry the parasites. TDR has supported research on this disease for many years, searching for new chemicals effective against the parasites, developing new drugs to treat the disease, identifying ways to prevent mosquitoes from transmitting the parasite, analyzing rapid diagnostic tests, and gathering evidence for the best ways to use the treatments in the harsh field conditions where malaria is endemic.

The four malaria species that produce human disease are *P. vivax, P. falciparum ,P. malariae* and *P.ovale*. [1]. Malaria is usually transmitted when a person is bitten by an infected female Anopheles mosquito. Only Anopheles mosquitoes transmit malaria and to do so the mosquito must have been infected by having drawn blood from a person already infected with malaria [1].

Malaria is a major cause of death equal with HIV/AIDS and tuberculosis. The mortality and morbidity associated with malaria have a crippling effect on the economies of endemic countries [1]. It afflicts more than 500 million people, causing from 1.7 million to 2.5 million deaths each year [2]. Malaria occurs in over 90 countries worldwide. According to National Antimalarial Programme (NAMP), total malaria cases in 2000 was 2.02 million globally, out of which 1.05 million was the total *P. falciparum* cases. Currently, more than two billion people live at risk of contracting malaria, and the estimated global annual incidence of clinical malaria is greater than 300 million cases. More than one million people die every year from the direct causes of malaria, with children at highest risk [3]. So there has been increase in *P. falciparum* percentage from 26% in 1965 to 50% in 2000 [4]. *Plasmodium falciparum* is responsible for most morbidity and mortality and it causes serious complications like jaundice, renal failure and cerebral malaria. Its prognosis is usually grave. Antimalarial drugs are targeted against the asexual erythrocytic stage of the parasite. The parasite degrades hemoglobin in its acidic food vacuole [5], producing free heme able to react with molecular oxygen and thus to generate reactive oxygen species as

toxic by-products. A major pathway of detoxification of heme moieties is polymerization as malaria pigment [6, 7]. Majority of antimalarial drugs act by disturbing the polymerization (and/or the detoxification by any other way) of home, thus killing the parasite with its own metabolic waste [8]. The main classes of active schizontocides are 4-aminoquinolines, aryl-alcohols including quinolinealcohols, antifolate compounds which inhibit the synthesis of parasitic pyrimidines. The newest class of antimalarials is based on the natural endoperoxide artemisinin and its hemisynthetic derivatives and synthetic analogs. Some antibiotics are also used, generally in association with quinoline-alcohols [9]. Few compounds are active against gametocytes, and against the intra-hepatic stages of the parasite.

Alkaloids related to Quinine are the most commonly used, in the treatment or prevention of Malaria. The main antimalarial drugs are the 4-aminoquinolines because they have proven to be the most highly successful class of compounds for the treatment and prophylaxis of malaria [10]. These include chloroquine, amodiaquine, hydroxychloroquine, mefloquine and piperaquine. 8-aminoquinolines are generally used as gametocytocides these includes primaquine, pamaquine and tafenoquine. Aryl alcohols like halofantrine and lumefantrine are useful in chloroquine resistant malaria. Artemisinin derivatives are the fastest active antimalarial drugs. These includes artemether, arteether and artesunate [11].

1.2 Drug resistance in malaria

Falciparum malaria is a mass killer that went out of control. The drug treatments for this potentially lethal infection that have been most widely recommended and provided over the past 50 (i.e. chloroquine and sulphadoxine - pyrimethamine) years no longer work in most tropical countries. Resistance to these drugs emerged in Asia and South America and spread to Africa. As resistance worsened, morbidity and mortality rose as direct consequences. Resistance to antimalarial drugs has been described for two of the four species of malaria parasite that naturally infect humans, *P. falciparum* and *P. Vivax* [12]. *P. falciparum* has developed resistance to nearly all antimalarials in current use, although the geographical distribution of resistance to any single antimalarial drug varies greatly [12]. It is the plasmodia that causes malaria that develops resistance to anti-malarial drugs not the mosquitoes that transmit the disease. Drug resistant malaria has become one of the

most important problems in malaria control in recent years [13]. Resistance in vivo has been reported to all antimalarial drugs except artemisinin and its derivatives [13]. Drug resistance necessitates the use of drugs, which are more expensive and may have dangerous side effects. In some parts of the world, artemisinin drugs are the first line of treatment, and are used indiscriminately for self-treatment of suspected uncomplicated malaria - so we can expect to see malaria forms resistant to artemisinin soon according to World Health Organization (WHO) [14]. The areas most affected by drug resistance are the Indo-Chinese peninsula and the Amazon region of South America. The main obstacle to malaria control is the emergence of drug resistant strains of *Plasmodium falciparum.* Emergence of resistance in *P. falciparum* to antimalarial drugs increases malaria morbidity, mortality and treatment cost. Chloroquine resistant *falciparum* malaria is more common in North eastern states of India. Chloroquine resistance is a major contributor to the increasing malaria-related morbidity and mortality. Malaria control efforts have been greatly affected by the emergence and spread of chloroquine resistance. Because of the continued increase of resistance to antimalarial drugs in many regions of the world, with the resultant effect or morbidity and mortality, it is essential to ensure rational deployment of the few remaining effective drugs, to maximize their useful therapeutic life while still ensuring that safe, effective and affordable treatment is accessible to those at risk. This requirement has resulted in a re-examination of the potential of combinations of existing products and the development of new combination drugs [15].

1.3 Combination therapy in malaria

Drug combinations, rather than monotherapy , are now seen to be the best solution for treating malaria , and artemisinin based drug combinations are highly effective, with cure rates similar to that of chloroquine 30 years ago [16,17]. Combination therapy with antimalarial drugs is the simultaneous use of two or more blood schizonticidal drugs with independent modes of action and different biochemical targets in the parasite [18]. Fixed-dose combination and multiple-drug therapies are used to exploit the synergistic and additive potential of individual drugs. The aim is to improve efficacy and to retard the development of resistance to the individual components of the combination. This concept has been realized in multiple-drug therapy for leprosy, tuberculosis and cancer and, more

recently, antiretroviral treatments. It has also already realized to some extent in the field of malaria with the development of such drugs as sulfadoxine - pyrimethamine, atovaquone - proguanil and mefloquine-sulfadoxine-pyrimethamine [19]. As per WHO to improve efficacy and delay the onset of resistance, artemisinin drugs should always be used in combination with another effective antimalarial. It is the most rational way to use the few antimalarials available, maximizing the benefits to the patients while minimizing the risk of losing efficacy, secondary to the development of resistance. When used in combination with other effective antimalarials, the artemisinin derivatives, most artesunate and artemether have constantly achieved very high parasitological cure rates even against multidrug resistant strains [20]. In these emergencies when mortality is high, artemisinin derivatives save lives because of their given speed of action. Given orally, they are superior to intravenous quinine, in patients with uncomplicated hyperparasitaemia [20]. Increasing resistance of *Plasmodium falciparum* malaria to antimalarial drugs is posing a major threat to the global effort to "Roll Back Malaria." [21]. Artemisinin derivatives are particularly effective in combinations because of their high killing rates (parasite reduction ratios RR circa 10,000 fold per cycle) lack of adverse effects, and absence of significant resistance, Because of the short half-life of artemisinin derivatives, their use as monotherapy requires daily doses over a period of 7 days. Combination of one of these drugs with a longer half-life partner antimalarial drug allows a reduction in the duration of antimalarial treatment while at the same time enhancing efficacy and reducing the likelihood of development of resistance to the partner drug [22]. Artemisinin- based combinations produce rapid clinical and parasitological cure. Its parasite resistance is yet to be documented. These combinations reduce gametocyte carriage rate, and are generally well-tolerated [22]. For treatment of malaria artemisinin, artesunate, artemether and dihydroartemisinin have all been used in combination with other drugs like amodiaquine, lumefantrine [23]. The major immediate effect of the artemisinin component is to reduce the parasite biomass. The residual biomass is exposed to maximum concentrations of the partner drug well above its minimum inhibitory concentration, resulting in a lesser likelihood of resistant mutations breaking through [22]. The use of antimalarial drug combinations with artemisinin derivatives is recommended to overcome drug resistance in *Plasmodium falciparum*. The fixed combination of oral artemether – lumefantrine, an

artemisinin combination therapy (ACT) is proved as highly effective, and better tolerated. It is the only registered fixed combination containing an artemisinin [24]. Artemether – lumefantrine is the most viable artemisinin combination treatment available now. In addition to its efficacy, safety and tolerance profile, it is available as a fixed – dose formulation. This increases the likelihood of patient compliance with the drug regimen [22]. Trials showed that addition of artemisinin derivative artesunate to existing antimalarial treatment for three days, a marked reduction in the number of gametocytes is observed in the month after following the treatment. Artemisinin derivatives are the most rapidly acting of all antimalarial drugs and produce the fastest clinical responses to treatment. They are noticeably better than antimalarial drugs. The ACTs are now accepted as the best treatments for uncomplicated falciparum malaria, and policy change has taken place in most countries so make these the first line recommended drugs. The evidence base for efficacy and safety has grown considerably in recent years as these have become the most studied of all antimalarial drugs. Together with improved diagnosis and appropriate vector control measures, the ACTs should have a significant effect in reducing the burden of malaria throughout the tropical world, but to achieve this they will need to become more affordable and more available. As the deployment of Acts increases, we will need to invest more in education, health service delivery, monitoring of rare adverse effect and assessment of resistance to optimize their use, there by ensure they have the greatest impact on malaria.

1.4 Pharmaceutical Dosage Forms

The growth of pharmaceutical industry in last three decades has been a pointer towards the requirement of quality medicines to alleviate diseases, ill health and suffering to animals and mankind. The simplest therapy of known organic molecules like those that aspirin, sulphadiazine and naturally occurring medicines from plant origin have been replaced now by complex organic molecules, which are analogues, derived from multiple synthetic routes. In the event of usage of such molecules, the establishment of analytical profile of these molecules together with the desired medicinal activity like safety and efficacy has been the focus of attention of world-renowned scientists and pharmacist.

Drug substances are seldom administered alone, but rather as part of a formulation. Each particular pharmaceutical product is a formulation unique into itself. In addition to the active therapeutic ingredients, a pharmaceutical formulation also contains a number of non-therapeutic agents. These agents are generally referred to as pharmaceutical adjuncts, excipients or necessities, and it is through their use that a formulation achieves its unique composition and characteristic physical appearance.

There are many different forms into which a medicinal agent may be placed for the convenient and efficacious treatment of a disease. Most commonly known dosage forms are capsules, tablets, injections, suppositories, ointments, aerosols and many more including modern drug deliver systems like use of prodrug.

1.5 Development of Drug Products

The development of drug & drug products in the pharmaceutical industry is a long-term process, often taking more than a decade from the start of a research project to appearance of the drug on the market. That process involves several decision points, such as the choice of the candidate drug after preclinical screening phase, the investigational new drug (IND) application before testing the compound for the first in man and finally the new drug application (NDA) which summarizes the data obtained from all the studies needed for marketing approval of the drug as a medicine.

Analytical chemists take part in many of the studies that constitute this documentation. Drug analysis and assay play important role in the development, manufacture and therapeutic use of drugs. Drug quality and its specifications are based on drug analysis, and that knowledge is later used for quality control during full-scale production.

1.6 Pharmaceutical Analysis

Analytical Chemistry

Analytical chemistry is a scientific discipline that develops and applies methods, instruments and strategies to obtain information on the composition and nature of matter in space and time [25]. The branch of analytical chemistry, which may be termed as an "infant", has grown in leaps and bounds and has stood to be the greatest tool and support

to the other branches of chemistry like organic chemistry and biochemistry and other branches of chemistry. Analytical chemistry differs from other branches of chemistry in both, its scope and approach. While the other disciples are aimed at acquiring knowledge and creating theories in their respective fields, analytical chemistry develops method and tools necessary to acquire information about the chemical composition, its changes over time, its spatial arrangement and the structure of molecules and crystals. The methods of analytical chemistry incorporate not only the knowledge from chemistry, physics, physical chemistry and related technical fields but also from mathematics viz: probability theory and mathematical statistics [26].Thus, analytical chemistry is more a multidisciplinary field of natural sciences than pure chemical branch nevertheless; analytical chemistry is an area of chemistry because it deals with the determination of chemical composition. Chemical scientists, physical scientists, biological scientists and engineers have an impressive array of powerful and elegant tools for gathering qualitative and quantitative information about the composition and structure of matter. The correct choice and efficient use of modern analytical instruments requires an understanding of the fundamental principles of operation of these measuring devices [26]. Only when the student –analyst attains such understanding that, reasonable choices be made among the several alternative means of solving an analytical problem; only then the student- analyst will be aware of the pitfalls that accompany physical measurements, and only then will the student be sufficiently attuned to the limitations in sensitivity, precision and accuracy of an instrumental measurement[27]. Analytical chemistry is the science, which deals with methods for detection, identification and quantification of chemical, biological and microbiological species in matrices of chemical, biological and environmental importance. A qualitative method yields information about the identity of atomic or molecular species or the functional groups in the sample; a quantitative method provides numerical information as to the relative amount of one or more of these components [28]. Pharmaceutical analysis is traditionally defined as analytical techniques in chemistry dealing with drugs both as bulk drug and pharmaceutical products i.e. formulation. In pharmaceutical industry, generally a bulk drug is referred to as drug substance or API (active pharmaceutical ingredient) where as pharmaceutical product is referred to as drug product or finished product.

However in academia, as well as in the pharmaceutical industry, other branches of analytical chemistry are also involved viz. bio-analytical chemistry, drug metabolism studies and analytical biotechnology. Drug analysis means identification, characterization and determination of drugs. Drug assay refers to determination of drugs in mixtures such as dosage forms and biological fluids. Drugs may be gases, liquids or solids. Drugs used in formulations such as syrups, tablets and aerosol preparations are referred to as bulk drugs. Prior to the formulation and manufacture of dosage forms, bulk drugs must be properly identified (qualitative analysis) and analyzed for drug content (quantitative analysis). Qualitative and quantitative determination of drugs and related compounds (metabolites) in biological fluids are also necessary once drugs are used in animals and humans during experiment development and treatment of patients.

1.6.1 The Analytical Methods

These analytical techniques can be broadly classified as follows

1) Classical techniques
2) Instrumental techniques

1) Classical techniques

They are group of analytical methods that require the use of chemicals, a balance, calibrated glassware and other commonplace laboratory apparatus such as funnels, burners or hot plates, flasks and beakers. The basic principle of classical techniques involves the measurement of mass or the volume. These are further classified as gravimetric technique and volumetric technique [29, 30].

a) Gravimetric analysis

It involves the critical measurement of mass change during a chemical reaction to determine the amount of chemical reactant in the sample. A classical gravimetric analysis consists of precipitation of a salt of assayed substance. The precipitation is filtered, dried and weighed e.g. Estimation of Ni present in an ore.

b) Volumetric analysis

It involves the critical measurements of volume of chemical reactant to determine the concentration of sample. Volumetric analysis is titration in which a solution of one of the chemical reactant in a burette is added to a solution of a second chemical reactant. The

solution in the burette is titrant and the solution in the vessel is titrand. The sample can be either titrant or titrand. A classical volumetric analysis makes use of an indicator to locate the end point. Volumetric techniques depend upon the type of chemical process taking place and hence are divided further into sub classes such as neutralization, precipitation, complexometric, redox and non-aqueous methods.

2) Instrumental Techniques

With tremendous development in technology and the advent of computers, the instrumental methods of analysis are becoming the most popular methods in the field of qualitative and quantitative analysis. Instrumental techniques are especially preferred in cases where the amount of the analyte to be determined is small and the results have to be very accurate and precise. Analytical instruments are devices that measure some factors that enable us to measure the substance. Rationally instrumental techniques are divided in to three categories, according to the type of property of assayed substance that is measured or used during the assay. The spectral methods use or measure some form of radiations during the assay. The electro analytical methods apply an electrical signal to the sample and monitor an electrical property of the sample. The separative methods rely upon the separation of the sample prior to measuring a property of the sample [29, 30].

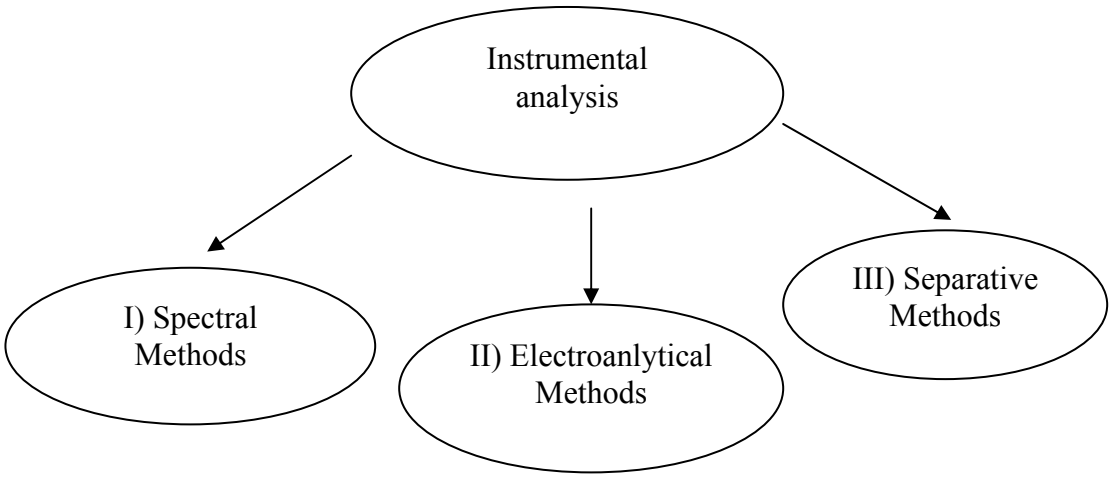

Figure - 1.1 Instrumental methods

I) Spectral Methods:

In the spectral methods, an instrument is used to measure the amount of radiation that is absorbed, emitted or scattered by the sample. Following are the various spectral instrumental methods:

1) Atomic Absorption.
2) Atomic Fluorescence and Ionization.
3) Flame and Atomic Emission.
4) UV-Visible absorption.
5) Chemiluminescences and Electrochemiluminiscence.
6) Fluorescence and Phosphorescence.
7) Infrared Spectroscopy.
8) Photoacoustic Spectrometry.
9) Scattering Spectroscopy.
10) Refractometry.
11) Nuclear Magnetic Resonance.
12) Electron Spin Resonance.
13) X-ray Methods.
14) Electron Methods.
15) Radio Chemical Methods.

The most commonly used methods in pharmaceutical analysis of drug product are briefed below

Atomic Absorption Spectroscopy

If the amount of radiation is measured, the technique is atomic absorption spectrophotometery. Except for naturally occurring radioactive materials radiation can be emitted from a sample only after the sample has absorbed energy from some outside source.

Fluorescence and Phosphorescence

If the absorbed energy is electromagnetic radiation in the X-ray, Ultra violet or visible region of the spectrum, the subsequently emitted electromagnetic radiation is a form of Luminescence termed either Fluorescence or Phosphorescence depending upon the

manner in which the excitation takes place. Fluorescence measurements are usually restricted to excitation in the X-ray and UV-Visible region. Phosphorescence measurements normally used to assay polyatomic species after excitation in the UV-Visible region.

UV – Visible absorption spectroscopic methods of analysis

Spectroscopy is a technique covering the absorption spectra of UV region (190-400nm) and color measuring visible range (400 – 800 nm). In the development of UV assay procedure for a particular compound, a solution of known strength between 5 to 100 µg mL^{-1} is made in suitable solvent, which is transparent to UV region. The solution is scanned in UV region for the absorption maxima against the reagent blank to find out the wavelength maxima. Certain organic molecules bearing known functional group when reacted with organic reagents or organometalic reagents form colored species in presence of acidic or alkaline media. Examples of such kinds are primary aromatic amines , which undergo diazotization in presence of hydrochloric acid, and sodium nitrite to yield species in solution, which are called aryl, salts which when reacted with amino phenols or naphthalene derivatives forms soluble colored complexes, which can be measured in the visible region.The following equation, is widely accepted.

Chromogen + Auxochrome = Colored complex or dye.

A molecule containing a chromophore is called a chromogen. A group that does not itself absorb radiation but when present in molecule enhances the absorption by the chromophore or shifts the wavelength of absorption when attached to the chromophore, is called an auxochrome.

Spectral changes are of the following types: Bathochromic shift (Red shift) - absorption maximum shifted to longer wavelength, hypsochromic shift (Blue shift) – absorption maximum shifted to shorter wavelength, hyperchromism - an increase in molar absorptivity, hypochromism- a decrease in molar absorptivity.

Infra- red Spectroscopy

A molecule is constantly vibrating; its bonds stretch (and contract), and bend with respect to each other. Changes in vibrations of molecules are caused by absorption of Infra red light: Light lying beyond (lower frequency, longer wavelength, less energy) the red of the visible spectrum. Like the mass spectrum, an infrared spectrum is a highly characteristic

property of an organic compound and can be used both to establish the identity of two compounds and to reveal the structure of a new compound. The infra red spectrum helps to reveal the structure of new compound by telling us what groups are present in or absent from the molecule. A particular groups of atoms gives rise to characteristic absorption band e.g. -OH group of alcohols absorbs strongly at 3200-3600 cm^{-1}; the C=O groups of ketones at 1710 cm^{-1}; the C≡N group at 2250cm^{-1}; the CH_3 group at 1450 and 1375cm^{-1}.

II) Electroanalytical Methods

The instrumental method of chemical analysis in which either an electrical signal is applied to one of the electrode dipping in the sample solution or an electrical property of the solution is measured is called as Electroanalytical method. Most electroanalytical methods apply an electrical signal while different electrical parameters of the solution are monitored. These methods are further classified as follows. Fig.1.2 shows schematic diagram of types of electroanalytical methods.

Amperometry

It is the method in which potential between the two electrodes is controlled and the current is measured.

Potentiometry

It is the method in which current between the two electrodes is controlled and potential is measured.

Electrogravimetry

In this method, mass of a reaction product (usually a metal) after exhaustive electrolysis is measured.

Voltammeter

A potential is applied to one of the electrodes while the current flowing through the electrode is measured in a technique.

Conductometry

The electrical conductance of the sample solution is measured in electro analytical method. Conductance is defined as inverse of electrical resistance.

Coulometery

When the quantity of electricity that is consumed during the electrochemical reaction is measured, the technique is known as Coulometery.

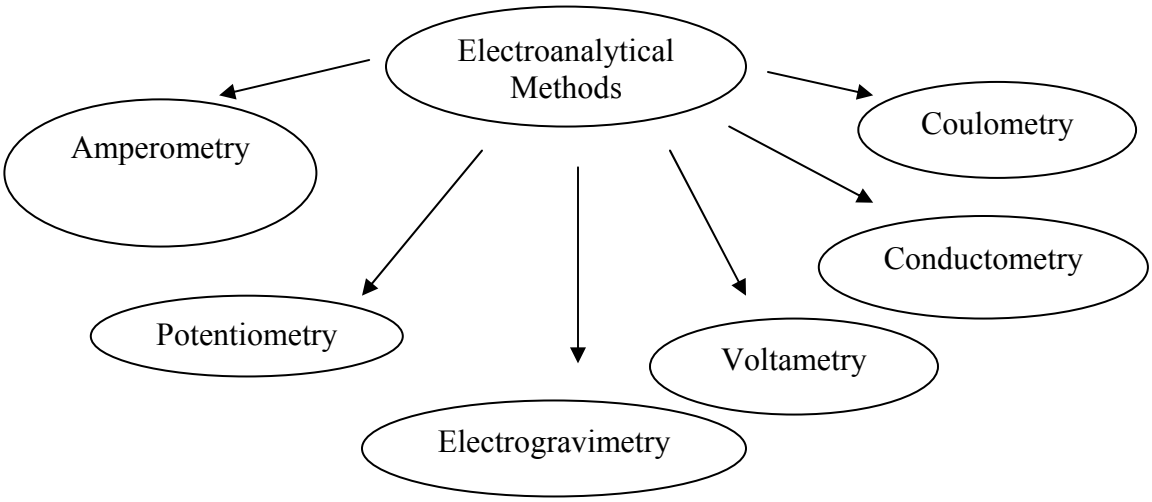

Figure 1.2 – Types of electro analytical methods

III) Separative Methods

Separative method takes advantage of physical or chemical properties of the components of a mixture to separate the components. After separation, the components can be individually assayed either qualitatively or quantitatively. The separative methods can be further classified as follows. Figure 1.3 shows schematic diagram of instrumental separative methods.

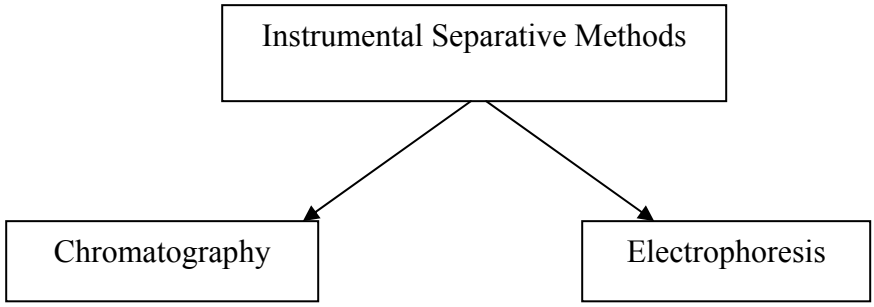

Figure 1.3 – Instrumental Separative Methods

Electrophoresis

The separative method takes advantage of the relative mobility of the ions towards an electrode of opposite charge and away from the electrode of similar charge. The buffered

solution through which the ions normally travel is supported by either porous paper or gel.

Chromatography

The first person to use chromatography was Tswett (1872-1919), the Russian scientist. He used chromatography a Greek word for Colors Chroma and write Graphein to describe his work on separation of colored plant pigments into bands on a column packed with chalk powder [31]. The chromatography is the science of separation, which involves the flow of mobile phase over a stationary phase. Chromatography is mainly divided into two parts,

1) Based on development

There are three types of methods e.g. elution development, frontal development and displacement development. Out of this elution, development is used in most separations while other two are used in some instances.

a) Elution Development

In this method, a sample is injected into the mobile phase as a discrete plug or band of material and is swept through the column by a continuous stream of mobile phase, which has a lower affinity to stationary phase than any components in the sample. If the sample components have different rates of migration through the column, they may be separated into separate bands with zones of pure mobile phase between them. If the composition of mobile phase remains unchanged then during the analysis, the technique is called Isocratic elution.

This technique is also used in GC as a flow and as an oven temperature and called as Isothermal. The only function of carrier gas is to transport the sample through the column. In case of complex mixtures, it may be preferable to change the composition of the mobile phase during the course of analysis. By changing, the composition of the mobile phase its strength or elution power can be changed and this changes the rate of migration of components through the column and the technique is called gradient elution.

b) Frontal development

In frontal development, the sample is swept continuously on to the column by the mobile phase. All the sample components will be held on to the column until the column becomes saturated with respect to a particular component. That component is then eluted

from the column. The component with the smallest affinity for the column will be eluted first, initially in the pure form and then as a mixture with next component to be eluted and so on until all the components eluted.

c) Displacement Development

In this method, a sample is placed on the top of column as discrete plug but in this, the mobile phase has a higher affinity to stationary phase than another component in the mixture. The mobile phase or displacer thus pushes the component bands of the column and they move down the column. They are separated according to their affinity to the column each component acting as a displacer for the component ahead of it.

2) Based on Mode of Separation

Based on the nature of interaction between the sample components and the two phases, it is further classified. The interaction involves various physiochemical processes occurring in the system, reflecting the relative attraction and repulsion that the particles of competing phases show for the solute and for each other. The mechanism can be classified into number of types, as

Adsorption, partition, bonded Phase, ion exchange, size exclusion, affinity, micellar, complexation,ion-exclusion and counter current.

Due to versatility and wide variety of applications of HPLC in analytical chemistry techniques, few techniques are described in details, which are used for this present research work.

A) Adsorption or Normal Phase Chromatography

Normal phase chromatography alternatively termed as an adsorption or liquid-solid chromatography. It was first used in the classical open column form in 1906 by Tweet to separate plant pigment. In normal phase chromatography, the components are retained on the stationary phase through the interaction of permanent dipoles of the component with permanent dipoles on the stationary phase [32]. With this separation mode, the more polar solute will elute with greater retention. Increasing the polarity of the mobile phase retention of the polar solute can be decreased. Retention in Normal phase chromatography is due to interaction of polar functional groups of the solute with the discrete site on the stationary phase surface. The selectivity of the separation depends upon the relative

strength of these polar interactions for different solute. Normal phase chromatography exhibits a unique ability to distinguish between the solute with different number of electronegative atoms, such as oxygen or nitrogen or molecules with different functional groups [32].

A wide range of stationary phase materials has been used in normal phase chromatography. These include sucrose, cellulose, starch, silica gel, charcoal, magnesium oxide and alumina. Silica is the preferred stationary phase for a number of practical reasons. Silica allows higher sample loading and is less likely to catalyze the decomposition of any sample components while alumina has been known to catalytically decompose many organic compounds. A much wider range of silica column is available commercially and there is greater coverage on the application of silica stationary phase for different analysis in the literature. However, it may be occasionally advantageous to use alumina, for particularly basic compounds, which are very strongly retained on silica column. Conversely, carboxylic acid is very strongly retained on alumina sometimes irreversibly and hence is best chromatographer on silica [32].

In general, the retention and separation characteristics of Silica and Alumina are similar with more polar solvents being more strongly retained. The usual elution order is Hydrocarbons< Olefins < aromatic hydrocarbons < organic halides < sapphires < ether < nitro compounds < ester < baldheads < ketones < alcohols < amines < sulfones< sulphoxide < amides < carboxylic acids. In normal phase chromatography the adsorption sites on silica are hydroxyl groups (-OH) and the grouping (-Si-OH) is known as silanol groups. These silanol groups can exist on silica surface in a number of configurations depending upon the manner in which the silica has been treated prior to use [32]. This treatment of silica controls its activity, i.e. the number of active sites per unit surface area and the adsorptive strength (acidity) of these sites. Heating the silica in the presence of controlled quantities of water and acid varies the activity. Silica used in the HPLC is typically heated to 200-300^{o}C, which allows the formation of a fully hydroxylated surface. The individual silanol groups can exist in three general forms, with free silanol being the most acidic and geminal silanol groups being the least acidic. Free silanols generally occurring at relatively low concentration on the silica surface. Their highly acidic nature causes strong retention of basic solutes; hence, their presence is undesirable

for the separation of basic compounds. In addition to the type of material and its activity other factors that affect the retention of sample molecule are surface area and average pore diameter or particle size. Sample retention is directly proportional to surface area. Porous silica materials typically have surface areas in the range of 100-250m^2g^{-1} for spherical particles and about 300-400m^2g^{-1} for irregular particles. A practical optimum value of 400 m^2g^{-1} exists for silica particles. Probably the greatest drawback of normal phase chromatography is the lack of separation selectivity between different packing materials; virtually all compounds elute in the same order regardless of the column selected [32]. More recent alternative to the use of bare of silica for normal phase chromatography is the use of silica functionalized with diol, cynopropyl or amino groups. These functional groups are all significantly less polar than the silanol groups and hence give less retention of the polar solutes.

Mobile phase for normal phase chromatography

Adsorption separation is most commonly carried out on silica stationary phase and variation in retention time is generally achieved by altering the mobile phase composition. Therefore, the most important factor in optimizing a normal phase separation is the selection of the mobile phase composition.

The total interaction of a solvent molecule with a sample compound is the result of four interactions, these being dispersion, dipole, hydrogen bonding and dielectric polarity of the compound. Hence, polar solvents preferentially attract and dissolve polar compounds. In the same manner in the chromatography, the strength of solvent in normal phase system is related to its polarity. However, it has been shown that a better index of solvent strength for adsorption chromatography is given by the experimental adsorption strength parameter, ε^o. This parameter, which is measure of the adsorption energy per unit area of solvent, can be used to quantitatively define solvent strength for given adsorbent. A series in which the solvents are arranged in increasing order with both silica and alumina as adsorbents is termed as allotropic series. An increase in the value of ε^o means stronger solvents, leading to lower retention for a given solute. On the average, the values of ε^o for silica are 0.8 times less than that for alumina.

Appropriate mobile phase strength can be determined by selecting two solvents whose ε^o is too small (A) and too large (B) respectively. It is also necessary to take proper care to

ensure that two immiscible solvents are not used. In such cases, a co solvent (like methylene dichloride for hexane phase) should be added to the system to enhance the miscibility of two solvents. Further more the various chemical processes that the solvent undergoes like oxidation should also be considered and appropriate stabilizers (like 3-4 % ethanol), if necessary should be added.

The greatest problem associated with operation of normal phase chromatography is the effect of water, which has activity of polar adsorbents. Water is adsorbed on to strongest adsorption sites, leaving a more uniform distribution of weaker sites to retain the sample. This leads to a decrease in solute retention and adsorbent is said to be deactivated. This creates problem, since in practical terms it is very difficult to control the amount of water present in the mobile phase. The water content of mobile phase varies because of the Variation exists in the amount of water present in solvents, depending upon the humidity. Water is readily picked up and lost to the atmosphere and changes occur because of contact of the solvent with walls of the solvent reservoirs and the other containers.These problems are typically overcome by initial addition of small amount of water in the mobile phase. The beneficial effects of addition of water in mobile phase are it leads to less variation in the sample retention from run to run. It takes care of higher sample loading. It enhances column efficiencies and reduced peak tailing for basic compounds. It reduces batch to batch variation of the adsorbent.

B) Reversed Phase Chromatography

Reversed phase chromatography, alternatively termed as bonded phase chromatography, is the most widely used in liquid chromatography separation mode. It has been reported that over 75% of all high performance liquid chromatographic (HPLC) separations are performed using this approach [33]. The term reverse phase arises from the fact that this separation mode utilizes a non-polar stationary phase with a polar mobile phase, which is the reverse of the situation in normal phase chromatography. This approach was originally applied in liquid- liquid chromatography, in which stationary phase was film of liquid held on solid support. The mechanism of reverse phase chromatography is complex and sample components are retained through nonspecific hydrophobic interaction with the stationary phase [33]. In this separation mode, the more polar solute, the lower the

retention time where as non-polar solutes elute at greater retention time. Increasing the polarity of the mobile phase results in increased solute retention.

Separation in reverse phase chromatography is more difficult to explain than the normal phase chromatography. The interactions between solute molecules and the nonpolar stationary phase are much weaker to account for the degree of solute retention observed in reverse phase. The retention mechanism is much complex and could be best described as combination of partition and adsorption [33]. In a number of instances, the reverse phase chromatography has been referred to as partition chromatography, because the bonded organic surface layer can be regarded as a bound liquid film, although some conclude that the bonded phase acts more like a modified solid than liquid film. In addition, the heterogeneous nature of silica based materials means that different mechanism can operate over different regions of the surface.

It is generally accepted that the solvophobic theory offers the most valid interpretation of reverse phase chromatography retention. This mechanism, which is perhaps more appropriate when using aqueous mobile phase with low organic modifier content, is based on the assumption that the stationary phase is a uniform layer of a nonpolar ligand. The solvophobic theory assumes that the solute binds to the stationary phase, which then reduces the surface area of the solute exposed to the mobile phase. The solute is adsorbed as results of this solvent effect, i.e. the solute is adsorbed because it is solvophobic. Hence, solutes are retained more as result of interactions with the mobile phase rather than through specific interactions with the stationary phase. The fact that retention occurs primararily because of strong interactions between the mobile phase and the solute molecule means the mobile phase composition has more influence on separation selectivity than the stationary phase in this mode of chromatography [33].

Stationary Phase for Reverse phase Chromatography

There are two types of stationary phase available for reverse phase chromatography.While variations in the mobile phase composition is the most common means of altering the separation, selectivity in reverse phase chromatography, vast number of silica bonded phase columns are available and subtle differences in their selectivity often means that separation on apparently similar column types may show appreciable differences.

a) Bonded Phase Silica column

Factors such as base silica material and its pretreatment, type of stationary phase bonded on to the silica, amount of stationary phase material (carbon load) bonded on to the silica and any secondary bonding (end capping) reactions determine the performance of bonded phase silica column.

Generally, reverse phase columns are manufactured by functionalizing the same type of silica materials used for normal phase columns. Silica characteristics, such as pore size and distribution, particle size and shape, surface area and degree of activity will all affect the final performance of the bonded phase column[34]. The strength of interactions in reverse phase between the stationary phase and polar solutes depends upon the surface activity of the silica used as the support material. It also depends upon the pretreatment differences of the silica, in terms of degree of heating and water content. The most common method involves the reaction of silica with organochlorosilane to produce siloxane (Si-O-Si-R_3) reversed phase packing. Either mono- di- or tri-functional organochlorosilane-bonding reagent may be used [34].Reversed phase columns are identified and named by the nature of the bonded R groups. C_{18} is the most common functional group on reversed phase column. Other functional groups include C_8, Phenyl, C_4, C_2, CN, Diol, NH_2 and NO_2 [34]. Retention in reverse phase increases exponentially as the alkyl chain length of the bonded functionality increases. The term carbon load describes the amount of material bonded onto the silica, which is the amount of carbon expressed as a weight percentage of the bulk silica packing. Monofunctional C_{18} bonding reagents typically produce columns with carbon loads in the range of 7- 15% (w/w). Monofunctional bonding reactions are generally preferred as they can be carefully controlled, producing monolayer functionalized column with less batch-to-batch variation than bi- or tri- functional bonding reagents are used. Monofunctional C_{18} dimethylsilanes are the most widely used bonding reagents. The use of bi- or tri- functional bonding reagents results in both cross-linking and linear polymerization reactions and produces 'polymeric' C_{18} stationary phases, columns produced in this fashion can have carbon loads as high as 25% although they often exhibit low chromatographic efficiency as a result of poor mass-transfer characteristics.

Generally, the higher the carbon load of stationary phase, the greater its reverse phase retention. Column with different functional group may show different separation selectivity for certain solute mixtures. The final factor that affects the performance of bonded phase silica columns is the degree of end capping. Under optimum conditions, approximately 60% of total silanol groups being functionalized; however the reminder are effectively shielded by the dense layer of trimethyl silane group [34]. The longer the chain of bonded phase the retention time increases. The length of bonded phase chain also affects the column efficiency. A decrease chain also appears to increase column efficiency, particularly for well-retained solutes. One concern over the performance of bonded phase column is the reproducibility of some commercially available packing. For majority of reverse phase separation a good general-purpose column would be one 15 to 30 cm in length packed with C_{18} functionalized with 3 to 5µ spherical silica with pore size of 60 to 120 A^o and carbon load of 7 to 10%. The column should be end capped and have an efficiency of 5000 to 10,000 theoretical plates. For the stability of bonded phase column, generally the mobile phase should be within pH range of 2 to 8. Below pH 2.0, hydrolysis of bonded functional groups occurs, resulting in decreased retention. Above pH 8.0, silica, a weak acid dissociates and silica support to dissolve. This creates void in packing material, resulting in decreased column efficiency. The majority of bonded phase column can be operated with wide range of organic solvents at elevated temperature of approximately 80^oC [34].

b) Polymeric Reverse Phase Columns

Some polymeric materials are sufficiently hydrophobic to enable the reverse phase retention. The major advantages of these polymeric reverse phase columns, are that they can be operated over the pH range of 2.0 to 12.0 and have different selectivity when compared with silica-based columns. The greatest disadvantages of polymeric columns are they are usually less efficient than silica based column and tend to be less retentive [34].

Mobile phase used in the reverse phase chromatography

In reverse phase chromatography, the most important factor in optimizing the separation is selection of mobile phase. The great utility of reverse phase arises from the fact that the wide range of solvents available provides the much scope for easily altering the separation selectivity.

When developing a reverse phase separation, first step is to select a solvent mixture, which permits optimal sample retention, i.e. $1 < K' < 20$[35]. Choosing other solvents with similar retention characteristic, but form a different selectivity group can be then varying the separation selectivity. Solvent strength in reverse phase chromatography can be explained as a function of polarity and the eluting strength of reverse phase solvent is inversely related to its polarity. Therefore, polar solvents are weak eluting solvents in reverse phase chromatography while non-polar solvents are strong eluting solvents. Water is generally as the best solvent for most of the reverse phase chromatographic separation and the mobile phase strength is determined by mixing water with an appropriate volume of another solvent (termed as organic modifier). In practice, methanol is most widely used organic modifier as it has relatively low UV cut off (205 nm), reasonable eluting strength and is inexpensive. Acetonitrile, (which has allowed UV cut off (190 nm) better mass transfer properties is more expensive), is the next most commonly used solvent, followed by tetrahydrofuran [35]. Generally, these three solvents combine with water permits sufficient retention and selectivity variation for the majority of reverse phase separation.

Other factors, which affect the retention in reverse phase chromatography, are temperature, pH and mobile phase additives.

Generally, retention decreases with an increase in the temperature. Elevating temperature is used to decrease the viscosity of mobile phase in order to improve the chromatographic efficiency and to reduce the column back pressure and to get reproducibility of the retention time. The mobile phase pH requires considerations if solute molecules are affected by the pH, i.e. if they are acidic or basic compounds. The retention of such molecules can be manipulated through selective pH control. Thus, basic molecules elute faster in acidic mobile phase and retain in alkaline mobile phase.

Gel Permeation Chromatography (GPC)

GPC is alternatively termed as size exclusion chromatography or when applied to separation of water soluble polymers and proteins, is termed as Gel filtration chromatography and it is preferred method for separating high molecular weight neutral solutes (>2000 –3000). In GPC, solutes are separated because of their permeation into solvent filled pores within the column packing. Large molecules are excluded from some or all of the pores by virtue of their physical size, while smaller molecules permeates into greater proportion of the pores. GPC is mainly used to characterize the molecular weight distribution of polymer materials. The greatest advantage of GPC is that retention is very predictable; consequently, the retention time can be used to predict the molecular weight of unknown molecule. The major disadvantage of GPC is the lack of resolving power i.e. its limited peak capacity generally the solute must differ in molecular size by at least 10% in order to be resolved by GPC.

Ion exchange chromatography

The technique of ion exchange chromatography is one of the best-established forms of LC, having been used for many years for the separation of ionic solutes, usually in aqueous solutions. An ion exchanger in aqueous solution consists of anions, cations and water where either cations or anions are chemically bound to an insoluble matrix. The chemically bound ions are referred as fixed ions and the ions of opposite charge are referred as counter ions. Counter ions may move through the matrix by diffusion and in the ion exchange process itself, are replaced by the ions of same charge from the external solution. The ion exchanger is classified as cation exchange material when the fixed ions carrya negative charge and as an anion exchange material when the fixed ions carry a positive charge.

Thin Layer Chromatography (TLC) /High Performance Thin Layer Chromatography (HPTLC)

The basic principle of TLC is the transfer of mobile phase through the thin layer due to the capillary forces. The stationary phase and supports are all micro-porous solids showing high specific surfaces. In HPTLC, the particle size of stationary phase is around 6µm as compared to 12 to 20µm in TLC. A large variety of stationary phases like polar inorganic (Silica, Alumina), non-polar inorganic (Graphite, Charcoal), non-polar bonded

phases (C_2, C_8, C_{18} bonded phases), polar bonded phases (Amino propyl, Cyno propyl) and polar organic (Cellulose, polyamides) are used. The HPTLC instrumentation consists of three parts: the sample applicator, the development chamber and the scanner or detector [36, 37].

Gas Chromatography (GC)

Gas chromatography is that form of the general chromatographic technique in which the mobile phase is a gas (carrier gas). The stationary phase may either be a liquid of low volatility coated on an inactive solid support and packed into a long narrow column. In open tubular columns, the walls are coated with substrate or an active solid. According to stationary phase used, called gas liquid (GLC) or gas solid (GSC) chromatography. The adsorption on the solid is the main controlling factor in Gas Solid Chromatography. The essential parts of Gas Chromatography include supply of carrier gas at constant pressure, pressure regulator, monometer to measure the pressure at the inlet of column, sample introduction system, chromatographic column (thermo stated), detector and recorder [38].

Carrier gas

The carrier gases, which normally used, are helium, hydrogen, nitrogen, argon etc. The choice of gas depends upon availability, purity, inertness towards sample and stationary phase and the compatibility towards the detector used [38]. Usually carrier gas flow ranges from 20 to 100 ml/minute for packed column and 1 to 10 ml /minute for capillary column. The gases should be sufficiently pure such that dust particle, water and other volatile materials are excluded from the line.

Sample system

Sample transfer devices are available to introduce reproducible amount of sample into the system. The use of internal standard in the sample preparation helps to reduce the variation in sample introduction. Sample can be introduced either liquid syringe for liquid samples or gas tight syringe by converting sample matrix in gaseous phase. The sample technique using gas tight syringe is termed as headspace chromatography. The most common way to introduce a liquid sample is using a liquid syringe. The sample is injected through a self-sealing septum into the head of the column or into the vaporization chamber from which the sample is rapidly vaporized and swept by the carrier gas to the

head of column. The injector should also be at higher temperature than the column and have sufficient mass to supply the heat necessary for vaporization [38].

Columns

The column is the heart of Gas Chromatography for it is within the column that the process of separation takes place. Column used in Gas Chromatography are classified as Open Tubular (capillary column) and packed column. In capillary column, a film thickness 0.5 to 3 μ is desirable. The usual size for internal diameter of capillary column is in the range 0.25 to 0.50mm and length of 1to100 m. The packed columns are usually 1 to 5m in length and internal diameter about 1/8 inch [38].

Temperature Control

In GC, temperature plays very important role in separation as well as peak shape. The temperature of injector port is usually maintained at slightly above the boiling point of components, to provide sufficient and instant heat for vaporizations of sample. Among the detectors, TCD is the most sensitive detector to temperature programming. Usually the detectors are maintained at a temperature slightly higher than the highest temperature at which column is operated. The retention time of a component is inversely proportional to column length and column temperature. In isothermal operation, column temperature is maintained at middle point of the boiling range of sample. In a complex sample, where individual sample components have different range of boiling point, chromatography separation is started at relatively low temperature and during the later stage of analysis; the temperature is increased at a gradual rate to attain a fixed final temperature. Such kind of analysis is called as temperature programmed analysis.

Stationary Phase

The liquid used as stationary phases in packed and capillary column are closely related. Properties desirable of an ideal liquid phase are a low vapour pressure, thermal and chemical stability, low viscosity, non reactivity towards sample components and wide operating temperature range extending from $-80^{o}C$ to $450^{o}C$. The volatility or the thermal stability of the phase determines the upper temperature limit of the operating range that is referred as maximum allowable operating temperature. Exceeding this temperature value will accelerate the degradation of the phase. Stationary phase can be divided into non-polar, polar and specialty phases. The non-polar phases contain no functional group

capable of specific interaction with the sample. Here the interactions between the solute and stationary phase are limited to dispersive forces and components, therefore, separate according to their volatility with the elution order following their boiling point. Compounds that cannot be separated based on their boiling point require a different stationary phase for separation. To obtain the separation of solutes by forces other than dispersive forces, polar stationary phases are selected. The elution now depends upon a combination of boiling points and specific polar-polar interactions.

Mass Spectroscopy

In the mass spectrometer, molecules are bombarded with a beam of energetic electrons. The molecules are ionized and broken up in to many fragments, some of which are positive ions. Each kind of ion has a particular ratio of mass to charge or m/z value. For most ions, the charge is 1, so that m/z is simply the mass of the ion. The set of ions is analyzed in such a way that a signal is obtained for each value of m/z that is represented; the intensity of each signal reflects the relative abundance of the ion producing the signal. The largest peak is called as base peak. A plot or even a list showing the relative intensities of signal at the various m/z values is called mass spectrum and is highly characteristic of a particular compound. Mass spectra can be used in two general ways one is to prove the identity of two compounds and another is to establish the structure of new compounds [39].

1.7 Development of a New Analytical Method

In the second half of the last century, the technological developments of instrumental analysis were so wide and rapid that today the field of analytical chemist has expanded towards “computer based analytical chemistry”.

One of the major decisions to be made by an analytical chemist is the choice of the most effective procedure for a given analysis. In order to arrive at the correct decision, he must be familiar with the practical details of the various techniques and of theoretical principles upon which they are based. The techniques have differing degrees of sophistication, of sensitivity, of selectivity, of cost and of time requirements.

In view of the existing approaches for the development of degradation profile of drug product, it can be said that the technique of Liquid Chromatography can be developed into an invaluable laboratory tool for the separation and identification of compounds.

The technique of HPLC is over 30 years old and is in mature stage of development and application. HPLC is a chemistry-based tool for separation, quantification and analysis of mixtures of chemical compounds. Because of the enormous development of the analytical technology in the last two decades, entirely new possibilities have been created for the determination of the purity of drug materials. Nearly all organic impurities are determined by chromatographic or related methods of which HPLC has been the most important for over a decade. HPLC is regarded as the most important analytical method in pharmaceutical analysis as it provides a number of highly selective variants to resolve almost every type of separation problem [40]. Derivatization of the drugs prior to analysis is normally not required. HPLC can be operated in both modes i.e. reverse phase and normal phase mode. Reverse phase analysis involves use polar mobile phase (e.g. water, methanol, acetonitrile, etc.) along with stationary phase like C_8, C_{18}, phenyl etc. Normal phase analysis involves use of non-polar solvents (e.g. hexane, dichloromethane, ethyl acetate etc.) along with silica as a stationary phase. Reverse phase analysis is useful for polar compounds (egg. amines alcohols, acids etc) while normal phase provides separation of non-polar compounds. In reversed-phase liquid chromatography, increasing the molecular size increases the hydrophobicity of solutes and results in a greater retention volume. This indicates that the van der Waals volume is an important property in optimization. Increasing the number of substituents with n-electrons and hydrogen bonding increases the solubility in water, i.e., they increase the polarity of the solutes. This indicates that dipole-dipole and hydrogen-bonding interactions contribute to hydrophobicity. Therefore, these properties are important in controlling the retention volume in reversed-phase liquid chromatography. However, the n-electrons of stationary phase materials such as polystyrene gel and the hydrogen bonding of non-end capped bonded silica gels also contribute to the retention. Many compounds can be analyzed by both the methods. For a preparative-scale separation, normal-phase chromatography is suitable due to the easy removal of solvent

Gradient elution (change in mobile phase composition with respect to time), temperature and wavelength programming techniques provide valuable information regarding the undetected components of a given drug substance. UV-absorbing components are easily detected, if present in sufficient quantity. Multiple wavelength UV detection program is capable of monitoring several wavelengths simultaneously.

Photodiode-array (PDA) detectors are used to record spectro-chromatograms simultaneously. PDA allows simultaneous collection of chromatograms at different wavelengths during a single run. Following the run, a chromatogram at any desired wavelength can be displayed. The UV spectrum of each separated peak is also obtained as an important tool for selecting an optimum wavelength for HPLC analysis. By examining UV spectrum for a peak from beginning to end, peak purity can be evaluated.

Fluorescence (FL) detectors are exquisitely sensitive and selective, making it ideal for trace analysis. The detection is based on analyte fluorescence by applying monochromatic light of desired wavelength for excitation of sample molecule. Refractive index (RI) detector is considered as a universal detector since the RI is a physical property of all compounds, any compound can be detected at moderate levels.

Electrochemical (EC) detectors commonly used in HPLC can be classified according to their operation: (i) direct- current amperometry (DCA) and (ii) conductivity. If the analyte is EC active, a DCA detector is usually preferred because sample derivatization and related problems are usually avoided. EC detection can be performed in either oxidative reductive mode depending on the nature of analyte. Conductivity detectors are mainly used in ion chromatography [41].

Method development and optimization of chromatographic method is an attractive challenge in analytical research.

Method development is based on several considerations. It often follows the series of steps [34].

In short on what is required of the method should be clearly understood before method development begins

1.7.1 Sample Pretreatment and Detection

Before the first sample is injected during HPLC method development, we must be reasonablysure that the detector selected will sense all sample components of interest. For this reason, information on the UV spectra can be important aid for method development. UV spectra can be found in the literature, estimated from the chemical structures of sample components of interest, measured directly or obtained during HPLC separation by means of photodiode-array (PDA) detector. Variable wavelength UV detectors (PDA) are the first choice, because they are convenient and applicable for most of the sample. If UV response is inadequate then other detectors are available e.g. Fluorescence, electrochemical etc. or sample can be derivatised for enhanced detection [42].

1.7.2 Analytical Tests included in finish product Analysis

For any drug product, there are the typical methods to be developed for intended use to establish the quality of product like determination of content of active in the dosage form i.e. assay secondly determination of related impurities in dosage form, determination of drug release i.e. dissolution and stability indicating method to establish stability of the product[43].

Determination of Assay

Assay is a measurement of active content in the drug product. It is one of the important quality tests for a finished product. It indicates purity of the product. It can be determined by quantitative method like Titrimetry, Spectrophotometric and by HPLC. We will discuss more on HPLC methods as these are now widely used techniques. For developing assay by HPLC following steps are involved in determination of assay

a) Good resolved peak of the active drug
b) A measurable response of the active peak.
c) Extraction of the active drug from the placebo matrix.
d) Validation of the method.

Determination of related impurities in Dosage form.

This is again most important test for purity of any dosage form. We require to study from the literature or chemical abstract which are the known or unknown impurities present in the active and which are the degradants. Techniques like Differencial Scanning Calorimetry (DSC), Thin layer chromatography (TLC) and HPLC (High performance liquid chromatography) are commonly used techniques for determination of impurities. TLC and DSC are not quantitative techniques so amongst all of them HPLC is the best suitable technique which is precise, accurate as well widely applicable and acceptable technique. Chromatography has revolutionized our ability to determine analyte purity and is currently the most important check giving essentially the fingerprint of a synthesis. Impurities and degraded products require separation method and usually studied from detection level to 150% of working concentration. This means that the analytes have to be quantified up to 1000 folds excess of major compound. This sometime creates a problem in chromatographic methods as minute amounts of related substance may be hidden under the peak of drug itself. This is background for the interest in peak purity test. With the help of diode array detection in liquid chromatography, compounds with different chromophores may be differentiated, either through spectral comparison or by absorbance rationing at selected wavelengths. However, a peak impurity present at below 1% may be difficult to detect using PDA detector. The use of mass spectral data for the verification of peak purity is still better. For qualitative studies, LCMS is beginning to establish its role as a primary online analytical tool for the elucidation of unknown structures among the impurities. From the toxicological point of view, the impurity profile of the analyte batch used in safety studies should form the reference for full-scale production material. This means that in the batches impurities in amounts that deviate from those found in the batches used for toxicology should be avoided. There are some other tests that also contribute to the general impression of the quality of analyte, i.e. test for photolytic impurities, content of chloride, supplanted ash or residue upon ignition that gives the inorganic impurities content. The general specifications for related known impurities are based on its toxicology. Where as limit for unknown impurities are set in accordance to maximum daily dose.

For drug product impurity limits will be as described in ICH guidelines [44, 45].

A) Reporting Level

Maximum daily dose	Threshold
≤ 1 gm	0.1%
> 1 gm	0.05%

B) Identification Level

Maximum daily dose	Threshold
<1 mg	1.0% or 5µg of total daily intake whichever is lower.
1–10 mg	0.5% or 20µg of total daily intake whichever is lower.
>10 mg – 2 gm	0.2% or 2 mg of total daily intake whichever is lower.
> 2 gm	0.1%

C) Qualification Level:

Maximum daily dose	Threshold
<1 mg	1.0% or 50 µg of total daily intake whichever is lower.
10 –100 mg	0.5% or 200µg of total daily intake whichever is lower.
>100 mg – 2 gm	0.2% or 2 mg of total daily intake whichever is lower.
> 2 gm	0.1%

It involves following steps

a) Good resolution of all possible impurities and the active drug

b) Scanning for suitable detectable wavelength.

c) Extraction of all impurities from sample matrix.

d) Limit of detection and quantification of all impurities

e) Linearity of impurities as well as the active.

f) Validation of the method.

Determination of drug release (Dissolution)

Dissolution testing is an integral component of new drug application to regulatory bodies. Dissolution is most often associated with assessment of in vivo performance. In vitro dissolution testing provides lot of information at all the stages of the drug product development process. It helps formulator to select appropriate excipients.

As per BCS classification drugs are classified in to four classes [46]

Class I - Highly soluble highly permeable drugs

Class II –Highly Soluble Low permeable drugs

Class III – Low soluble High permeable drugs

Class IV – Low soluble low permeable drugs

It is always easy to have dissolution method to be developed for Class I category drugs due to high solubility. Dissolution is also informative test for bioequivalence or pharmacokinetic Study. It is always dissolution profile, which determines quality of the drug product. Dissolution also keeps track on batch-to-batch variation. Dissolution testing guides to what extent the drug will be available to the body. There are statistical factors that are similarity factor and difference factor, which determines comparative dissolution profile. These factors play very important role in development of drug product for regulated markets. It helps the formulator to design the product as to match with reference product. The solubility of the actives is the most important challenge to achieve the sink condition in the dissolution vessel. In short, dissolution conditions should be chosen in such a way that will allow maximum discriminatory power or steepest dissolution profile during dissolution testing. In most cases, the dissolution apparatus tends to become less discriminating when operated at faster speeds that result in a flatter drug release profile. Use of a low rotation speed could result in a variation in the data due to poor hydrodynamics in the dissolution vessel and can become more a reflection of system design such as coning rather than true formulation changes. Visual observations such as incomplete dosage form disintegration, erosion or pellicle formation are especially useful during method development to understand the behavior of the tablet in the dissolution vessel. The agitation speed providing optimum hydrodynamics in the vessel can be determined by comparison of the dissolution profiles obtained by making small variations in paddle speed (robustness experiments) as well as by challenging the testing procedure through the use of mismanufactured tablets (discriminatory power experiments). The final dissolution procedure should be robust and should be able to distinguish small but real changes in the product formulation.

1.8 Analytical Method Validation

The analytical method validation is required in the pharmaceutical product development process to establish the confidence in the analytical methods used for testing the final product (release and stability testing), raw materials, in-process materials and excipients.

The analytical method validation is the process of establishing the scientific evidence through well-planned studies that an analytical method is acceptable for its intended application.

The recent guidelines for methods development and validation for new non-compendial (In-house) test methods are provided by the FDA draft document, "Analytical Procedures and Methods Validation: Chemistry, Manufacturing, and Controls Documentation". This recent document applies to the method development and validation process for products included in investigational new drug (IND), new drug application (NDA) and abbreviated new drug application (ANDA) submissions. Therefore, the regulatory agencies prospects for the analytical method development and validation are comprehensive [47-49].

The success in analytical method can be attributed to several important factors, which in turn contributes to regulatory compliance. In order to ensure compliance with quality and safety standards, the United States, Europe, Japan, India and other countries have published national or compendial (pharmacopoeial) guidelines that describe official test methods for many marketed drug products. In recent years, a great deal of effort has been put into the harmonization of pharmaceutical regulatory requirements in the United States, Europe, and Japan. As part of this initiative, the International Conference on Harmonization (ICH) has issued comprehensive guidelines for analytical method validation. The recent FDA methods validation draft guidance document as well as USP both refers to ICH guidelines [47-49].Validation of analytical method is an integral part of product development. The objective of validation of analytical procedure is to demonstrate that it is suitable for its intended purpose. As per the ICH, guideline for Method development the official methods need not to be validated for entire validation parameters but merely to be verified for their suitability and feasibility.

1.8.1 Characteristics of Analytical Procedures

Validation

Validation is the process of evaluating products or analytical methods to ensure compliance with product or method requirements. When equipment or a particular method has been selected and found to be validated, the equipment and particular method go through a system suitability test before and between the sample analysis. Validation

requires for equipments and analytical methods. The equipment including both instrument and computer controlling it needs to be validated prior to routine use.

1) Instrument validation: It includes following:

a) Installation Qualification: When installing the instrument in the laboratory the completeness of the shipment should be checked against the purchase order, the environment should be checked against the manufacturer specifications and the connection cables and power supply should be checked as recommended by the manufacturer.

b) Operational Qualification: This step is to verify that the instrument performs the function according to the defined specifications for accuracy, linearity and precision.

c) Performance Qualification: Before and during the routine use of the instrument should be tasted and calibrated to perform the analytical task properly. The frequency of these tests depends on the type and use of the instrument.

The characteristics that may need to be specified for analytical procedures are listed below and defined, with an indication of how they may be determined. Not all the characteristics are applicable to every test procedure or to every material. Much depends on the purpose for which the procedure is required. A tabular summation of the characteristics applicable to various analytical procedures is given in table 1.1.

Characteristics that should be considered for different types of analytical procedure.

Class A*:* Tests designed to establish identity, whether of bulk drug substances or of a particular ingredients in a finished dosage form.

Class B: Methods designed to detect and quantify impurities in a bulk drug substance or finished dosage form.

Class C: Methods used to determine quantitatively the concentration of a bulk drug substance or of major ingredients in a finished dosage form.

Class D*:* Methods used to assess the characteristics of finished dosage forms, such as dissolution profiles and content uniformity.

Validation of Analytical procedures is directed to the four most common types of analytical procedures.

• Identification Tests - Are intended to ensure the identity of an analyte in a sample. This is normally achieved by comparison of a property of the sample (e.g. spectrum, chromatographic behavior, chemical reactivity, etc.) to that of a reference standard.

• Quantitative test for impurities' content – This test is intended to accurately reflect the purity characteristics of the sample.

• Limit test for control of impurities

• Quantitative test for the active moiety in samples of drug substance or drug product or other selected component (s) in the drug product. Assay procedures are intended to measure the analyte present in a given sample. The assay represents a quantitative measurement of the major components in the drug substance. For drug product, similar validation characteristics apply when associated with other analytical procedures e.g. dissolution. The objective of analytical procedure should be clearly understood since this will govern the validation characteristics, which need to be evaluated.

Table 1.1 Summaries Of Characteristics Applicable To Various Analytical Procedures

Characteristic	Class A	Class B		Class C	Class D
		Quantitative tests	Limit tests		
Accuracy		✓		✓	✓
Precision		✓		✓	✓
Robustness	✓	✓	✓	✓	✓
Linearity and Range		✓		✓	✓
Selectivity	✓	✓	✓	✓	✓
Limit of detection	✓		✓		
Limit of Quantification		✓			

Typical validation parameters which should be considered are as listed below [47-49].

a. Specificity
b. Accuracy
c. Linearity and range
d. Limit of detection
e. Limit of quantitation
f. Precision
    - Repeatability
    - Intermediate Precision
g. Ruggedness
h. Robustness

This list is for typical analytical procedure but occasional exceptions should be dealt with on a case to case basis. It is also to be noted though robustness is not listed in the list it should be considered at an appropriate stage in the development of analytical procedure.

Further revalidation may be required in the situations where there are changes in the synthesis of the drug substance or changes in the composition of the finished product or changes in the analytical procedure.

1.8.1.1 Specificity / Selectivity

The selectivity or specificity of a procedure is its ability to measure an analyte in a manner that is free from other components in the sample being examined. For example, impurities arising from manufacture or degradation or ingredients other than the analyte, whether these are pharmacologically active or inert. Selectivity may be expressed in terms of the bias of the assay results obtained when the procedure is applied to the analyte in the presence of expected levels of other components, compared to the results obtained on the same analyte without added substances. When then other components are well known and available, selectivity may be determined by comparing the test results obtained on the analyte with and without the addition of the potentially interfering materials. When such components are either unidentified or unavailable, a measure of selectivity can often be obtained by determining the recovery of standard addition of pure analyte to a material containing a constant level of the other components.

1.8.1.2 Accuracy

The accuracy of an analytical method is the closeness of the test results obtained by that method to the true value. Accuracy may often be expressed as percent recovery by the assay of known, added amounts of analyte.

The accuracy of method for determination of active in the dosage form is determined by adding known amount of active in placebo to cover both above and below the normal levels (80%, 100% and 120 %) expected in samples. The peak area responses and assay of active in the three ranges were calculated and the accuracy of the results was compared to the actual amount of drug added and the amount of drug recovered. This is to know how accurately the active is retrievable from the sample matrix. It is carried out at three different levels. The amount recovered is calculated. Percentage recovery at each level is calculated and statistical calculation gives you percentage recovery, which should be within 98-102%. Then it is concluded that the method is accurate.

1.8.1.3 Linearity and range

The linearity of an analytical method is its ability to elicit test results data directly proportional to the concentration of the analyte in samples within a given range.

This parameter checks the detector performance for the selected method. Series of dilutions are prepared and analysed as per the method.

The graph is plotted for Concentration vs. response. The detector response is said to be linear if Correlation coefficient of the graph when calculated is in the range of 0.9999 - 1.0.

1.8.1.4 Precision

The precision of the procedure is the degree of agreement among individual test results. It is measured by scatter of individual results from the mean and it is usually expressed as the standard deviation or as coefficient of variation (relative standard deviation) when the complete procedure is applied repeatedly to separate identical samples drawn from the same homogenous batch of material.

1.8.1.4.1 Repeatability (within-laboratory variation)

This is the precision of the procedure when repeated by an analyst under the same set of conditions (same reagents, equipments, settings and laboratory) and within a short interval of time. The repeatability of a procedure is assessed by carrying out complete, separate determinations on separate identical samples of the same homogenous batch of material and thus provides a measure of the precision of the procedure under normal operating conditions.

1.8.1.4.2 Reproducibility

This is the precision of the procedure when it is carried out under different conditions – usually in different laboratories, on separate, identical samples taken from the same homogenous batch of material. Comparisons of the results obtained by different analysts, by the use of different equipments, or by carrying out the analysis at different times can also be provided valuable information.

1.8.1.5 Sensitivity

Sensitivity is the capacity of the test procedure to record small variations in concentration. It is the slope of the calibration curve. A more general use of the term to encompass limit of detection and/or limit of quantification should be avoided.

1.8.1.5.1 Limit of Detection

Limit of detection is a parameter of the limit test. It is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Thus limit test merely substantiate that the analyte concentration is above or below a certain level. The limit of detection is usually expressed as the concentration of analyte (e.g. percentage or $\mu g\ mL^{-1}$) in the sample. The limit of detection is the lowest concentration of analyte that can be detected by the given method.

The limit of detection of active and its related impurities is determined by serially diluting at lower concentration range, extended fairly close to the expected LOD. The concentrations were selected based on the logic that the method must be enough sensitive to at least quantify less than 0.5 times LOQ.

Duplicate injections of .Linear concentration of impurities are injected .The detection responses were calculated from the calibration curve using the formula i.e. DL = [3.3∗ SyX/Slope]. Based on the above calculation minimum detection limits are established.

1.8.1.5.2 Limit of Quantitation (LOQ)

Limit of quantitation is a parameter of quantitative assay for low levels of compound in sample matrices. It is lowest concentration of analyte in a sample that can be determined with acceptance precision and accuracy under stated experiment condition".

Duplicate injections of each impurity /active in the concentration range of 1- 150% of the specified concentration (approx.) is made to establish limit of detection and limit of quantitation. The detection responses were calculated from the calibration curve using the formula i.e. QL = [10∗ SyX/Slope]. Based on the above calculation minimum quantitation limits are established.

1.8.1.6 Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of the test results obtained by the analysis of the same samples under a variety of normal test conditions i.e. different instrument, analysts, days, etc.

The ruggedness of Assay test method of the dosage form is carried out by two different analytical persons on two different instruments on different dates to know the degree of reproducibility of results obtained by the analysis of the same sample.

1.8.1.7 Robustness

The robustness of an analytical method is measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The robustness of analytical method was evaluated by checking parameters such as effect on changing column oven temperature (± 5°C), effect on changing wave length, (± 1nm), effect of pH of the mobile phase (± 0.2), effect on changing mobile phase composition by ±.30% of the minor component but not more than 10% of the absolute and effect on changing the flow rate of the mobile phase (± 0.2ml/min.).

Performing a thorough method validation can be a tedious process, but the quality of data generated with the method is directly linked to the quality of this process. Time constraints often do not allow for sufficient method validations. Many researchers have experienced the consequences of invalid methods and realized that the amount of time and resources required to solve problems discovered later exceeds what would have been expended initially if the validation studies had been performed properly.

1.8.1.8. Summary Statistics

Summary statistics are used to make sense of large amounts of data. Typically, the mean, sample standard deviation, range, confidence intervals and measures for skewness and spread of the distribution are reported [50].

The Mean

The mean, x can be shown to be the best estimate of the true value 'μ'; it is calculated as the arithmetic mean of n observations.

$X = \Sigma (x_i) / n$

The Median

This is the value or observation, which subdivides the numerical, ordered data into two halves. If the number of observations (n) is odd, (n-1)/2 observations are smaller than the median and the next higher value is reported as the median If n is even then, the average of the middle two observations is reported. The most useful characteristic of the median is small influence exerted on it by extreme values, that is, its robust nature.

Standard Deviation

The Standard Deviation is a measure of the spread of data (dispersion) about the mean. It is the positive square root of the variants.

Standard Error

The standard error is the standard deviation of an estimate. Thus, the standard error of the mean (SEM) is the standard deviation of the mean. If *s* is the standard deviation of a sample then the SEM is given by the formula as follows:

$SEM = SD / \sqrt{n}$

Coefficient of Variation or Relative Standard Deviation

The ratio of the standard deviation to the mean is the relative standard deviation (RSD). It is expressed as a percentage.

RSD = 100 (SD / x)

Regression and Calibration

Calibration is fundamental to achieving consistency of measurement. Often calibration involves establishing the relationship between an instrument response and one or more reference values. Once the relationship between input value and the response value is established, the calibration model is used in reverse i.e. to predict a value from an instrumental response.

Regression:In statistics, the term regression is used to describe a group of methods that summarize the degree of association between one variable and another variable. The most common statistical method used to do this is least-square regression, which works by finding the "best curve" through the data that minimizes the sum of squares of the residuals. There are number of least-square regression models, for e.g., linear, logarithmic, exponential and power.

1.9 Literature Survey

Literature survey shows various analytical methods for separation of artemisinin products, artesunate, artemether, amodiaquine and their metabolite(s) using high-performance liquid chromatography –mass spectrometry, gas chromatography –mass spectrometry, liquid chromatography with electrochemical detector [51-62].Fusari and others [63] reported thin layer chromatography elution method for the assay of primaquine bulk drug and its tablets. In their investigation, a quantitative thin layer chromatography followed by quantitative elution and spectrophotometry was used for assay determination. Detection of chloroquine in urine by TLC without preliminary extraction is available in the literature [64]. A quantitative TLC method for chloroquine, primaquine and other quinoline derivatives is reported by Zheng and coworkers [65]. Determination of antimalarial drugs under field conditions using thin-layer chromatography is available in the literature [66]. Simplified TLC system for qualitative and semi-quantitative analysis of chloroquine and other pharmaceuticals is also reported by few workers [67]. Spectrophotometric determination of primaquine phosphate and amodiaquine

hydrochloride in pharmaceutical preparation was reported in the literature [68, 69]. Simultaneous spectrophotometric determination of amodiaquine and primaquine mixture in dosage forms is also reported in the literature [70]. A spectrophotometric method based on the formation of complexes with iodine for the determination of chloroquine, primaquine, amodiaquine and pyrimethamine has been described by Salam and coworkers [71]. Spectrophotometric micro-determination of some pharmaceutically important aminoquinoline antimalarials as ion-pair complexes is carried out by Amin and coworkers [72]. This method was based on interaction of amodiaquine, chloroquine and primaquine with calmagite indicator to give highly colored ion-pair complexes. A simple and rapid spectrophotometric method for the assay of amodiaquine hydrochloride, chloroquine phosphate and primaquine phosphate is described in the literature [73]. The method is based on the interaction of the drugs with tetracyanoethylene to give a stable charge transfer complex. Colorimetric assay determination of some antimalarials like quinine sulphate, primaquine diphosphate, amodiaquine hydrochloride and pyrimethamine is available in the literature [74]. The method is based on the interaction of the drugs and p-chloranilic acid to give a stable product with an intense color. Extractive spectrophotometric determination of some antimalarials using fast green FCF or orange II reagent is reported [75]. A report on quantitative separation of primaquine from amodiaquine by selective precipitation followed by UV measurement is available [76]. Verma and others [77] reported spectrophotometric determination of periodate with amodiaquine dihydrochloride and its application to the indirect determination of some organic compounds via the malaprade reaction. A Difference-spectrophotometric method for estimation of primaquine phosphate in tablets is also described [78]. Spectrophotometric methods using different reagents like cerric ammonium sulfate, tetracyanoquinodimethane, ammonium molybdate, ninhydrin, 4-dimethylaminocinnamaldehyde, amidopyrine, MBTH (3-methylbenzothiazolin-2-one hydrazone), quinine and chloranil for the determination of different quinoline antimalarial like primaquine, amodiaquine and chloroquine are reported in the literature [79-88]. Refractometry, colorimetry and a technique combining both processes was evaluated as simple and accurate field assays determination of commonly available antimalarial drugs, artesunate, chloroquine, quinine, and sulfadoxine [89]. Kinetic and spectrophotometric

methods are reported for the determination of amodiaquine and chloroquine based on their oxidation with potassium iodate and potassium bromate [90]. Spectrophotometric assay of chloroquine phosphate in the presence of spectral interference in suspensions and syrups is reported [91]. Pyrimethamine is determined by flurometry from combination formulation. [92].Reports are also available on electro-analytical determination of antimalarial drugs. La-Scalea [93] described the dissociation and electro-oxidation of primaquine diphosphate using voltametry. Assay determination of amodiaquine using polymeric membrane electrode is available in the literature [94]. Berka and coworkers reported the chlorocoulometric determination of primaquine [95]. Electrochemical oxidation and voltametric determination of primaquine in tablets is available [96]. A report on poly vinyl chloride matrix-membrane electrodes for manual and flow-injection analysis of chloroquine in pharmaceutical preparations is also available in the literature [97]. Flow-through chloroquine sensor and its applications in pharmaceutical analysis was reported by few workers. A polarographic method for the determination of chloroquine phosphate by single-sweep oscillopolarography is described by Zhang [98]. Conductometric and indirect atomic absorption spectrometric determination of antimalarials is described by Amin [99]. The method was described for the determination of amodiaquine, chloroquine and primaquine based on formation of their ion associates with certain metal ions viz. [Cd^{2+}, Co^{2+}, Mn^{2+} and Zn^{2+}] thiocyanate.GC-MS studies on biologically important 8-aminoquinoline derivative was carried out by Baty and co-workers [100]. In this study 5, 6 –Dihydroxy -8-aminoquinoline, a possible metabolite of primaquine metabolite was detected by single ion monitoring after conversion to trimethylsilyl ether derivative. A GC-MS has also been applied for analysis of primaquine in plasma and urine using a deuterated internal standard [101]. Identification of a chloroquine artifact by gas chromatography - mass spectrometry is reported in literature [102]. Das and others [103] described the quantitation of chloroquine phosphate and primaquine phosphate in tablets using high-performance liquid chromatography. HPLC method determination of amodiaquine hydrochloride and primaquine phosphate in dosage forms is available [104]. Estimation of primaquine in its formulations (tablets) using RP-LC is described by few workers [105]. Chromatographic study on the isolation of peroxydisulfate oxidation products of primaquine is also described [106]. A sensitive and

reproducible high performance liquid chromatographic as well as a high performance thin layer chromatographic assay method for the simultaneous estimation of chloroquine, primaquine and bulaquine has been developed by Dwivedi and co-workers [107]. Liquid-chromatographic assay for amodiaquine in tablets and biological fluids is also reported [108]. High-performance liquid-chromatographic determination of chloroquine phosphate in dosage forms using metronidazole as an internal standard is reported [109]. Determination of chloroquine and its decomposition products in various brands of different dosage forms by liquid chromatography is described [110]. A report on photo-degradation studies of chloroquine phosphate by high-performance liquid chromatography is also available [111]. Dual-mode gradient HPLC procedure for the simultaneous determination of chloroquine and proguanil is available [112]. Quality control of piperaquine in pharmaceutical formulations by capillary zone electrophoresis. [113].Chiral separation of primaquine by capillary electrophoresis using β-cyclodextrine [114] polymer and by cyclodextrine modified micellar electro kinetic capillary chromatography [115] are reported. Chiral separations of chloroquine using heparin as a chiral selector in HPLC [116], heparin and dextran sulfate in capillary zone electrophoresis [117] are explained.Bio-analytical methods are mainly focused on the determination of antimalarial drugs and their metabolites in biological fluids using different analytical techniques. Spectrometric assay of primaquine in plasma is reported by few workers [118]. Ward [119] reported the determination of primaquine in biological fluids by reversed-phase high-performance liquid chromatography. Photochemical and fluorimetric determination of primaquine in a flowing solvent with application to blood serum has been reported by Tsuchiya and co-workers [120]. Nora and co-authors [121] reported an electrochemical assay method for the simultaneous determination of primaquine and its metabolites from plasma and urine samples obtained after oral administration of primaquine diphosphate. Determination of primaquine in whole blood and urine by normal-phase high-performance liquid chromatography has been reported in the literature [122]. HPLC determination of pamaquine, primaquine and carboxyprimaquine in calf plasma using electrochemical detection was also reported [123]. Simultaneous determination of an antimalarial agent, CDRI compound 8053, and its metabolite primaquine in serum by HPLC has been described [124]. Reports are

available on HPLC determination of primaquine and carboxy-primaquine in human plasma and blood cells [125]. High throughput assay for determination of piperaquine [126], lumefantrine [127] in plasma is reported. J. Training and others developed and validated an automated solid extraction and liquid chromatographic method for determination of piperaquine in urine [128]. Many methods for determination of antimalarial compounds by bulk, UV, HPLC are reported [129-136].Some of the antimalarial drug substances and the drug products like artemether, artesunate, arteether are official in International Pharmacopoeia [137] but not all these are official in Indian Pharmacopoeia [138], British Pharmacopoeia [139] or US Pharmacopoeia [140]. Reversed phase High Performance Liquid Chromatographic method for determination of dihydroartemisinin that is major degradant of artesunate and artemether is reported [141-147]. A thin layer chromatography (TLC) method is developed to analyze artemisinin and its derivatives, artemether and arteether, using RP 18[148]. Literature survey shows various analytical methods for separation of artemisinin products, artesunate, artemether, amodiaquine and their metabolite(s) using high-performance liquid chromatography – mass spectrometry, gas chromatography –mass spectrometry, liquid chromatography with electrochemical detector. All these methods are for determination of individual drug substance and its metabolite [149-153]. To our best of knowledge, no methods are available in the reviewed literature for simultaneous determination of degradants and drug substances of fixed dose combination drug products.

1.10 Objectives Of Present Research Work

It is clear from the above literature review that some of the methods are reported for individual determination or single component determination but there is no research work on simultaneous determination of artemether and lumefantrine or artesunate and amodiaquine and their degradants like dihydroartemisinin in fixed dose combination dosage form. As per the latest guidelines of World Health Organization and ICH, all the drug products should be controlled for degradants [154]. Determination of degradants is most important test for purity of any dosage form. Therefore, the need for development of analytical methods for consistent quality establishment through out the shelf life of the product arises. When such fixed dose combination formulations are developed, the

development of the analytical method to evaluate the stability as well as the content of the drug substance becomes the attractive challenge in the analytical research. In this work, it is planned to develop stability indicating analytical methods for quantitative determination of artemether and lumefantrine, artesunate and amodiaquine simultaneously in a fixed dose pharmaceutical formulations. It is also planned to validate developed analytical methods as per standard guidelines [47-49]. It is further planned to develop and validate stability indicating analytical methods for related substances using HPLC/UPLC . Developed methods are planned to utilize for studying various dosage forms including tablets (tab in tab, bilayer, dispersible) , dry powder suspension etc.

These methods shall be used for the monitoring the consistent quality of the drug product through out the shelf life.

2.1. Artemether

CAS Reg. No. 71963-77-4

Pharmacopoeial Status: Artemether is official in International Pharmacopoeia, china pharmacopoeia. The drug substance Artemether used in the development studies was manufactured by M/s Ipca Laboratories, and was micronised.

Chemistry Review

Common Name: Artemether

IUPAC Name: (3R, 5aS, 6R, 8aS, 9R, 10S, 12R, 12aR)-Decahydro-10-methoxy-3, 6, 9-trimethyl-3, 12-epoxy-12H-pyrano [4, 3-j]-1, 2-benzodioxepin [155].

Molecular Formula: $C_{16}H_{26}O_5$

Molecular Weight: 298.4

pKa : Not reported in the literature.

Log p : Not available in the reviewed literature.

Structure:

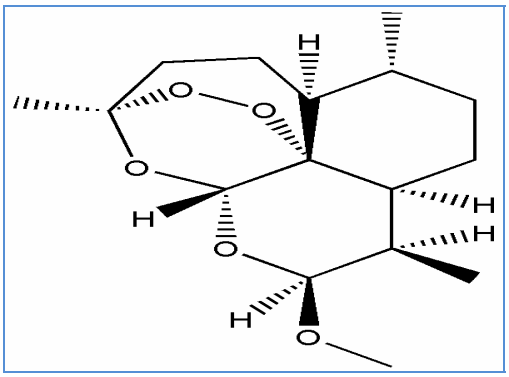

Figure 2.1 Structure of Artemether

General Properties

Application: Anti-malarial. Used as a IM/IV injection.Used in the combination therapy along with lumefantrine. Available as fixed dose tablets, dry powder suspension in combination with lumefantrine.

Description: It is a white crystalline powder with a bitter taste, practically insoluble in water, very soluble in dichloromethane and acetone, freely soluble in ethyl acetate, and dehydrated alcohols. Malaria is one of leading re-emerging infectious disease due to

mutative malarial parasite developed resistance to chloroquine. Sulfadoxine Pyrimethamine is an alternative can be used in the treatment of the disease resistant to chloroquine, but resistance to sulfadoxine-pyrimethamine has been reported also in some areas. Artemisinin is an alternative used in areas where the disease has become highly resistant to sulfadoxine-pyrimethamine. Dihydroartemisinin, its methyl ether (artemether), its ethyl ether (its (Arteether) and its hemisuccinate ester (artesunate) are known as more effective than its Parent material - artemisinin. Artemisinin is a peroxide-bridged sesquiterpene lactone compound, which has no N atom, unlike quinine class anti-malarial drugs.

Pharmacokinetic

After oral administration it is rapidly absorbed and reaches Cmax within 2 hrs. The product is metabolized in the liver by hydrolysis; giving rise to dihydroartemisinin that is also effective against malaria by the same mechanism of action elimination half-life is approx 2 to 3 hours.

Action

A potent and rapidly acting blood schizoticide .It is concentrated in the food vacuole. It then splits its endoperoxide bridge as it interacts with heam blocking conversion to haemozoin, destroying existing heamozoin and releasing heam and a cluster of free radicals into the parasite and destroys the parasite.

Adverse Effect & Precaution

Artemether has been remarkably well tolerated, and appears less toxic than quinine or chloroquine; adverse effects include bradycardia, electrocardiogram abnormalities, gastrointestinal disturbances (nausea, abdominal pain, and diarrhea - oral therapy only), dizziness, injection site pain, skin reactions, and fever. Transient decreases in neutrophils and reticulocytes have been reported in some patients treated with artemether.

Caution is required in patients with cardiovascular disease, Hepatic impairment, and renal insufficiency.

Major Degradants

Artemisinin, anhydroartemisinin and dihydroartemisinin are the major degradents.

2.2 Lumefantrine

CAS Reg. No. -82186-77-4

Chemistry Review

Common Name: Lumefantrine

IUPAC Name: (1R, S)-2- Dibutylamino-1-{2, 7-dichloro-9-[(Z) (4-chlorobenzylidene)-9H-fluoren-4-yl}-ethanol . [155].

Molecular Formula: $C_{30}H_{32}Cl_3NO$

Molecular Weight: 528.94

pKa: 8.7

Log p : Not available in the reviewed literature.

Structure:

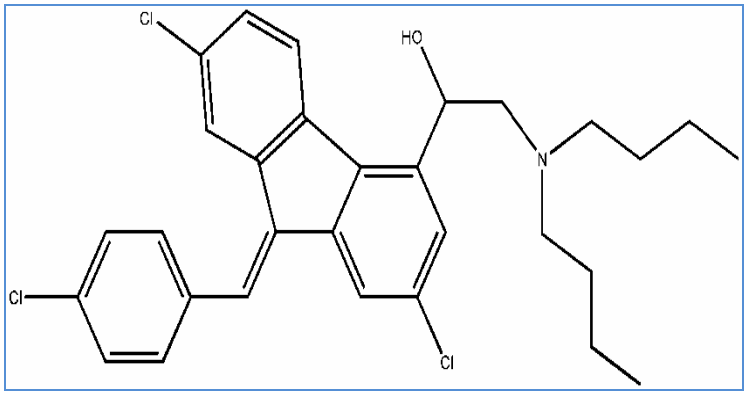

Figure 2.2 Structure of Lumefantrine

General Properties

Description: It is a yellow crystalline powder, odorless or almost odorless & has a bitter taste, soluble in water, sparingly soluble in alcohol: very slightly soluble in benzene, chloroform and ether.

Pharmacokinetic

After oral administration, absorption of lumefantrine, a highly lipophilic compound, starts after a lag time of up to 2 hours, with peak plasma concentration about 6 to 8 hours. The product is metabolized in the liver by CYP3A4 to desbutyl-lumefantrine,

elimination half-life of lumefantrine is approx 2 to 3 days in healthy volunteers and 4 to 7 days in falciparum malaria.

Mechanism of Action:

Its antimalarial effects involve lysosomal trapping of the drug in the intra-erythrocytic parasite, followed by binding to toxin haemin that is produced in the course of hemoglobin digestion. This binding prevents the polymerization of haemin to non-toxic malaria pigment.

Application: Anti-malarial. Used in the combination therapy along with artemether. Available as fixed dose tablets, dry powder suspension in combination with artemether.

Adverse Effect & Precaution:

Common adverse effects reported included headache, drowsiness, sleep disorder, abdominal pain, anorexia, nausea, vomiting, diarrhea, cough, arthralgia, asthenia, and ataxia.

Major degradants

Lumefantrine is stable molecule and do not have any significant degradant.

2.3 ARTESUNATE

CAS Reg. No. 88495-63-0.

A fine, white crystalline powder. Very slightly soluble in water, very soluble in dichloromethane R, freely soluble in ethanol and acetone R. It is anti-malarial drug. In the present work, a fast and an accurate method has been developed and validated for the simultaneous determination of artesunate and amodiaquine and its degradaent dihydroartemisinin, artemisinin in a single analysis. Developed method is applied for different formulation approaches and different combination strengths.

Nomenclature

(3R,5aS,8aS,9R,10S,12R,12aR)-Decahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano [4,3-/]-1,2-benzodioxepin-10-ol,hydrogen succinate,2-propyl l-pentanoic acid [155].

Formula

Empirical Formula – $C_{19}H_{28}O_8$

Chemical Structure

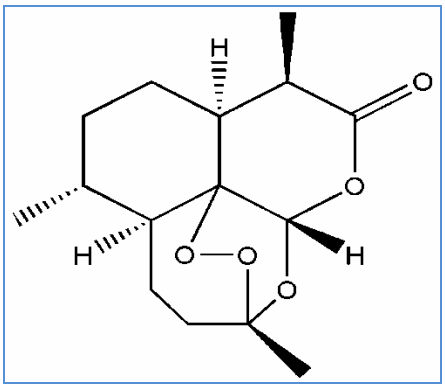

Figure -2.3 Structure of Artesunate

Pharmacopoeial Status

Artesunate is official in International Pharmacopoeia, china pharmacopoeia. The drug substance Artesunate used in the development studies was manufactured by M/s Ipca Laboratories, and was micronised.

Physical Properties

Molecular weight – 384.4

Appearance and color – White crystalline powder,

Solubility – soluble in ethanol, acetone

pKa: 4.6

Log p : Not available in the reviewed literature.

Therapeutic Category

Antimalarial.

Application

Initially for oral administration artesunate may be given as 3 days course if used in combination for five days if used alone.. Dosage forms available are 50 mg, 100 mg, 150mg & 200 mg. Also available in combination with amodiaquine (in the ratio of 1:3 or 1:2.7), in combination with sulphadoxine and pyrimethamine. The following doses are those suggested by WHO for treatment of malaria in the areas of multidrug resistance. Larinate –Ipca Laboratories Limited, Arsumax- Novartis, Falcigo –Themis Pharma.

Pharmacokinetics

Artesunate is rapidly metabolized to its active metabolite dihydroartemisinin. After oral administration, bioavailability of dihydroartemisinin is 82%.

Major Degradants

Very sensitive molecule. Dihydroartemisinin is the major degradants. Glycan is another major degradant. Artemisinin is minor degradant.

2.4 AMODIAQUINE HYDROCHLORIDE [155]

CAS Reg. No. 6398-98-7

Pharmacopoeial Status

Amodiaquine HCl is official in International Pharmacopoeia, USP. The drug substance Amodiaquine HCl used in the development studies was manufactured by M/s Ipca Laboratories.

Chemistry review

Common Name: Amodiaquine Hydrochloride

IUPAC Name: 4-[(7-Chloro-4-quinolyl)amino)-α-(diethylamino)-o-cresol;4-(7-Chloro-4-quinolyl)amino-2-[(diethylamino)methyl]phenol;

Molecular Formula: $C_{20}H_{22}ClN_3O$, 2HCl, $2H_2O$

Molecular Weight: 464.8

pKa : 5.8

Chemical Structure

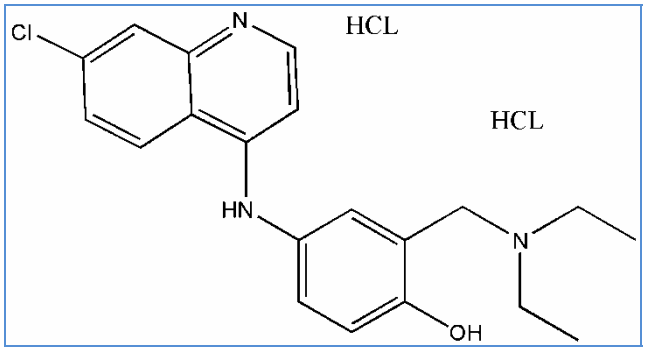

Figure 2.4 Structure of Amodiaquine Hydrochloride

General Properties

A yellow crystalline powder; odorless (or almost odorless); with a bitter taste. Soluble 1 in 22 parts water 1 in 70 parts ethanol (96%) Practically insoluble in benzene, chloroform and ether. A 2% solution in water has a pH of 2.6 - 4.6. PH of 1% aqueous solution: 4.0 to 4.8. It is anti-malarial drug. In the present work, a fast and an accurate method has been developed and validated for the simultaneous determination of artesunate and amodiaquine and its degradaent dihydroartemisinin, artemisinin in a single analysis. Developed method is applied for different formulation approaches and different combination strengths.

Therapeutic Category

Antimalarial.

Pharmacokinetics

Amodiaquine hydrochloride is readily absorbed from gastrointestinal tract. Amodiaquine is rapidly converted in the liver to the active metabolite desethylamodiaquine. Only a negligible amount of amodiaquine is being excreted unchanged in the urine. The plasma elimination half-life of desethylamodiaquine has varied from 1 to 10 days or more. About 5% of the total administered dose is recovered in urine, while the rest is metabolised in the body. Amodiaquine and desethylamodiaquine have been detected in the urine several months after administration.

Application

Dosage available 153.1 mg tablets. Also given in combination with artesunate.

Available as dry powder sachets.

Major degradant

No major degradants reported. It is the stable molecule.

3.1 Rational for Development of Stability Indicating Assay Methods By Using High Performance Liquid Chromatography.

3.1.1 Introduction

A stability-indicating assay method is a validated quantitative analytical procedure that can detect the changes with time in the pertinent properties of the drug substance and drug product. A stability-indicating assay accurately measures the active ingredients, without interference from degradation products, process impurities excipients, or other potential impurities [156].

Method development and optimization of chromatographic method is an attractive challenge in analytical research. The technique of HPLC is over 30 years old and is in mature stage of development and application [29, 30]. Chromatographic methods are ideal assay techniques for such applications because they are sensitive and simple, they can be considered as complete methods including separation and quantitative determination and they can be applied without previous separation.

Therefore, high performance liquid chromatographic (HPLC) and high performance thin layer chromatographic (HPTLC) methods were developed as stability indicating assay methods for the quantification of Drug Substances and its formulations.

Method development

The philosophy of method development is based on several considerations. The systematic approach to HPLC method development is based on the knowledge of chromatographic process and variations occurring with the sample and experimental conditions. Method development often follows a series of steps as summarized in figure-3.1.Usually results of each chromatographic run must be assessed before proceeding with the next experiment. Before beginning method development, we need to review what is known about the sample. Sample information such as number of compounds present, chemical structure, functionality of compounds, molecular weight, pKa values, UV spectra of the compound, concentration range of compounds in samples of interest and lastly sample solubility [149].

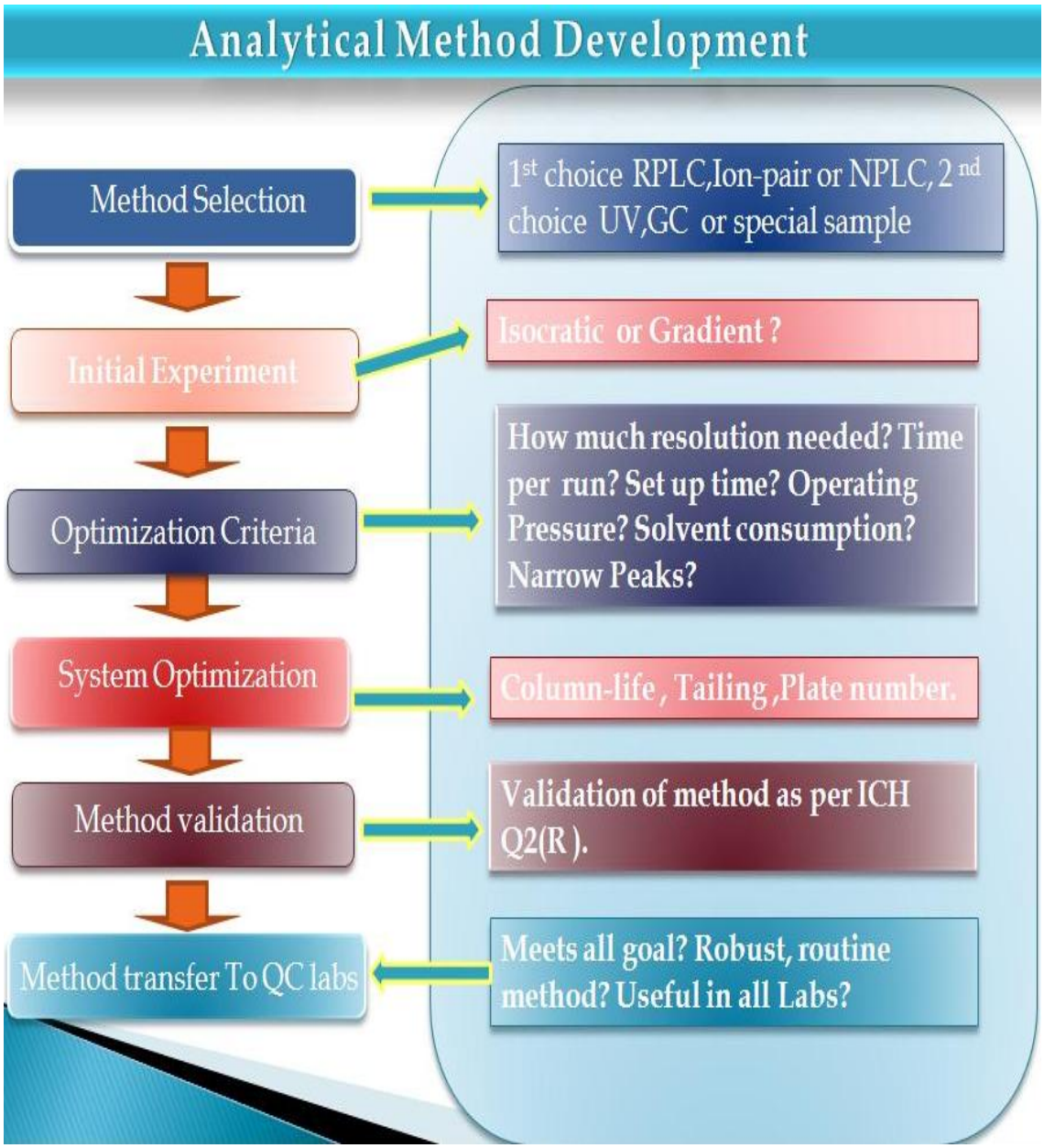

Figure-3.1. A Tree Of Steps Involved In Method Development

Depending on the use made of this sample information, two somewhat different approaches to HPLC method development are possible. Some chromatographers try to use chemistry of the sample to a best choice for initial HPLC conditions and others rely on their experience. Blending of two approaches make method development most successful with minimizing the number of required experimental runs. One of the major decisions to be made by analytical chemists is the choice of most effective procedure for given analysis. In order to arrive at correct decision, he must be familiar with the practical details of various techniques and theoretical principles upon which they are based. The techniques are differing in degrees of sophistications, of sensitivity, of selectivity, of cost and the time requirements.

One should be very clear before hand for separation goals and for that, he/she should follow below sequence [157].

Steps 1 - Define the problem and purpose of analysis

In order to define the problem, one should know whether primary goal is quantitative analysis, the characterization of unknown sample components or isolation of pure component. Further, one should decide whether it is necessary to resolve all sample components, degradants, and impurities as some times only main component and impurities separation is, sufficient or other time it is required main component should be separated from degradants or impurities from each other. If quantitative analysis is looked for then quantitation up to what level is required that should be known. Method development should be known to be done on how many different types of sample matrices that are raw material, one or more formulations, and an environmental sample. Whether one or more HPLC procedures require? alternatively, is a single or similar procedure for all samples desirable? Chromatographer should consider number of samples to be analysed at one time. Sometimes for large number of samples to run, one has to opt for shorter run time by shortening the column length or increasing the flow rate.

In short, what is required of the method should be clearly understood before method development begins. From the answer to the above questions, the goal of the experiment will be cleared.

Step 2- Information of sample

More the gathered information about a sample, the better is the beginning for the method development. The important information concerning sample composition and properties like number of compounds present in the sample, concentration range of compounds in the sample preparation, the other properties like the chemical structure, molecular weight, pKa value, UV spectra, solution stability and solubility enhances the method development.

Based on the composition of the sample, choice of the initial conditions for an HPLC separation will be decided.

Step 3- Sample preparation and detection

Sample comes in various forms such as solutions ready for injections, solutions that require dilution, addition of internal standard or other volumetric manipulation, solids must be dissolved or extracted from formulation matrix and sample that requires pretreatment to remove interferences and to protect the column from damage.

Sample preparation is an essential part of HPLC analysis to provide a reproducible and homogenous solution that is suitable for injection onto the column. Best results are obtained when the composition of the sample solvent is close to that of the mobile phase, since this minimizes baseline disturbance and does not affect the sample retention and resolution. Before the first sample is injected during HPLC method development, the detector is selected based on the information of UV spectra.

Step 4- Selecting the HPLC method

The HPLC method selection will be decided from the tree diagram in figure-3.2.

During HPLC method development, before the sample is injected we must be reasonably sure that the detector selected will sense all sample components of interest. For this reason, information on the UV spectra can be important aid for method development. UV spectra can be found in the literature, estimated from the chemical structures of sample components of interest, measured directly or obtained during HPLC separation by means of Photodiode Array (PDA) detector. Vriable wavelength UV detectors are the first choice, because they are convenient and applicable for most of the sample. If UV response is inadequate then other detectors are available e.g. fluorescence, electrochemical etc. or sample can be derivatised for enhanced detection.

Step 5- Selection of stationary phase

The column is the heart of the HPLC separation process. The availability of stable, high-performance column is essential in developing a rugged and reproducible method. The three important parameters involved in the stationary phase that can be varied in HPLC separation.

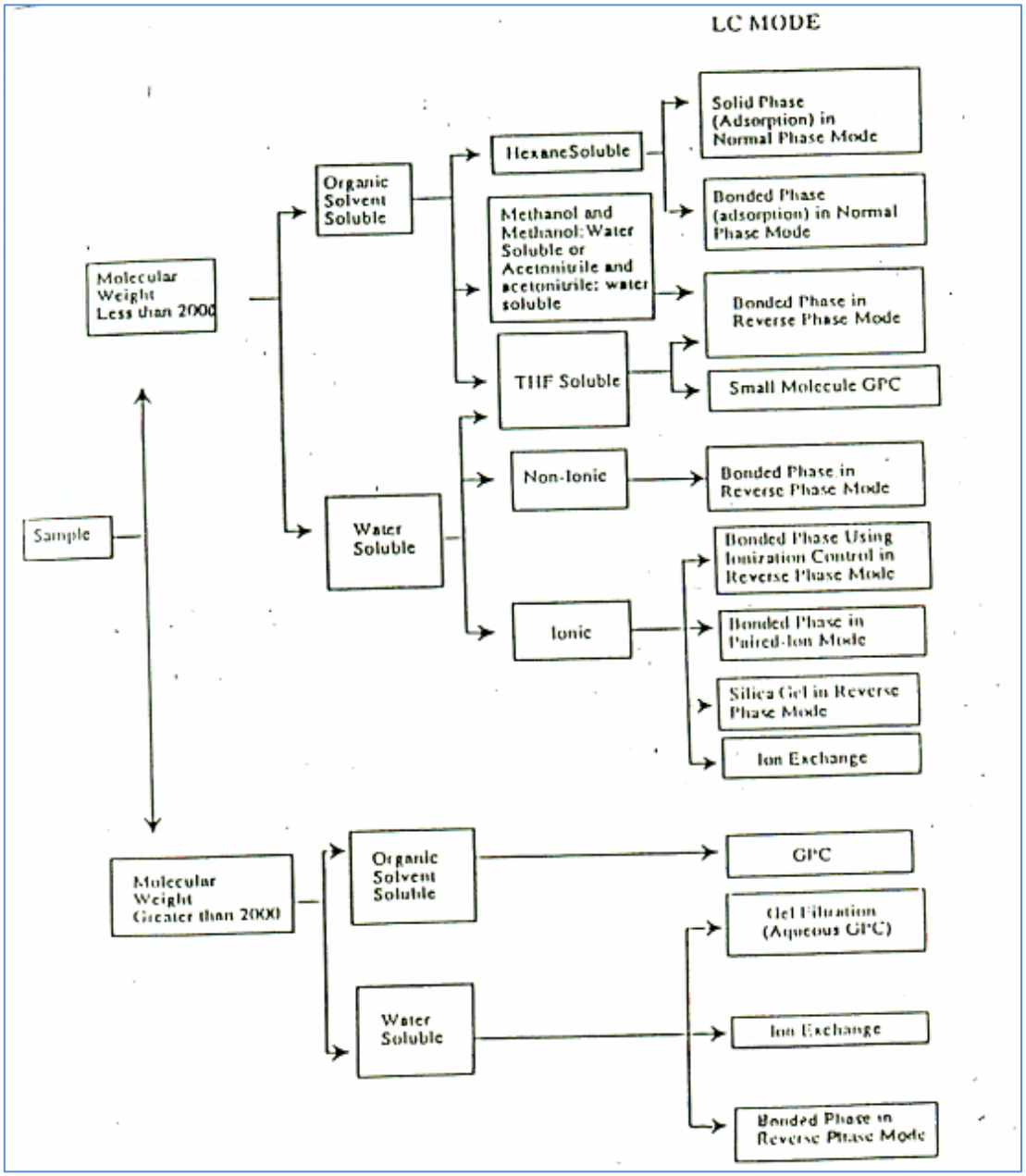

Figure 3.2. Approach for HPLC method selection.

a) Type of Bonded Phase

The basis of reverse phase chromatography (RPC) retention is shown schematically in figure 3.3. Separation by reverse phase chromatography is similar to the extraction of different compounds from water into an organic solvent such as octanol, where more hydrophobic (non-polar) compounds preferentially extract into the non-polar octanol phase. The column typically, a silica support modified with C_8 and C_{18} bonded phase is less polar than water-organic mobile phase. Sample molecules are partitioned between the polar mobile phase and non-polar C_8 or C_{18} stationary phase.

Sample retention normally increases for bonded phases of greater length (C_{18}>C_8>C_6>C_2). The stability of bonded-phase packing is especially important in method development. Good column minimizes the need for further adjustments of separation conditions or replacement of the column. When used under the same conditions, longer-chain alky-bonded-phase packing is more stable than short-chain compounds.

Silica based packing, has a favourable characteristic of high mechanical strength that is stable under high operating conditions for a long period.

To avoid problem from irreproducible sample retention during method development, it is important that column be stable and reproducible. Therefore, silica support modified with C_{18} or C_8 bonded phase are preferred as the stationary phase.

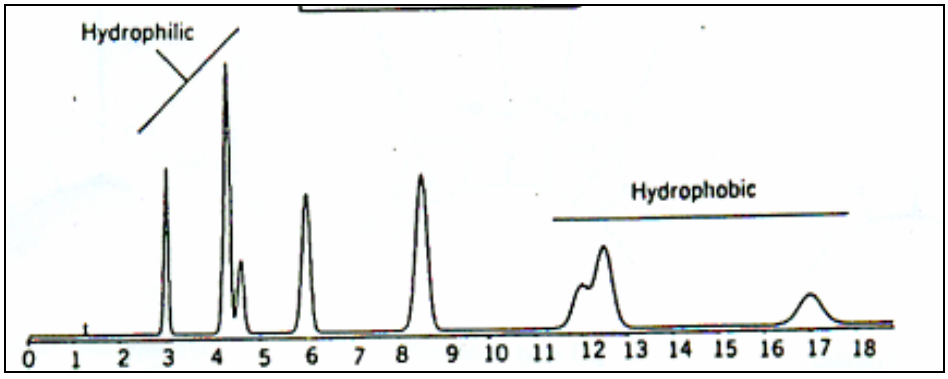

Figure 3.3: - Schematic representation of reversed-phase process for non-ionic compounds.

b) Column dimensions and particle size

During method development stage, the column should provide reasonable resolution. Short run time (including column equilibration and duplicate runs), an acceptable pressure drop for different mobile phases [158]. Analytical methods are best developed with 4.0, 4.6 or 3.9 mm internal diameter columns. Columns with 3-5µm particles generally give the best compromise of efficiency, reproducibility and reliability. Column of 3µm particles allow faster separations or higher efficiencies, but they have a tendency to plug more easily, which greatly reduces column life. The analytical column with 3-5µm particle size stationary phase and of 10-15cm length and 4.6mm internal diameter, with a flow of 0.8 - 2 cm^3/ min of the mobile phase, is a good initial choice. These conditions provide reasonable plate number or column efficiency, a run time of < 15 minutes and a minimum pressure < 2500 psi for any mobile phase made from mixtures of water, acetonitrile and/or methanol.

c) Surface coverage

Modified absorbents for reverse phase chromatography are prepared by chemically bonding an organic group to the silica surface. According to their structure, chemically bonded phases are classified as monomer and polymeric phases. The monomer type involves the formation of a monomolecular organic layer on the surface of the silica particles as shown in figure 3.4 Monomeric type column have excellent synthesis reproducibility, very good lot-to-lot reproducibility and short mobile phase equilibration time[158].

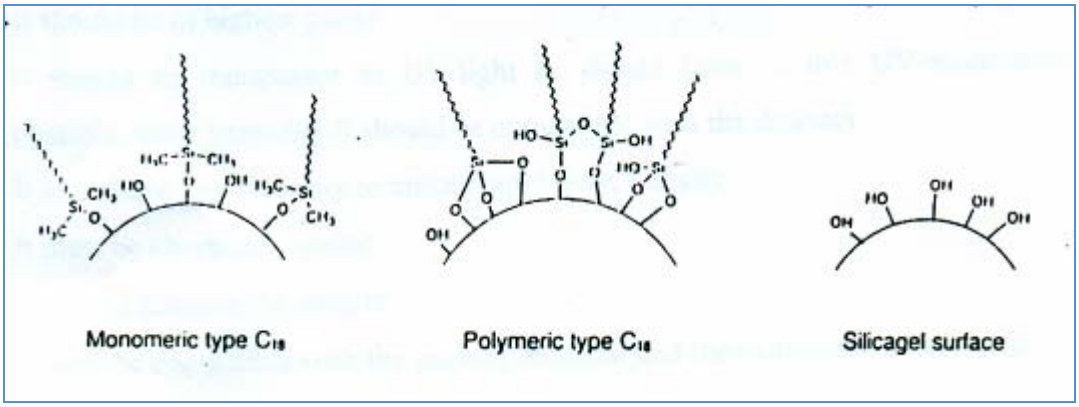

Figure 3.4- Diagrams of each stationary phase construction (before end-capping treatment)

Due to stearic hindrance, not all sites react and some silanol groups are left free or unbounded. Ionic samples, especially basic compounds, can interact with the silanols. This can lead to increased retention, peak tailing, and column-to-column irreproducibility. To minimize unwanted interactions of this free silanol groups with solutes, they are made to react by the process of end capping with a small silane such as trimethylchlorosilane, dimethyldichlorosilane, or hexamethyldisilazane.

Step 6- Selection of Mobile Phase

One of the great advantages of liquid chromatography is the versatility afforded by the liquid mobile phase. Not only can many different parameters be varied when the mobile phase is liquid, but the solutes can also interact with the mobile phase as well as with the stationary phase. Sufficient solubility of solute molecules in the mobile phase must be ensured in order to prevent precipitation [159]. The general requirements for a particular solvent to be useful for liquid chromatography are it should be of highest purity, should be transparent to UV-light or should have as low UV-absorance as possible: more generally, it should be compatible with the detector. It must have low viscosity to ensure rapid mass transfer. It must be chemically stable. It should dissolve the sample and it must be compatible with the packing material and the instrument's hardware.

Acetonitrile and methanol are most common organic solvents for reverse phase mobile phases, because of their low UV cutoff and low viscosity. It is preferable in RP-HPLC for the analyte to migrate through the column in neutral form. If the analyte possesses no ionisable group then the mobile phase containing water and organic solvent may be sufficient. If the analyte contains ionisable groups then the mobile phase requires pH adjustment to control the ionization. When pH = pKa for a compound, it is half ionized. Almost all of the pH-related change in retention occurs for pH value within ± 1.5 units of the pKa value. In order to develop the rugged separation that remains the same for small changes in pH. The pH of the mobile phase should be out of the range of $\pm$ 2.0 unit of pKa value, so that the molecules are either in completely ionised or non-ionised form [159].All silica based column contain accessible silanols. For basic sample, silanol interactions can lead to poor peak shape and reduced column efficiency. Their

effect on sample retention can be reduced by using a low-pH mobile phase (2.0 <pH<3.5) to minimize the concentration of ionised silanols:

$H^+ + SiO^-K^+ \leftrightarrow K^+ + SiOH$

Silanol effects can be further reduced by using a higher buffer concentration (> 10 mM) and choosing buffer cations that are strongly held by silanols ($Na^+ < K^+ < NH^{4+} <$ teriethylammonium) and therefore, block sample retention by ionized silanols. A 25 mM concentration of potassium phosphate and 1mM sodium dihydrogen phosphate is usually adequate for most basic samples. Buffers in the potassium form are more soluble in organic-water mobile phases than are buffer in sodium form, which makes mobile phase formulations more convenient for potassium buffers.Closely eluting components can be well resolved by the addition of ion-pairing agent such as sodium-pentane sulphonate, sodium-hexane sulphonate,sodium-deccane sulphonate, tetrabutyl ammonium hydroxide etc. in the mobile phase. When sample contains components with extreme polarities, addition of optimum quantity of tetrahydrofuran helps to retain early eluting component and for faster elution of late eluting component. Addition of triethylamine in mobile phase helps in reducing the silanol interactions and improvement in peak tailing and peak height. Thus, triethylamine acts as modifier when added in optimum amount however, excess use of triethylamine may reduce the column life [159]. The measurement of pH (by a pH meter) for a mobile phase that contains organic solvent is imprecise, because electrode response tends to drift. Therefore, the pH of the buffer should be adjusted before the addition of the organic solvent. The buffer salts used in the pH range are as follows:

pH – 2 to 4: Potassium dihydrogen phosphate, Sodium dihydrogen phosphate, orthophosphoric acid, acetic acid, trifluro acetic acid and formic acid etc.

pH – 4 to 6: Ammonium acetate, acetic acid, ammonium hydroxide and ammonium dihydrogen phosphate etc.

pH – 6 to 8: Dipotassium hydrogen phosphate, disodium hydrogen phosphate, ammonium hydroxide, ammonium dihhydrogen phosphate.

The general concentration of buffer salts used in the mobile phase preparation is in between 0.01M to 0.1M per liter. Reducing the concentration of buffer tailing increases

and increasing in buffer salt concentration reduces the peak tailing and retention time for basic compounds in reverse phase chromatography.

Step 7- Optimization of selected parameters

One has to optimize the selected parameters merely by changing each parameter at a time keeping all other parameters constant. This will provide when, what and how much each parameter affect.

Step 8- Anticipating problems

What can give wrong results? Alternatively, what precautions one should take during the method application so that method can serve the intended purpose. Like if hardness of the tablets increases during the stability and if the method is with whole tablets then extraction of the active from the tablet may require more shaking or sonication time as time require to disintegrate the tablets may increase. If solution turns hazy after standing for long, time may lead to wrong results, so method design should take care of all the possible variables and manual errors.

Step 9- Validation of the method and release for QC lab.

Validation of analytical method is an integral part of product development. The objective of validation of analytical procedure is to demonstrate that it is suitable for its intended purpose. As per the ICH guideline (Q2 (R_2) for Method development and validation, the official methods need not to be validated for entire validation parameters, but merely to be verified for their suitability and feasibility [47-49,160].Typical validation parameters, which should be considered, are as listed below

a. Specificity
b. Accuracy
c. Precision
d. Repeatability
e. Intermediate Precision or Ruggedness
f. Limit of detection
g. Limit of quantitation
h. Linearity and range

This list is for typical analytical procedure but occasional exceptions should be dealt with on a case to case basis. It is also to be noted though robustness is not listed in the

list; it should be considered at an appropriate stage in the development of analytical procedure.Further revalidation may be required in the situations such as changes in the synthesis of the drug substance,changes in the composition of the finished product and /or changes in the analytical procedure. The changes of revalidation required depend on the nature of the changes. The validated method is successfully transferred at manufacturing QC/QA laboratory and applied for release as well as for stability of the drug product to establish the shelf life of the product.

As discussed in this chapter the each parameter is studied in detailed and the stability indicating assay methods are developed for following two fixed dosed antimalarial combinations.

- Artemether and Lumefantrine tablets
- Artesunate and Amodiaquine tablets.

3.2 Simultaneous Determination of Artemether and Lumefantrine by Reverese Phase HPLC Method in Fixed Dose Pharmaceutical Preparation.

3.2.1 Introduction

Artemether, chemically (3R,5aS,6R,8aS,9R,10S,12R,12aR)-Decahydro-10-methoxy-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin., its molecular formula is $C_{16}H_{26}O_5$

in addition, its molecular weight is 298.4 is used in the treatment of malaria and Lumefantrine, chemically (1R, S)-2-Dibutylamino-1-{2, 7-dichloro-9-[(Z) (4-chlorobenzylidene)-9H-fluoren-4-yl}-ethanol molecular wt is 528.4. (Artemether is a sesquiterpene lactone derived from artemisinin. Artemisinin is a compound derived from the sweet wormwood plant and has been used for centuries in traditional Chinese medicine to treat fever. Lumefantrine is a synthetic aryl-amino alcohol antimalarial (quinine, mefloquine and halofantrine are members of the same group) [161]. The structures of the two drugs are shown in figures 2.1, 2.2. Artemisinin and its derivatives are at present, only the effective drugs against drug resistant malaria. However, their use alone may result in development of resistance to these life saving drugs [162]. According to the new WHO malaria treatment guidelines, uncomplicated falciparum malaria must be treated with artemisinin combination therapy (ACT) and not by artemisinin alone or any other monotherapy. The WHO recommends this combination as first line therapy for falciparum malaria in endemic areas [163].Artemisinin when used correctly in combination with other anti-malarial drugs is not only effective in curing malaria, but also the parasite is highly unlikely to become drug resistant. Artemether is fast acting drug with a short half-life. Lumefantrine acts slowly and has a longer half-life. Artemether rapidly reduces parasite biomass and quickly resolves clinical symptoms, at the same time the long-acting activity of lumefantrine is thought to prevent recrudescence. This dual effect also appears to reduce the selective pressure on the parasite to develop resistance. The antimalarial activity of the combination of lumefantrine and artemether is greater than that of either substance alone drugs [2].

When the formulations are developed with such combination of drug substances, the development of the analytical method to evaluate the content of artemether as well as

lumefantrine the content of the drug substances becomes the challenge in the analytical research.

Literature survey reveals that there are many methods available for individual estimation, but only few stability-indicating methods are reported for artemether and lumefantrine combination drug products containing two or more active drug substances [47-56]. There is no official pharmacopoeial method for the simultaneous determination of artemether and lumefantrine in pharmaceutical preparations to apply for routine quality control analyses. International Pharmacopoeia's draft method has been reported for the determination of each of artemether and lumefantrine respectively [164]. The reported method is a gradient method, which is critical and time consuming. Simultaneous determination of artemether and lumefantrine in fixed dose combination tablets by HPLC with UV detection is reported using standard addition method. It lacks the stability indicating power and use of chloroform in the RP method. [165]. Chloroform being immiscible with aqueous solvents the separation of phase is major risk and it destroys the column packing hence not recommended to use for reversed phase chromatography for long term usage. The focus of the present study was to develop and validate a simple, isocratic HPLC method for the simultaneous determination of artemether and lumefantrine in the fixed dosage from which can be routinely used in the QC labs for intended purpose for establishing the quality of the product.

3.2.2 Experimental

Chemicals and Reagents

Artemether and Lumefantrine working standards were obtained from Ipca Laboratories Ltd, Mumbai. Combination product of artemether and lumefantrine (Label claim: artemether 80 mg and lumefantrine 480 mg), were obtained from R & D (Formulations), Ipca Laboratories Ltd, Mumbai. Acetonitrile, Potassium dihydrogen phosphate, orthophosphoric acid were purchased from Merck (India). Glacial acetic acid was obtained from Spectrochem, Milli-Q grade water was used throughout the work. All dilutions were performed in standard volumetric flasks.

Instrumentation and Chromatographic Conditions

Chromatographic separation was performed with Alliance 2695 pump, 2695 autosartemetherpler, 2996 PDA detector and 2695 separation module. Chromatograms and data were acquired and processed using Empower software version 1. A Waters Symmetry C_{18} column (150mm x 3.9 mm, 5 µm particle size) was used for the analysis. The mobile phase comprising of a mixture of buffer :(0.56% of hexane -1-sulfonic acid sodium salt & 2.75 g of sodium dihydrogen phosphate monohydrate in 995 ml of water + 5 ml Triethylamine, pH adjusted to 2.3 with orthophosphoric acid) and acetonitrile in the ratio of 25:75 v/v. The mobile phase flow rate was 1.0mL $min^{-1.}$ 10 µL of sample was injected in the chromatographic system and detection wavelength was set at 210 nm for artemether and 380nm for lumefantrine. A typical HPLC chromatogram for simultaneous determination of artemether and lumefantrine in pharmaceutical formulation is shown in figure 3.5.

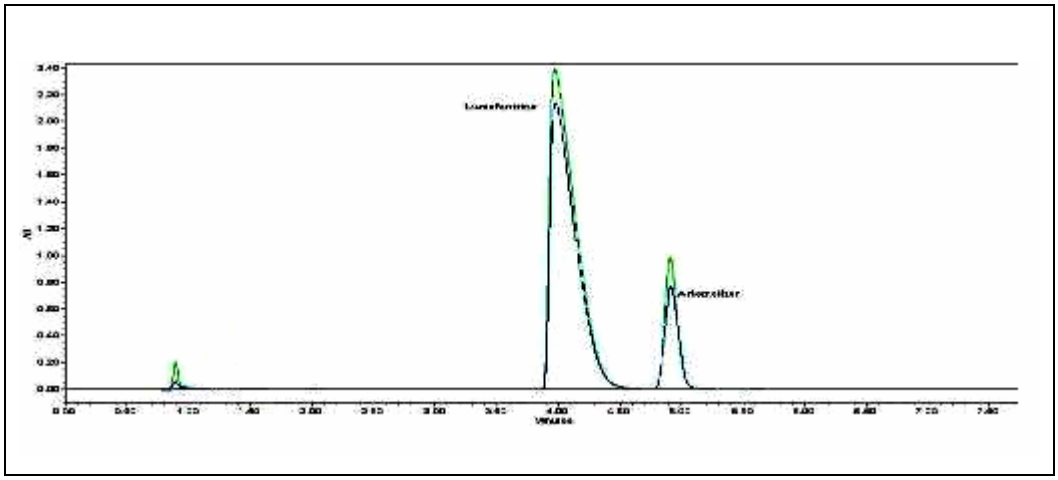

Figure 3.5 –Typical HPLC Chromtogram of standard and sample preparation.

3.2.3 Optimization of chromatographic conditions

Mode of Separation

In order to achieve simultaneous elution of the two components, different chromatographic conditions were attempted. Reverse phase chromatography is the first choice of separation for most regular samples, due to its simplicity and better column performances. In the normal phase chromatography, the separation selectivity is very less between different packing materials i.e. virtually all the compounds elute in the

same order regardless of the column selected. Hence, changes in the selectivity are primarily achieved by changes in the mobile phase. Even then, the selectivity changes are somewhat limited when compared to that obtained with reverse phase chromatography. Reverse phase chromatography columns are efficient, stable and reproducible. Due to their high reproducibility and stability coupled with wide versatility, reverse phase chromatography separations have become preferred mode of separation by HPLC. Aqueous eluents having high optical transparency as well as low flammability and toxicity can be used to accomplish most of separation goals.
Hence, a reverse phase mode of separation was chosen for HPLC determination.

Selection of Stationary phase / Chromatographic Column

Selectivity is most easily achieved in reverse phase chromatography by the variation of bonded groups (C_2, C_4, C_8, C_{18}, phenyl, cyano, nitro and amino). Selectivity may be further enhanced by variety of modifications possible in aqueous mobile phases such as variation in pH, addition of buffers and organic modifiers [159]. In the present development we have tried different stationary phases like C_8, C_{18} and cyano were used. Trial experiments indicated that using different concentration of acetonitrile or methanol with different pH of the buffers did not produce suitable retention and peak shape of Lumefantrine on a C_8 column. Waters Symmetry C_{18} was selected since this being packed with particles of silica gel, surface of which has been modified with chemically bonded octadecylsilyl groups was well fitted to reversed phase (RP) chromatogphy. It is with high carbon loading 19.1%, which helps for very good resolution [166]. Sample contains mixture of acidic artemether and basic lumefantrine. Due to basic nature of lumefantrine it is strongly retained on C_{18} column and tail so badly that barely observable. Increased retention time and tailing of basic compound is modified using solvent strength of mobile phase. Further, column with 5µm particle size gave better resolution between dihydroartemisinin and artemisinin that are degradants of artemether along with lumefantrine, compared to 10µm particle size column. The chromatographic conditions were optimized to obtain good base line separation and peak shapes. The method described herein was developed for the simultaneous determination of the artemether and lumefantrine in immediate released tablets.

Hence, a 150cm X 3.9 mm i.d. with a 5µm particle size, Symmetry C_{18} column was employed as the stationary phase for the present research work.

Selection of Mobile Phase

The formulation being fixed dose tablet, have a complex matrix and wide difference in the label claim of the two drugs. It required method specificity, to ensure accurate extraction, quantization and no interference of placebo. For this purpose, the influence of nature of the column, mobile phase composition, type of buffer type and its pH was systematically investigated [167]. Reference methods for assay for individual tablets were utilized for the development. In addition, references listed in this paper were taken as a base. The next goal was to improve chromatographic performance with respect to peak shape and resolution. Various mobile phases with different compositions were tried on Symmetry C_{18} column, for the simultaneous separation of artemether and lumefantrine. The mixture of acetonitrile and water or methanol was the first choice of selection as mobile phase. As a sample contains mixture of acidic and basic components it is desired to add ion-pairing agent. Ion pair agent is known to separate basic component. Higher molecular weight ion pair agent are more difficult to remove from the reversed phase, therefore, intermediate molecular weight ion pair agent i.e. hexane sulphonate is preffered. It is also known that higher concentration of ion paring agent sometimes preferentially form complex with free silanol groups of the column packing instead of target molecule. At higher pH, being acidic artemether will be ionised and retention will decrease significantly [167]. Hence, mobile containing ion pair, hexane sulphonate and pH 2.3 of phosphate buffer with combination of ACN is employed. Resolution and K' was satisfactory, but lumefantrine observed to tail and hence diethylamide was introduced in the mobile phase where we observed tailing of lumefantrine (<3.) and improves resolution (>3.0). Triethylamine forms a layer over the column material thus; it masks the activity of the residual silinol groups. This reduces hydrogen bonding of the drugs with residual uncovered silanol groups resulting in elution of the drug as sharp peaks without tailing [168]. Observed tailing of lumefantrine (<3.) and improves resolution (>3.0). Artemether and lumefantrine have pH 3.3 and 7.2 respectively. This indicates the separation was very sensitive to pH; hence, sodium dihydrogen phosphate buffer instead of water is used. The effect of pH

was studied within the pH range of 2.0 - 6.0 at the fixed buffer concentration. The mobile phase pH 2.3 , gave base-to-base separation between artemether and lumefantrine and hence the same pH was selected for mobile phase.

On the basis of above, the mobile phase comprises of buffer : (0.56% g of hexane -1-sulfonic acid sodium salt & 2.75 g of sodium dihydrogen phosphate monohydrate in 995 ml of water + 0.5% triethylamine, pH adjusted to 2.3 with orthophosphoric acid) and acetonitrile in the ratio of 25:75 v/v is optimized for the present work.

Selection of Detector wavelength

The target dosage form contains two active ingredients i.e. artemether : lumefantrine are in the ratio of 1:6 .This gives the challenge for selection of wavelengh due to its six fold concentration difference.UV absorption spectra of artemether, lumefantrine and its impurities recorded at targeted concentration of sample in HPLC system using PDA detection. In these spectras artemether and its impurities showed 210 nm as λmax where as lumefantrine showed λmax-approx.at 210 nm, 236 nm, 264 nm, 302 nm and 380 nm. The UV absorbance of lumefantrine at λmax at 210 nm is as high as beyond detector linearity where as at 380nm it is in the range of detector linearity. The response of artemether and its impurities is nil at 380 nm, therefore, the same is decided to select the wavelength for lumefantrine. Hence it is concluded to use wavelength 210 nm and 380 nm for simultaneous detection of artemether and lumefantrine respectively by dual wavelength technique.

Extraction of actives from sample matrix or sample pretreatment

The next development step was to extract both the drugs from the formulation. Artemether being highly soluble in organic and Lumefantrine being practically insoluble in all the organic and aqueous solvents, the extraction of both the drugs was optimized by using different combination of organic and aqueous solution. For finalizing the right concentration responses of active artemether , lumefantrine for 50 $\mu g\ mL^{-1}$, 100 $\mu g\ mL^{-1}$, 500 $\mu g\ mL^{-1}$ and 1000 $\mu g\ mL^{-1}$ were noted down and then to get detection of impurities equivalent to 0.1%, 100 $\mu g\ mL^{-1}$ and 600 $\mu g\ mL^{-1}$ concentration is finalized. Mobile phase is used as a diluent however, the limitations of solubility of lumefantrine were observed due to high concentration of ion pair reagent. Therefore, the diluent was prepared with sodium dihydrogen phosphate monohydrate in 100ml of

water, pH adjusted to 2.3 with orthophosphoric acid. Further, the diluent is prepared by mixing 250 ml of the phosphate buffer and 750 ml of acetonitrile and 1 ml orthophosphoric acid.. time of extraction is determined by weighing artemether and lumefantrine standard separately in a standard volumetric flask added 15-50 ml of the diluent slowly with swirling of flask and found out how the quantity is being dissolved in the diluent that is whether immediately or after shaking or it requires shaking and sonication. If it solubalise immediately that means little shaking and sonication will be sufficient, if standard itself takes time or needs more shaking or sonication then it indicates either to change the diluent or needs harsh extraction with increase shaking and sonication. When active lumefantrine is weighed 1200mg in 200 ml volumetric flask and 100 ml of diluent is added, it did not dissolve completely and immediately. It needs shaking for 10-20 minutes, so 30 minutes sonication from the placebo is sufficient to extract the Further when tablets are crushed and equivalent powder is taken at that time lumefantrine content was on lower side when investigated it was observed that lumefantrine has sticking tendency hence it was decided to use whole tablets. Further when various filter papers were used like Glass fiber filter,Whatman #41, #42 and polypropylene filter the actives were found to adsorb on the surface and low assay values hence filter paper usage is discouraged and centrifuged solution is used for estimation. So, after studying different columns make and composition of mobile phase of buffer and acetonitrile, diluents the following method has been finalized to optimize the retention time and estimation of artemether and lumefantrine.

3.2.4 Brief Description of The Method

Preparation of Working Standard Solutions

The solution of artemether (1000 µg mL^{-1}) was prepared by dissolving 200 mg artemether in diluent in a standard 200 mL volumetric flask (solution A). The stock solution of lumefantrine(1200 µg mL^{-1}) was prepared by dissolving 1.2g lumefantrine in diluent in a standard 200 ml volumetric flask (solution B). Pipette out 10ml each of solution A and solution B in standard 100 ml volumetric flask and dilute up to the mark with diluent. This gives 100 µg mL^{-1} of artemether and 600 µg mL^{-1} of lumefantrine.

Sample Preparation

Twenty tablets were weighed and their average weight was calculated. Five tablets were weighed and transferred into 1000 ml volumetric flask, 600 ml of diluent was added and the flask was sonicated for 30minutes with intermediate shaking. This solution was cooled to a room temperature and diluted upto the mark with diluent. Transferred 20ml solution in the centrifuge tube and centrifuged at 3000 RPM. 5 ml of the supernatant solution was diluted to 20 ml with the diluent. This gives final concentration of 100µg mL^{-1} and 600 µg $mL^{-1}$of artemether and lumefantrine respectively.

Table 3.1 describes the detailed optimized parameters of the developed method.

Table -3.1 Optimized Chromatographic Conditions

R No.	PARAMETER	DESCRIPTION
1.	Instrument	High performance Liquid Chromatograph, make- Waters
2.	Pump	Waters 2695 separation module quaternary pump
3.	Injector	Waters 2690 Alliance System
4.	Column	Symmetry C_{18}, 150cm x 3.9 mm i.d.,5µm.
5.	Detector	Waters 2996 Photo Diode Detector
6.	Wavelength	210 nm and 380 nm
7.	Recorder	Waters Empower data system
8.	Mobile Phase	Buffer (0.56% of hexane-1-sulfonicacid sodium salt and 0.01M of sodium dihydrogen phosphate containing 0.5%triethylamine, pH adjusted to 2.3 with orthophosphoric acid):Acetonitrile: in the ratio 25:75 (v/v).
9	Diluent	250 ml of the phosphate buffer + 750 ml of acetonitrile +1 ml orthophosphoric acid.
10.	Flow Rate	1.0 mL min^{-1}
11.	Injection Volume	10µl

3.2.5 Method Validation

The method validation is performed as per ICH guidelines Q2R. [47-49,160].

793.2.5.1 System suitability

System suitability tests are used to verify that the reproducibility of the equipment is adequate for the analysis to be carried out. System suitability tests are performed as per the USP 30/NF 25 [169] to confirm the suitability and reproducibility of the system. Prior to the acceptance of any validation data, the suitability of the system was determined by making six replicate injections of the working standard preparation. The % RSD was verified for each system suitability performed. The results are presented in Table-3.2 met the acceptance criterion. The test was carried out by injecting 10µl standard solution containing artemether and lumefantrine of 100 µg mL^{-1} and 600 µg mL^{-1} respectively. This was repeated six times. The %RSD values of artemether and lumefantrine were 0.47 and 0.20 respectively. The %RSD values were found to be satisfactory and meeting the requirements of less than 2. RSD of replicate injections, theoretical plates and tailing factor were determined and presented in table 3.2.

3.2.5.2 Specificity:

The specificity of the HPLC method was determined by injecting individual component and placebo preparation (containing all the excipients of the tablets namely croscarmellose sodium, colloidal silicon dioxide hypromellose, polysorbate 80, lactose anhydrous and magnesium stearate except both the actives), where in no interference was observed due to any of the excipients of tablet dosage form at the retention time of artemether and lumefantrine. Also the forced degradation study it was established that no degradant was found to interfere with the retention time of artemether and lumefantrine proving the specificity of the method.

Table 3.2 for System Suitability Study of Artemether and Lumefantrine

Parameters	Artemether	Lumefantrine
% RSD of replicate injections (n=6)	0.47	0.22
Tailing factor	1.19	2.23
Theoretical plates	8147	1991

Sample preparation for forced degradation study

a) Acid Hydrolysis : Weighed (20) tablets and calculated the average tablet weight. Weighed and transferred five tablets into a 1000-mL volumetric flask. Filled the

volumetric flask approximately with 600ml of diluent. Sonicated for 30 minutes with intermediate shaking. Shaken for 15 minutes. Again added 200 ml diluent for 15 minutes with intermediate shaking, added 50 ml 2N HCl, mixed well and neutralized with 2N NaOH , cooled at room temperature and diluted up to the mark with diluent. Centrifuged at 3000 RPM for 10 minutes.

(Acid Blank : Filled the volumetric flask approximately with 800 ml of Diluent add 50 ml 2N HCl mixed well and neutralized with 2N NaOH , cooled at room temperature and diluted up to the mark with diluent. Centrifuged at 3000 RPM for 10 min.)

b) Alkali Hydrolysis : Weighed (20) tablets and calculate the average tablet weight. Weighed and transfer 5 tablets into a 1000-mL volumetric flask.Filled the volumetric flask approximately with 600 ml of diluent. Sonicated for 30 minutes with intermediate shaking. Shaken for 15 minutes. Again added 200 ml diluent sonicated for 15 min with intermediate shaking, added 50 ml 2N NaOH mixed well and neutralized with 2N HCl, cooled at room temperature and diluted up to the marked with diluent. Centrifuged at 3000 RPM for 10 min.

(Alkali Blank: Filled the volumetric flask approximately with 600 ml of diluent, added 50ml 2N NaOH mixed well and neutralized with 2N HCl, cooled at room temperature and diluted up to the mark with diluent.Centrifuged at 3000 RPM for 10 min.)

c) Oxidation: Weighed (20) tablets and calculated the average tablet weight. Weighed and transferred 5 tablets into a 1000-mL volumetric flask. Fill the volumetric flask approximately with 600 ml of diluents. Sonicated for 30 minutes with intermediate shaking. Shaken for 15 minutes. Again added 300ml diluent, sonicated for 15 min with intermediate shaking, added 50ml 30 % H_2O_2 mix well, cool at room temperature and diluted up to the mark with diluent.

Centrifuged at 3000 RPM for 10 minutes)

(Oxidation Blank: Filled the volumetric flask approximately with 800 ml of diluent, added 50 ml 30 % H_2O_2 mixed well cooled at room temperature and diluted up to the mark with diluent. Centrifuged at 3000 RPM for 10 minutes.)

d) Heat Treatment: Active Artemether and Lumefantrine were exposed to heat at 60°C for stipulated period. Further sample preparation is same as per parent sample.

The result presented in table – 3.3 met the acceptance criteria proving the specificity. Figures 3.6 and 3.7 proved the peak purity of the both the actives alone and in the formulation under stressed conditions. It was demonstrated that no other components in the products (e.g. formulation excipients, other active, preservative, impurities, etc.) interfere with the determination of artemether and lumefantrine.This was achieved by separately chromatographing a pure sample of the analyte, control blend of excipients, both APIs separately and in combination. The peak purity study was carried out on forcefully degraded samples of actives, in combination and sample, which was achieved by acid hydrolysis, alkali hydrolysis and oxidation. Peak purity for artemether and lumefantrine peak from degraded sample was confirmed. Accelerated compatibility study between artemether, lumefantrine and other formulation excipients was carried out by exposing artemether, lumefantrine active and excipients mixtures alone and in combination at elevated temperature such as 55^0C for one week and photostability chamber as per ICH 1B guideline for photostability study and by chromatographing these preparations against the standard. Acid hydrolysis, alkali hydrolysis and oxidation for drug substance as well as for drug product showed significant change in assay value. Under thermal and photostability, condition the drug substance and drug product are stable. The method was found to be selective (specific), since no other formulation components, produced any chromatographic responses that would interfere with the determination of artemether and lumefantrine and the spectral homogeneity of the artemether and lumefantrine was maintained. It is concluded after assessing the data that the given method can be used for its intended purpose to establish the quality of artemether and lumefantrine tablets through out the shelf life.

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Table 3.3 - Forced Degradation Study of Drug Substance and Drug Product

Degradation Condition	**% Assay**		**Purity angle is < than purity threshold**
	Artemether	Lumefantrine	
Parent (API)- individual	99.82%	98.77 %	Pure
Parent Artemether & Lumefantrine (APIs in combination)	99.48%	99.11%	Pure
Parent sample (20+120)	98.22 %	97.87 %	Pure
ACID HYDROLYSIS			
Artemether and Lumefantrine(API) individual	95.99%	95.96%	Pure
Artemether & Lumefantrine (APIS in combination)	95.62 %	94.50 %	Pure
sample	86.62%	85.16 %	Pure
ALKALI HYDROLYSIS			
Artemether and Lumefantrine (API)Individual	90.72%	89.68 %	Pure
Artemether & Lumefantrine (APIS in combination)	90.82 %	88.18 %	Pure
sample	90.23 %	89.63 %	Pure
OXIDATION			
Artemether and Lumefantrine (API) individual	95.99%	98.23 %	Pure
Artemether & Lumefantrine (APIS in combination)	99.07 %	99.83 %	Pure
Sample	95.15 %	95.98 %	Pure
HEAT			
Artemether and Lumefantrine(API) Individual	99.82%	98.87 %	Pure
Artemether & Lumefantrine (APIS in combination)	98.79 %	98.65 %	Pure
sample	97.10 %	99.30 %	Pure
PHOTO STABILITY			
Artemether and Lumefantrine(API)Individual	99.23%	98.57 %	Pure
Artemether & Lumefantrine (APIS in Combination)	98.50 %	98.45 %	Pure
Photo stability sample (20+120)	97.82 %	98.23 %	Pure

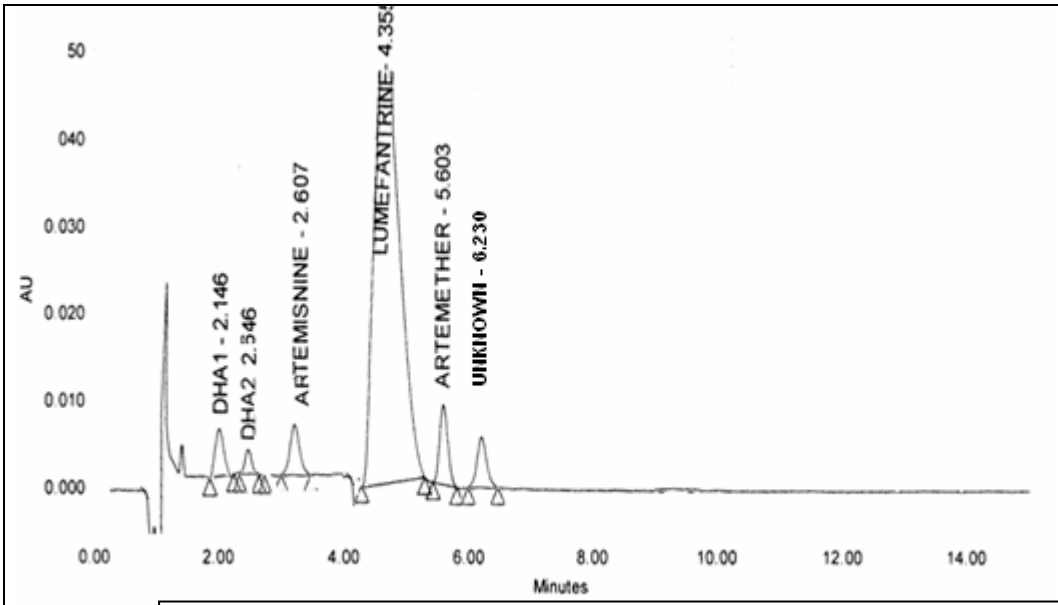


Figure 3.6- Typicaql Chromatogram of forced degraded sample of alkali hydrolysis

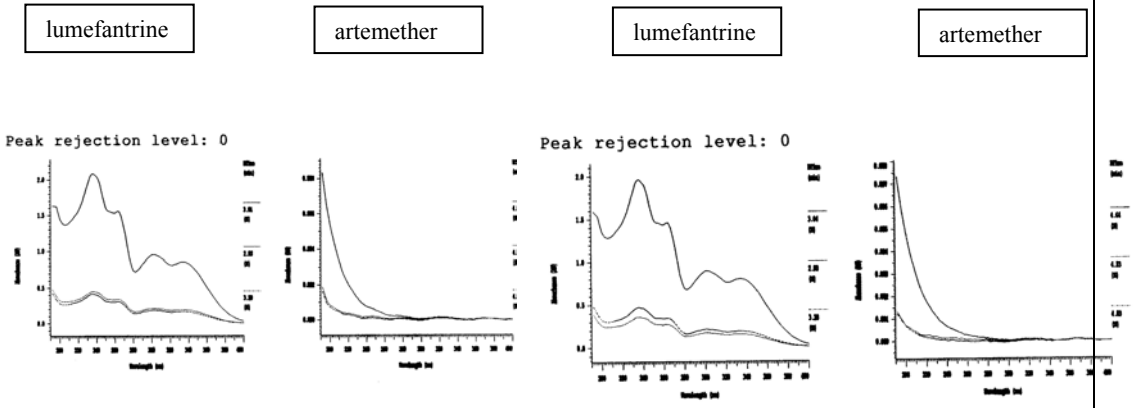


Figure 3.7- Peak purity graphs of degraded sample

3.2.5.3 Linearity of The Detector Response

Linearity was evaluated by analysis of working standard solutions of artemether and lumefantrine of five different concentrations. The range of linearity was from 60 - 140 µg mL^{-1} for artemether, 360 - 840 µg mL^{-1} for lumefantrine. Curves were obtained by plotting the peak area of respective drugs against concentrations. The slope, Y-intercept and regression coefficient (r) were obtained from linear regression analysis. The

regression data obtained for the two pharmaceuticals actives are represented in table 3.4 and 3.5. Figure 3.8 and figure 3.9 represent the linearity graph of artemether and lumefantrine respectively. The result shows that with-in the concentration range mentioned above there was an excellent correlation between peak area ratios and concentration of each drug.

Table 3.4 Linearity Study of Artemether

Sr. No.	Conc. of standard µg mL^{-1} (x-axis)	Mean area (y-axis)
1	60	25209
2	80	31236
3	100	40805
4	120	48296
5	140	56824

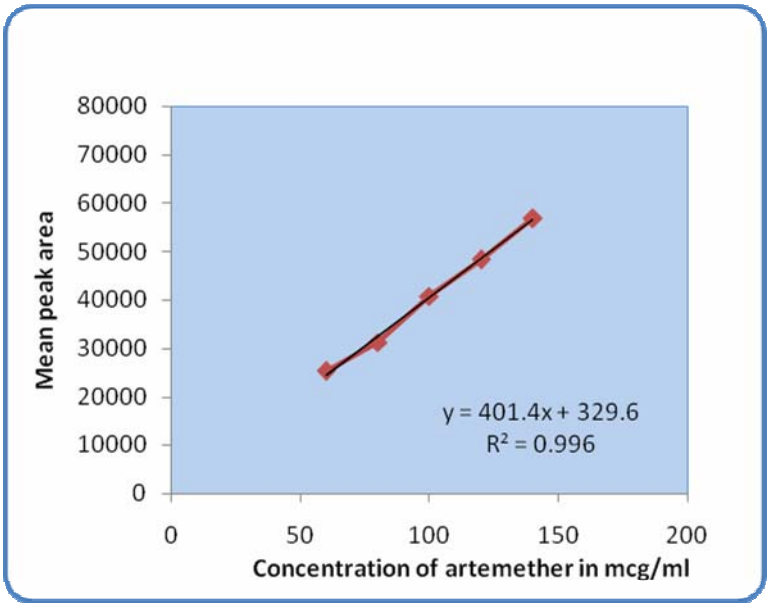


Figure -3.8 Linearity of Artemether

Table 3.5 Linearity Study Of Lumefantrine

Sr. No.	Conc. of standard µg mL^{-1} (x-axis)	Mean area (y-axis)
1	360	1439497
2	480	1891714
3	600	2386398
4	720	2831117
5	840	3319476

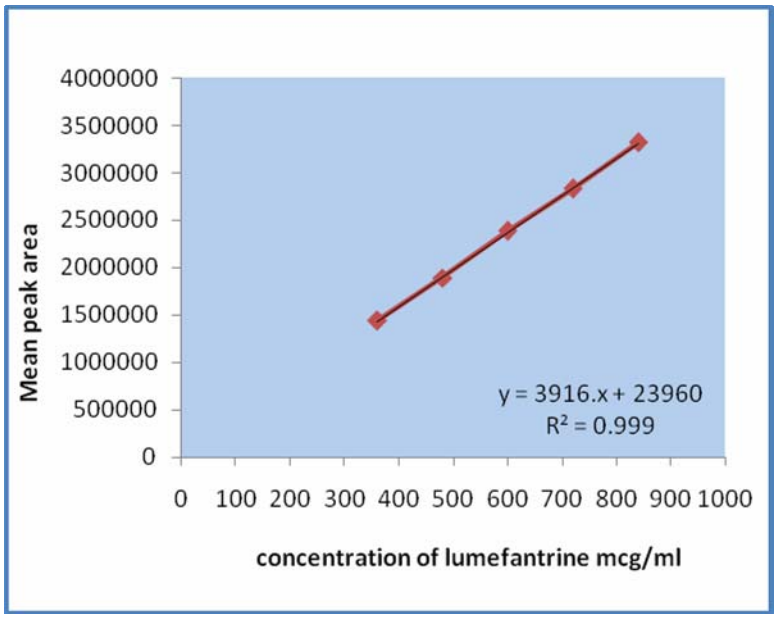


Figure -3.9 Linearity of Lumefantrine

3.2.5.4 Limit of Detection and Limits of Quantitation

The Limit of Detection was determined by making serial dilutions of standard solutions of artemether and lumefantrine followed by HPLC analysis. The Limit of Detection data was evaluated from the calibration curve using the formula i.e. DL = [3.3∗ SyX/Slope]. The LOD of artemether and lumefantrine were found to be 2.03 µg mL^{-1} and 2.04µg mL^{-1} respectively.

The Limit of Quantitation was determined by making serial dilutions of standard solutions artemether and lumefantrine followed by HPLC analysis. The Limit of Quantitation data was evaluated from the calibration curve using the formula i.e. QL = [10∗ SyX/Slope]. The LOQ of artemether and lumefantrine were found to be 6.168 µgmL^{-1} and 6.202 µg mL^{-1} respectively. Figure 3.10 represent specimen chromatogram of LOD and LOQ of artemether and mefantrine.

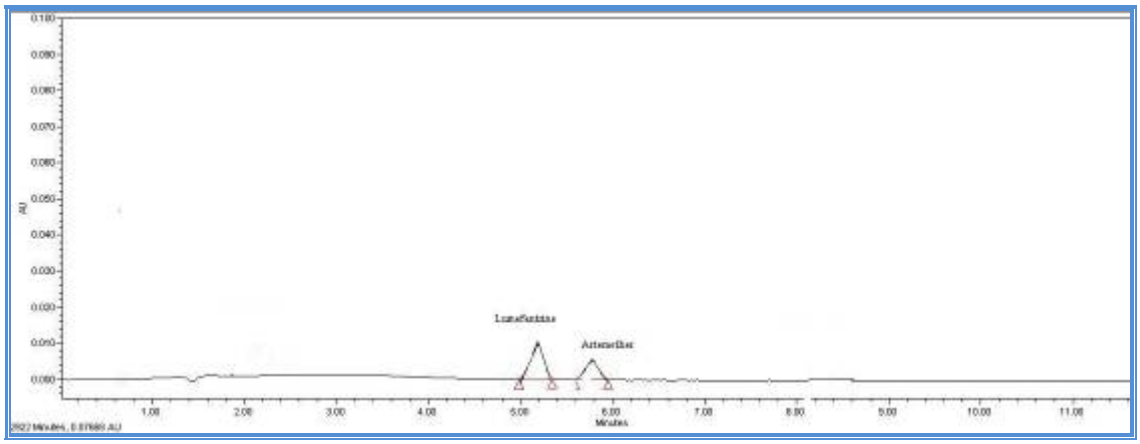


Figure 3.10 Chromatogram of LOQ of Artemether and Lumefantrine

3.2.5.5 Accuracy

To study accuracy of the method, recovery experiment was determined by applying the standard addition method in which known quantities of each drug substance corresponding to 80%, 100%, and 120% of the assay concentration of each drug were added to the placebo. The accuracy was expressed as the percentage of analytes recovered by the assay.

The accuracy / recovery of the assay method of determination of assay content of artemether and lumefantrine was determined by testing 3 sets of 3 samples of the control blend according to the assay method containing of the active content per tablet,

80%, 100% 120% of the range. Placebo or control blend consisted of mixture of excipients namely croscarmellose sodium , colloidol silicon dioxide hypromellose, polysorbate 80, lactose anhydrous and magnesium stearate. Each set of additions was repeated three times the accuracy was expressed as percent recovered at each level of concentration and as overall accuracy when the data are normalized to percent theoretical. Table 3.6, lists the recoveries of the two drugs from a series of spiked concentrations. The recovery results obtained were between 99.40% to 100.87% for artemether and 99.02% to 100.36% for lumefantrine.The recovery values obtained for both the actives showed % bias within 1%. This indicates the method is highly accurate for simultaneous determination of the artemether and lumefantrine in fixed dose pharmaceutical dosage forms.

Table 3.6 - Accuracy Study

Level	Amount of Placebo (mg)	Amount of std. added X(mg)	Amount Recovered Y (mg)	%RSD (n=3)	% Recovery	% Bias
Artemether						
80%	680.50	80.17	79.69	1.70	99.40	-0.60
100%	540.40	100.20	99.72	0.70	99.52	-0.48
120%	400.20	120.10	121.15	0.22	100.87	+0.87
Lumefantrine						
80%	680.50	480.20	481.94	0.51	100.36	+0.36
100%	540.40	600.20	600.61	0.69	100.06	+0.06
120%	400.20	720.40	713.15	1.10	99.02	-0.98

3.2.5.6. Precision

Precision study was assessed by injection repeatability and sample repeatability. Injection repeatability was confirmed by performing replicate injection of the standard solution and calculating the % RSD of the peak area responses for both the content. The data showed good precision of the system with RSD of 1.88% and 0.34% respectively for artemether and lumefantrine, which is ≤ 2.0% .Table 3.7, represented data of system precision. The sample repeatability was studied by analyzing the same sample for six times and calculating the % assay value. RSD of assay of artemether and lumefantrine were 0.58% and 0.38% respectively. These are well within the limit of 2.0%. The results listed in table 3.8, indicated that the amount of each drug in the tablets is within the requirements of 95 -105% of the label claim and the method is precised.

Table 3.7- System Precision

Sr. No.	Peak Area Response of Standard (Artemether)	Peak Area Response of Standard (Lumefantrine)
1	39837	2384100
2	39683	2395226
3	39769	2384868
4	39978	2401102
5	40446	2403612
6	41673	2394904
Mean	40231	2393968.7
SD	756.12	8081.42
%RSD	1.88	0.34

Table 3.8 Method Precision (Repeatability)

Sr. No.	Weight of sample (mg)	Mean Peak Area Response of		% Assay	
		Artemether	Lumefantrine	Artemether	Lumefantrine
1	1248.0	39225.5	2407427.0	98.45	100.04
2	1252.3	39747.5	2415855.0	99.42	100.04
3	1253.8	39395.0	2431521.5	98.42	100.57
4	1250.6	39286.5	2411191.5	98.40	99.99
5	1249.4	39649.0	2420628.0	99.41	100.47
6	1247.2	39653.5	2427419.0	99.59	100.93
Mean				98.95	100.34

3.2.5.7 Solution stability

The stability of the analytical solutions of the method was studied by analyzing the standard and sample solution immediately as well as until 24 hrs with two intermediate time point. The stability was assessed by comparing the area response for standard preparation and percentage assay in case of sample preparation. The study of stability of sample solution concluded that the sample solution was stable for 24 hrs when kept at ambient temperature .The results are tabulated in table 3.9 and 3.10. Figures 3.11 and 3.12 represent solution stability data of standard solution and sample solution.

Table 3.9- Solution Stability Study of Standard (At Ambient Temperature)

Hours	Artemether			Lumefantrine		
	Mean Peak Area Response of Standard	Difference	% Difference	Mean Peak Area Response of Standard	Difference	% Difference
0	39187.0	0	0	2389422.5	0	0
2	39144.0	-43.0	-0.1097	2380374.0	-9048.5	-0.3787
4	39790.0	603.0	1.5388	2377277.0	-12146.0	-0.5083
6	39368.5	181.5	0.4632	2382485.0	-6938.0	-0.2904
12	39445.0	258.0	0.6584	2385838.0	-3584.5	-0.1500
24	39194.5	7.5	0.0191	2397286.0	7863.5	0.3291

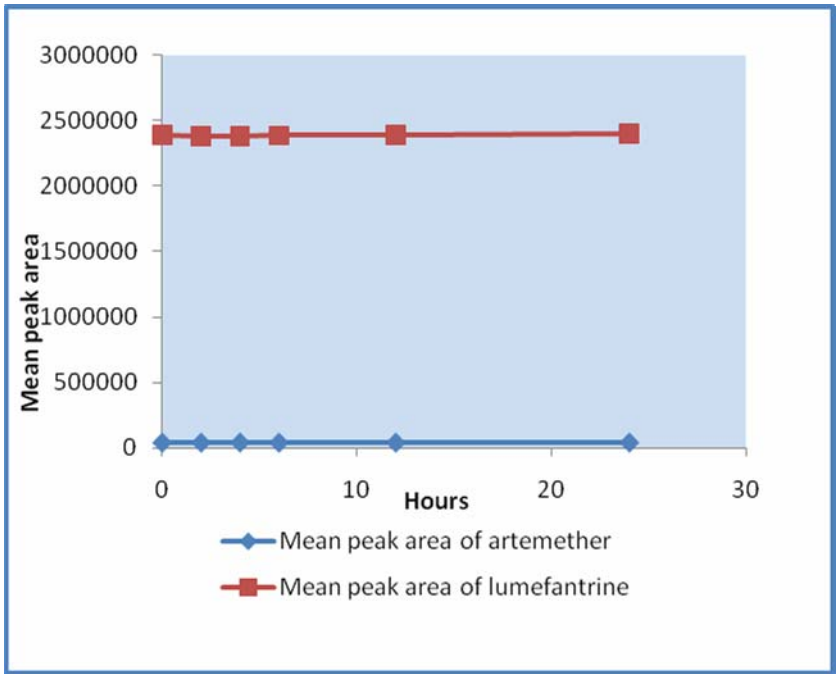


Figure -3.11 Solution Stability Curve of Standard Artemether and Lumefantrine

Table 3.10 Solution Stability Study Of Sample At Ambient Temperature

Time in Hours.	Weight of Sample (mg)	% Assay of Lumefantrine	% Difference from the initial assay of Lumefantrine	% Assay of artemether	% Difference From the initial assay of artemether
0	1248	99.79%	0.00	99.72%	0.00
2	1248	99.43%	- 0.36	99.07%	- 0.66
4	1248	99.49%	- 0.29	99.10%	- 0.62
6	1248	99.27%	- 0.52	98.87%	- 0.85
12	1248	99.95%	0.17	98.82%	-0.90
24	1248	100.15%	0.36	99.88%	0.16

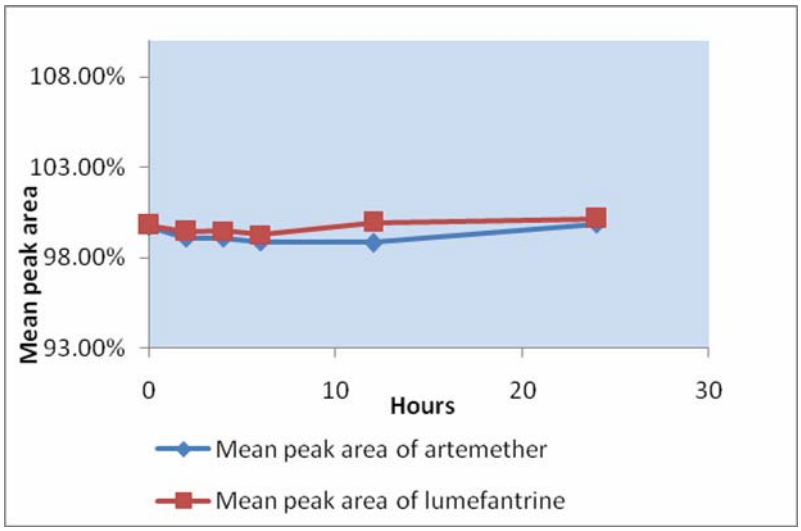


Figure 3.12 Solution Stability Curve of Artemether And Lumefantrine Tablets

3.2.5.8 Ruggedness or Intermediate Precision

The ruggedness or the Intermediate Precision of stability indicating artemether and lumefantrine was carried out by two different analytical persons on two different instruments on different dates to know the degree of reproducibility of results obtained by the analysis of the same sample. Also, % RSD for the set of samples (n =12) was observed to be 0.94 for artemether and 0.55 for lumefantrine. Intermediate Precision or Ruggedness data for assay method of product by two different analysts on different dates and different instruments is tabulated in table 3.11 and 3.12.

3.2.5.9 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but detectable variation in method parameters and equipments. This was studied by deliberately changing parameters like flow rate (± 0.2ml),composition of mobile phase and column . The system suitability parameters were studied for these changes. All system suitability parameters were found within the acceptance criteria. The method was found to be robust for above changes in the flow rate and the pH of the mobile phase.The method was critical with respect to mobile phase composition and column, hence both should be critically followed as per the method description.The result presented in tables 3.13 represents robustness data.

Table 3.11 Intermediate Precision or Ruggedness Study for Artemether Content in Artemether and Lumefantrine Tablets

Sr. No.	Day- I Instrument & Model: Waters X Location: ADL,Mumbai			Day- II Instrument & Model: Dionex II Location: ADL,Mumbai		
	Wt. of sample	Mean peak Area Response	Assay %	Wt. of sample	Mean peak Area Response	Assay %
1	1248.0	39225.5	98.45	1257.3	639.40	97.16
2	1252.3	39747.5	99.42	1245.8	647.19	99.25
3	1253.8	39395.0	98.42	1250.2	659.84	100.83
4	1250.6	39286.5	98.40	1249.1	654.41	100.09
5	1249.4	39649.0	99.4	1244.5	644.67	98.97
6	1247.2	39653.5	99.59	1248.2	646.41	98.94

Table 3.12 - Intermediate Precision or Ruggedness Study for Lumefantrine Content in Artemether & Lumefantrine Tablets

Sr. No.	Day: I Instrument & Model: Waters X Location: ADL,Mumbai			Day: II Instrument & Model: Dionex II Location: ADL,Mumbai		
	Wt. of sample	Mean peak Area Response	Assay %	Wt. of sample	Mean peak Area Response	Assay %
1	1248.0	2407427.0	100.04	1257.3	34573.96	99.09
2	1252.3	2415855.0	100.04	1245.8	34336.86	99.32
3	1253.8	2431521.5	100.57	1250.2	34475.76	99.37
4	1250.6	2411191.5	99.99	1249.1	34559.38	99.70
5	1249.4	2420628.0	100.47	1244.5	34380.66	99.55
6	1247.2	2427419.0	100.93	1248.2	34574.13	99.82

Table 3.13 Robustness Study

Parameters		**Artemether**		**Lumefantrine**	
	Changes	% RSD (NMT 2%)	% Assay ± 2%	% RSD (NMT 2%)	% Assay ± 2%
A : Flow rate of mobile phase (ml/min)					
0.8	-0.2	1.17	99.65	0.21	99.85
1.0	0	0.58	99.56	0.38	100.15
1.2	0.2	1.45	99.84	0.42	99.65
B :Composition of mobile phase (Buffer: ACN)					
20:80	-5%	1.03	99.12	0.33	99.21
25:75	0%	0.58	99.56	0.38	100.15
30:70	+5%	1.16	99.63	0.41	99.77
C: pH of Buffer					
2.2	-0.1	0.15	99.13	0.38	99.72
2.3	0	0.54	99.56	0.37	100.15
2.4	0.1	0.75	98.78	0.64	99.12
D: Change in column					
Kromasil C_{18}	-	1.31	98.68	1.67	98.35
BDS Hypersil	-	0.34	99.54	0.30	99.54

With Buffer: Acetonitrile proportion 30:70, there is no proper separation of artemether and lumefantrine. This is true even in case of Buffer : Acetonitrile, 28 : 72 proportions.

NOTE : With BDS Hypersil C_{18}, Artemether is eluted first and then Lumefantrine gets eluted. In addition, the peak of Artemether is distorted in standard. Hence not recommended to use and concluded that Waters Symmetry C_{18} is the best choice.

3.2.6 Application of The Validated Method

The validated method was applied in the QC lab for product release and for stability testing. Stability batches at different conditions (25^{o}±2 ^{o} C,60% ± 5% RH, 30^{o} C, ±2 ^{o} C, 65% ± 5% RH 40^{o} C±2 ^{o} C 75% ± 5% RH) were withdrawn at definite time intervals (1,3,6 months) by formulator R & D (Formulation), and analysed. Stability batches were analysed by validated stability indicating method and results showed the assay of artemether decreases at 40^{o} C±2 ^{o} C 75% ± 5% RH with increase in DHA hence it is also concluded that product is stable only at or below 30^{o} C, ±2 ^{o} C, 65% ± 5% RH. Figure 3.13 and 3.14 represent typical chromatogram of one of the stability batches at all conditions.

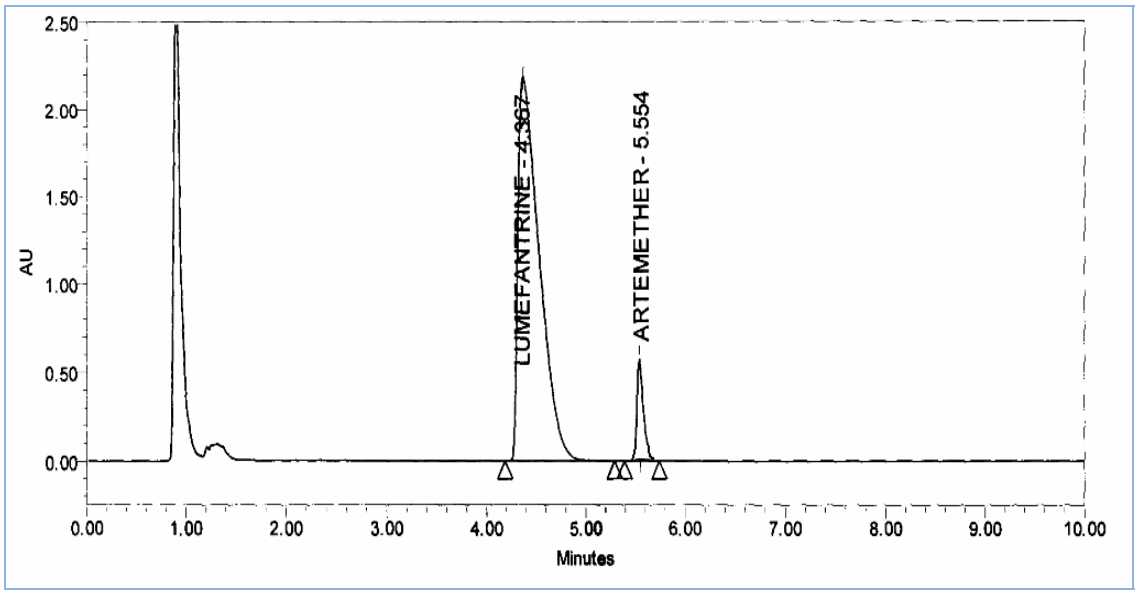


Figure 3.13 : Chromatogram of Sample at 25^{0}C 60% RH at 3 Months.

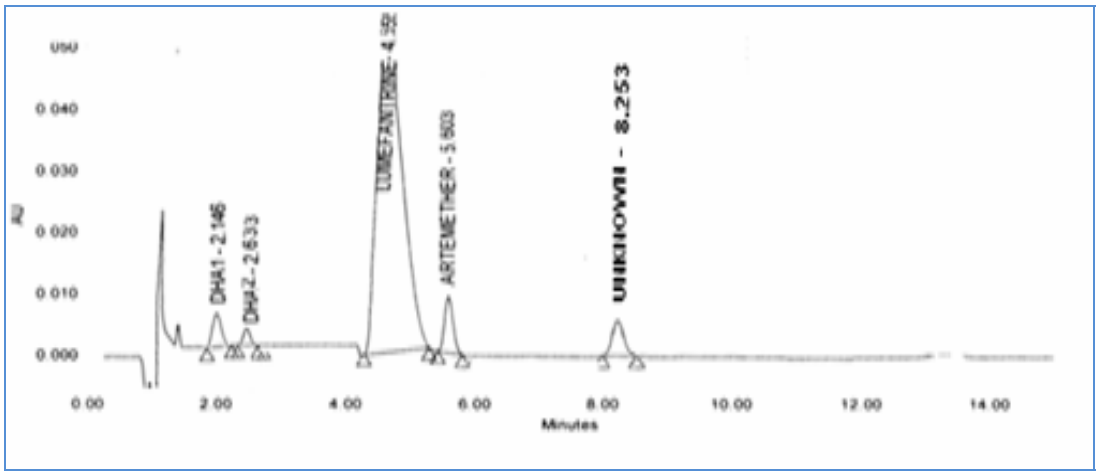


Figure 3.14 : Chromatogram of Sample at 40^0C 75% RH at 3 Months.

3.2.7 Conclusion

The developed method is speedy, easily reproducible , with simple sample preparation step, improved sensitivity and a short chromatographic run time. The method validation was performed as per the ICH Q2R guidelines and found to be accurate and rugged for the quantization of artemether and lumefantrine from the formulation. Although there is a difference in the polarity between the two compounds, the simultaneous determination could be achieved successfully. Method was validated for its performance parameters such as specificity (placebo interference), linearity and range, recovery, precision and ruggedness .It was concluded that the developed method offers several advantages such as fast and easy mobile phase, and simple sample preparation steps, improved sensitivity and comparatively short run time, made it specific and reliable easily reproducible in any quality control setup, provided all the parameters are followed accurately for its intended use. Beauty of the method is that it can be extrapolated to use for the other strengths (20+120, 40+240mg and 80+480 mg of artemether and lumefantrine tablets respectively) different formulations like dispersible tablet. dry powder suspension also different types of dosage forms like tablet in tablet, bilayer , single granulation of the same combination etc.

3.3 Development and Validation of Stability Indicating Reverse Phase HPLC Assay Method for Simultaneous Determination of Artesunate and Amodiaquine in Fixed Dose formulations.

3.3.1 Introduction

Artesunate, chemically (3R,5aS,8aS,9R,10S,12R,12aR)-Decahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-/]-1,2-benzodioxepin-10-ol,hydrogen succinate, is used in the treatment of malaria and Amodiaquine Hydrochloride chemically 4-[(7-Chloro-4-quinolyl)amino)-α-(diethylamino)-o-cresol;4-[(7-Chloro-4-quinolyl) amino-2[(diethylamino)methyl]phenol; is also used as antimalarial drug [161]. The structures of the two drugs are depicted as shown in figure 2.4 and 2.5.

Malaria, the most important parasitic disease of humans, remains a major health and economic burden in most tropical countries. Malaria is a major cause of death equal with HIV/AIDS and tuberculosis [2]. It afflicts more than 500 million people, causing from 1.7 million to 2.5 million deaths each year [3]. It occurs in over 90 countries worldwide. Because of the continued increase of resistance to antimalarial drugs in many regions of the world, with the resultant effect of morbidity and mortality, resulted in a re -examination of the potential of combinations of existing products and the development of new combination drugs [4]. Combination therapy with antimalarial drugs is the simultaneous use of two or more blood schizonticidal drugs with independent modes of action and different biochemical targets in the parasite [170]. When the formulations are developed with such combination of drug substances, the development of the analytical method to evaluate the efficacy as well as the content of the drug substance becomes the attractive challenge in the analytical research.

Literature survey reveals that only few stability indicating methods are reported for assay of combination drug products containing two or more active drug substances [171-174]. An analytical method with a single extraction procedure and two separate high performance liquid chromatographic systems for the determination of artesunate, dihydroartemisinin and mefloquine in human plasma for application in clinical pharmacological studies of the drug combination. The combination of two sensitive, selective and reproducible reversed phase liquid chromatographic (RP-HPLC) methods

was developed for the determination of artesunate (AS), its active metabolite dihydroartemisinin (DHA) and mefloquine (MQ) in human plasma. The method was found to be suitable for use in clinical pharmacological studies[175]. Several methods using HPLC and mass spectrometry are reported in the literature for chloroquine and its metabolite in biological fluids like blood, plasma and urine[176-189]. Determination of chloroquine and desethylchloroquine in hair by GC-MS and TLC has been reported [190]. Methods for determination of chloroquine and its metabolite by GC and GC-MS are reported [191-197] .Methods for quantitative determination of chloroquine and desethylchloroquine by HPTLC are given [198-202]. Reports on determination of chloroquine and its metabolite by HPLC with fluorescence detection are also available [203]. Continuous determination of chloroquine in plasma by laser-induced photochemical reaction and fluorescence has been reported by few workers [204]. Capillary electrophoretic method for determination of chloroquine and its metabolite are explained [205].

There is no Pharmacopoeial method for the simultaneous determination of artesunate and amodiaquine in pharmaceutical preparations to apply for routine quality control analysis. Pharmacopoeial methods have been reported for the determination of individual content of artesunate and amodiaquine respectively.

The present research work describes development of a stability indicating high performance liquid chromatographic method for the simultaneous determination of artesunate and amodiaquine from the combination tablets. It makes use of a reversed phase mode of separation. Rapid, precise, accurate, rugged and robust high performance liquid chromatographic method is developed for simultaneous determination of Artesunate and Amodiaquine. The separation of two actives was achieved by using BDS C_{18}, 100 X 4.6 mm, 3µ column using mobile phase consisted of acetonitrile and buffer containing 1.36 g monobasic potassium phosphate in 1000 ml of water and pH was adjusted to 3.0 with orthophosphoric acid and detection was carried out at dual wavelength 210 nm for artesunate and 300 nm for amodiaquine. External standard method was used for quantization. The proposed HPLC method was validated and applied for simultaneous determination of artesunate, amodiaquine, from its

combination pharmaceutical dosage preparation. All the analytical data was subjected to statistical analysis.

3.3.2 Experimental Conditions

Instruments

Waters High performance Liquid Chromatograph equipped with Waters Empower data system, Waters 2996 Photo Diode (PDA) Detector and Waters 2695 separation module was used. The column used was stainless steel, 10 cm x 4.6mm id packed with 3µm base deactivated (BDS) LC18 bonded material was used for all practical purpose.

Reagents

Artesunate (B.No.5057ASJI and Amodiaquine Hydrochloride B.NO.5009Q2RJ pure drug samples provided by IPCA Laboratories Ltd. Ratlam (API Division). A drug product containing 100 mg of artesunate and 300 mg of amodiaquine per tablet were obtained from R & D (Formulations), Ipca Laboratories Ltd.

All preparations were carried out using the following reagents

Milli-Q grade water, acetonitrile(ACN) HPLC grade (Merck), monobasic potassium phosphate reagent grade (Merck), orthophosphoric acid (Merck) and glacial acetic acid HPLC grade (Spectrochem LTD) and double distilled water were employed throughout the work and quantitative determination.

Preparation of Stock and Working Standard Solutions

The stock solution of artesunate (1000 µg mL^{-1}) was prepared by dissolving 25 mg artesunate in mobile phase in a standard 25mL volumetric flask (solution A). The stock solution of amodiaquine (4000 µg mL^{-1}) was prepared by dissolving 100.00 mg amodiaquine hydrochloride in mobile phase in a standard 25 ml volumetric flask (solution B). Pipette out 2.5 ml each of solution A and solution B in standard 10 ml volumetric flask and dilute up to the mark with mobile phase. This gives 250 µg $mL^{-1}$of artesunate and 1000 µg mL^{-1} of amodiaquine.

In order to decide the linear range for the solutes to be separated and quantified, it was necessary to take in to consideration the amount and the proportion of these components in the formulation under study. The formulation contains 100 mg artesunate and 300 mg of amodiaquine. The contents are in 1:3 or 1:2.7 ratio with respect to artesunate.

3.3.3 Optimisation of Chromatographic Conditions

Before developing any chromatographic method, one has to review the nature of sample, and goals of the separation defined. The sample related information that needs to be known prior to HPLC method development is given below [206,207]

Number of components present in the sample were two namely artesunate and amodiaquine.Chemical structures (functionality of components) are as given in figure 2.3 and 2.4. Molecular weights of compounds.are 384.42 and 464.8 for artesunate and amodiaquine hydrochloride.respectively. pKa values of compounds are 4.6 and 5.8 respectively.Concentration range of compounds in sample of interest is in the ratio of 1:3 and 1:2.7. solubility of both ythe components differ drastically. Artesunate have poor aqueous solublility where as amodiaquine hydrochloride is hihgly water soluble. Sample fall into category of regular regular.

The various parameters that were considered in the development process such as mode of separation, selection of stationary phase, selection of mobile phase, selection of detection method, extraction of actives from the sample matrix and finally validation of the developed method.

Mode of Separation

In HPLC there are two types of samples that need separation; that is the regular and special samples. Regular samples are usually the mixtures of small molecules (<2000Da), while special samples are those, which require specialized methodology for separation. Regular samples are those that do not require special sample treatment and can be separated using a regular method. Special samples are usually large macromolecules, enantiomers and various inorganic ions that require special sample pretreatment and specialized type of chromatography for their separation. Regular samples can be further classified as ionic or neutral. Ionic solutes can be generally defined as organic molecules that contain one or more functional groups capable of acidic or basic behavior in the pH range of 2 to 8.

Artesunate and Amodiaquine fixed dose tablets are all regular samples with molecular weights 384.42 and 464.8 of artesunate and amodiaquine respectively. Reverse phase chromatography is the first choice of separation for most regular samples, due to its simplicity and better column performances. In the normal phase chromatography, the

separation selectivity is very less between different packing materials i.e. virtually all the compounds elute in the same order regardless of the column selected. Hence, changes in the selectivity are primarily achieved by changes in the mobile phase. Even then, the selectivity changes are somewhat limited when compared to that obtained with reverse phase chromatography. Reverse phase chromatography columns are efficient, stable and reproducible. Due to their high reproducibility and stability coupled with wide versatility, reverse phase chromatography separations have become preferred mode of separation by HPLC. Aqueous eluents having high optical transparency as well as low flammability and toxicity can be used to accomplish most of separation goals. Selectivity is most easily achieved in reverse phase chromatography by the variation of bonded groups dimethyl silane (C_2), butyl silane (C_4), octylsilane (C_8), octadecylsilane (C_{18}), phenyl, cyanopropyl,nitro, amino etc). Selectivity may be further enhanced by variety of modifications possible in aqueous mobile phases such as variation in pH, addition of buffers and organic modifiers. In the present research work, a reverse phase mode of separation was employed taking into account the semi polar to polar nature of artesunate, amodiaquine, and their solubility in acetonitrile and distilled water.Hence a reverse phase mode of separation was chosen for HPLC determination.

Selection of Stationary phase / Chromatographic Column

The column is the heart of HPLC separation process. The availability of stable, high performance column is essential in developing a rugged, reproducible HPLC method [153]. The appropriate choice of separation column includes three different approaches,Selection of separation system. The particle size and the nature of the column packing. The physical parameters of the columns, i.e. the length and the diameter.The column is selected depending on the nature of the solute and the information about the analyte. Reversed phase mode of chromatography facilitates a wide range of columns like dimethyle silane (C_2), butyl silane (C_4), octylsilane, octadecylsilane (C_{18}), phenyl, cyanopropyl, nitro, amino etc. Cyano, phenyl are more polar and weak columns hence C_{18} was chosen for the study since it is the most retentive, rugged and widely available.

Additionally, it is least polar and more functions as retaining or as a stronger column in reversed phase. Generally, a longer column provides a better separation due to higher

theoretical plate numbers, but it is reported that only a few centimeters of the columns exerts the dominant effect of separation and hence short columns can provide seperation that are achieved on longer columns, if the mobile phase composition is suitably adjusted. Different concentration of acetonitrile or methanol with different pH of the buffers did not produce suitable retention and peak shape of amodiaquine on a C_8 column. End capping minimised unwanted interaction with silanol . Using base deactivation process, the surface of the column is made much more homogenous, so that all silanols are of same type [208]. Special care is taken to ensure a high density of coverage followed by thorough end capping in order to further reduce possibility of any silent interaction. Because of deactivation process, the silanlols that are still present after dramatization becomes much more " friendly" towards basic and acidic compounds and the packing material becomes excellent choice to develop highly reproducible bands with polar analytes with this silent interaction. Base Deactivated (BDS) columns are well suited for wide range of analytes, including both acids and bases with peak shape and column performance significantly improved. Further, BDS column with 3µ particle size gave better retention time as compared to 5µ particle size column. Shorter columns packed with the smaller particles are often used for shorter analysis times. The smaller the particle , the higher the efficiency. Column with 3µm particles gave higher efficiency than 5 and 10 µm particles columns. This efficiency is delivered over greater range. Columns of 3µm particles usually provide a larger number of plates per unit time than columns with large particle size. This gave good separation between dihydroartemisinin II and artesunate, artesunate and artemisinin, and glycan, which are major degradants of artesunate. Therefore, a 10 cm column was employed for the analysis . For analytical purposes a 0.4 –0.6 cm internal diameter column having a particle size between 3 – 10 µm are used. As particle size decreases the surface area available for coating increases. Trial experiments indicated that using columns with 3µm particle size gave the best suited of efficiency, reproducibility . In this case, the column selected had a particle size of 3µm and an internal diameter of 4.6 mm. The column plate number (N) is an important characteristic of a column. N- Defines the ability of the column to produce sharp, narrow peaks for achieving good resolution of band pairs with small α values. N is dependent on specific experimental factors. Peak

shape is equally important in method development. Columns that provide symmetrical peaks are always preferred while peaks with poor asymmetry can result, in low plate number, poor resolution, imprecise quantitation ,degraded and undetected minor bands in the peak tail and poor retention reproducibility. A useful and practical measurement of peak shape is peak asymmetry factor and peak tailing factor. Peak asymmetry is measured at 10% of full peak height and peak tailing factor at 5%. Reproducibility of retention times and capacity factor is important for developing a rugged and repeatable method. Hence, a 10cm X 4.6mm i.d. with a 3µm particle size, base deactivated C_{18} column was employed as the stationary phase for the present research work.

Selection of Mobile Phase

In liquid chromatography, the solute retention is governed by the solute distribution factor, which reflects the different interactions of the solute – stationary phase, the solute – mobile phase and the mobile phase - stationary phase. For a given stationary phase, the relation of the given solute depends directly upon the mobile phase, the nature and the composition of which has to be judiciously selected in order to get appropriate a required solute retention. The mobile phase has to be adapted in terms of elution strength (solute retention) and solvent selectivity (solute separation). Solvent polarity is the key word in chromatographic separations since the polar mobile phase, which gives rise to low solute retention in normal phase and high solute retention in reversed phase liquid chromatography [159].

The choice of mobile phase for a given separation constitutes a very important stage in producing a good separation in HPLC. The requirements for solvents to be used as mobile phase in HPLC are they should be high purity to avoid introduction of peaks that may overlap with the analyte peaks. They should be readily available, should have low viscosity and reactivity to avoid chemical interaction with the analytes. They should be immiscibility with the stationary phase. Also, should be compatible with the detector Thus, for absorbance detection, the solvent should not absorb at wavelengths to be used. For refractive index detectors, the solvent refractive index must be significantly different from that of the solutes.Last but not least they should be with limited flammability and toxicity.

Acetonitrile and methanol are the most popularly used organic solvents in HPLC. Acetonitrile is the best initial choice of organic solvent for the mobile phase. Acetonitrite – water mixtures are used because it has lower UV cut-off (185 to 210 nm) and low viscosity leading to higher plate numbers and lower column backpressures. However, methanol which has a relatively lower UV cut-off (205nm) is a reasonable alternative. The components of interest for separation need to separate at 210 nm , very close to UV cut off methanol and hence methanol is not considered in this present research work. Initially mobile phase comprising distilled water and acetonitrile in the volume ratio 50:50 was employed. It was observed that amodiaquine eluted at with K'<1 and artesunate eluted out rapidly with no resolution between dihydroartemisinin and artesunate < 1.5. Mobile phase composition has major effect on separation or band spacing. No other variable will generally prove as powerful for controlling values of α and K'. A trial and error approach to the selection of best strong solvent, pH, and additives is best possible with a regular sample,which takes more time and is inefficient. It is better to try a minimum number of mobile phases that are most likely to provide maximum changes in α. In present research work water/ACN mobile phase adjusted to optimum solvent strength. 50% ACN gave K'>1 for amodiaquine and poor resolution between dihydroartemisinin II and artesunate. Substituting ACN by tetrahydrofuran (THF) at concentration of 30-32% gave K'>1 for amodiaquine where as dihydroartemisinin II and artesunate peaks showed poor resolution. Further use of THF is discouraged due to its oxidizing nature, which gives drift in the base line. Solvent strength is usually varied by changing strong and weak solvent in RP chromatography. Organic solvent is a strong solvent and water is a weak solvent. RP systems are characterized by strong interactions between the polar mobile phase and various sample molecules. Interaction between sample molecule and non polar stationary phases are weak, this effect suggests that interaction between sample and solvent molecules will mainly determine relative retention values and α. Mobile phase or solvent selectivity can be characterized by solvent dipole moment, solvent basicity (proton acceptor) , solvent acidity (proton donor) . The resolution Rs is determined by three separate factors α, N and capacity factor K'. Though all these are interrelated it is good practice to first adjust the conditions for an optimum k' value and then focus attention on

combination that affect α and N. In this approach K' values for sample are held roughly constant and conditions are changed for improved values of α and N. Elution of bands with K'<1 leads separation that will be unsatisfactory. K' of individual band increases or decreases with changes in the solvent strength. RP solvent increases with % increase of organic solvent. Different combination of water and acetonitrile did not help for better resolution. Both artesunate and amodiaquine are acidic , therefore, change in mobile phase with buffering capacity can play major role. Both being acidic and as being unstable in alkaline pH acidic mobile phase is selected. pKa of both artesunate and amodiaquine are 4.6 and 5.8 respectively. It is advisable to have the pH of mobile phase within approximately ± 2 units of pKa. Whenever acidic or basic samples are to be separated it is advisable to control mobile phase pH by adding a buffer solution. Reversed phase HPLC separations are generally carried out with C_8 or C_{18} bonded phase silica based columns that are stable in pH ranges 2.0 and 8.0. Thus, buffers able to control pH between 2.0 to 8.0 are desirable. For reverse phase chromatography, a buffer concentration of 10-50 mM is usually adequate. Phosphate buffers have better buffer capacity between pH 1.9-3.5 and 5.8-6.0, hence it is selected. Varying mobile phase pH ranges from 2.5 to 6.0 were tried out. Very high pH environment decompose artesunate. Hence, acidic mobile phase would protect basic chemical nucleus of artesunate without breaking lactone ring [209]. Further, the best resolution is achieved between dihydrartemisinin and artesunate with mobile pH 3.0 and hence the pH of the mobile phase was selected as 3.0. The pH of the mobile phase was adjusted in such a way that it gave satisfactory separation between the drug peaks in short analysis time.

0.01M monobasic potassium phosphate with pH adjusted to 3.0 was selected in combination with acetonitrile, as a mobile phase.The compounds were separated isocratically with mobile phase composed of buffer, (1.36 g of monobasic potassium phosphate in 1000 ml of water. adjusted pH to 3.0 with orthophosphoric Acid.) in addition, ACN in the ratio of 560:440 , with 0.80 mLmin $^{-1}$ (run time of 20 minutes) . Trials were taken with 50% buffer and 50% ACN in this case K' of amodiaquine <1 hence concentration of buffer increases in such a way that last peak i.e. gleeman impurity will have K'<20 and amodiaquine >1 and finally 560:440 composition of buffer and ACN yields desired separation. A mobile phase comprising buffer:

acetonitrile (56:44 v/v) gave a satisfactory separation between the drug peaks and run time of 20 minutes.

Selection of Detector

The detector is chosen depending upon some characteristic property of the analyte like UV absorbance, fluorescence, conductance, oxidation, reduction etc. Characteristics that are to be fulfilled by a detector to be used in HPLC determination are, high sensitivity, to facilitate trace analysis. It should have negligible baseline noise, to facilitate lower detection. It should have large linear dynamic range. It should be independent of variations in operating parameters, such as pressure, flow rates, etc. It's response should be independent of mobile phase composition. It should have low dead volume. Further, it must be non destructructive to the sample and stable over a long periods of operation. It also should be convenient and reliable to operate along with this it should inexpensive to purchase and operate. Most important it must be capable of providing information on the identity of the solute.

Multi component pharmaceutical preparations contain more than one active ingredient with variable concentrations. Detecting each component at their maximum absorbance, the purpose of using HPLC for their simultaneous determination is lost. Hence, in such cases one has to explore the possibility of selecting the wavelength at which all the components are detected. This single wavelength is referred to as "most suitable wavelength". While selecting the wavelength, the interest of minor component in the formulation or component with low extraction co-efficient needs special consideration. Variable wavelength detector or a PDA detector is used. In the present research work, challenge for selection of wavelength was due to the vast concentration difference in the dosage form that is 1:3. The UV absorption spectra of targeted concentration of sample artesunate and amodiaquine were recorded using PDA detector. Artesunate showed maxima only at 210 nm where as amodiaquine showed maxima at approx. 210 nm, 224 nm and 300 nm. Amodiquine showed response as high as beyond detector linearity. Whereas at 300 nm it is in the detector linearity range. Therefore,it is concluded to use wavelength 210nm and 300nm for simultaneous detection of artesunate and amodiaquine respectively by dual wavelength technique.Table 3.14 represent optimized

chromatographic conditions and figures 3.15 and 3.16 shows overlay spectras of artesunate and amodiquine and typical HPLC chromatogram of sample.

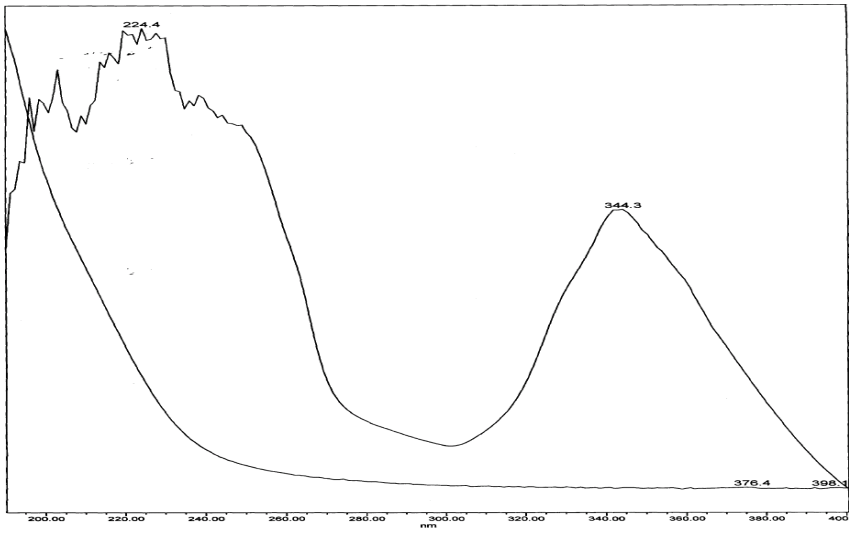


Figure 3.15- Overlay Spectra Of Artesunate And Amodiaquine.

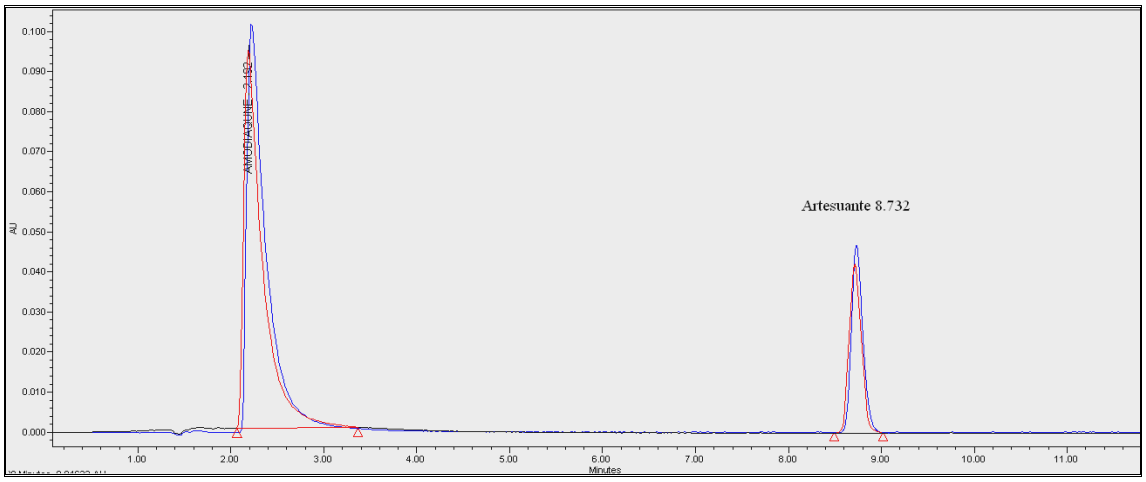


Figure 3.16 -Typical HPLC chromatogram for simultaneous determination of Artesunate (at 210 nm) Amodiaquine (at 300 nm) from pharmaceutical preparation. Along with placebo ,standard and sample.

Table 3.14 Optimized Chromatographic Conditions

SR No.	PARAMETER	DESCRIPTION
1.	Instrument	HPLC
2.	Pump	Separation module quaternary pump
3.	Injector	Auto injector
4.	Column	Base deactivated C_{18}, 10cm x 4.6 mm i.d.,3µm.
5.	Detector	PDA
6.	Wavelength	210 nm and 300 nm
7.	Recorder	Waters Empower data system
8.	Mobile Phase	Buffer (0.01M monobasic pottasium phosphate pH adjusted to 3.0 with orthophosphoric acid): Acetonitrile in the ratio 56:44 (v/v)
9.	Flow Rate	0.8 mL^{-1} min
10.	Volume	20 µl

3.3.4 Method Validation

Validation of the method is a process by which it is established by laboratory studies that the performance characteristics of the method meet the requirements for its intended application. To demonstrate that the proposed method is adequate for its intended use, the method was subjected to statistical validation.

The newly developed method was subjected to rigorous method validation as per ICH guideline Q2R to determine its suitability for intended use. The parameters that were considered in method validation were as specificity, accuracy, precision, repeatability, intermediate precision or ruggedness, linearity and range and robustness.

3.3.4.1 System Suitability Test

A system suitability test should be carried out to determine to see if the operating system is performing properly. System suitability tests are used to verify that the reproducibility of the equipment is adequate for the analysis to be carried out. These

tests are based on the concept that equipment, electronics, analytical operations and samples to be analysed constitute an integral system, which can be evaluated as such.

System suitability tests are performed as per the USP 31/NF [210] to confirm the suitability and reproducibility of the system. The test was carried out by injecting 20 µl standard solution of Artesunate Amodiaquine of strengths 250 µg, 1000 µg respectively. This was repeated six times. The standard deviation and % RSD values for peak area of these six determinations theoretical plates, resolution, tailing factor were determined and presented in table 3.15 .

Table 3.15- Result Of System Suitability

Parameters	Artesunate	Amodiaquine
% RSD of replicate injections (n=6)	0.54	0.07
Tailing factor	1.104	2.23
Theoretical plates	380	7883

3.3.4.2 Specificity

The ability of analytical method to unequivocally assess the analyte in the presence of other components (impurities and degradants) can be demonstrated by evaluating specificity. The specificity of the HPLC method was determined by injecting individual component, placebo preparation, where in no interference was observed for any of the components and no interference of any of the excipients is observed from the placebo preparation at the retention of artesunate, dihydroartemisinin remaining and amodiaquine hydrochloride. Forced degradation studies of the actives and the tablets were also performed using following conditions.

Procedure for force degradation study.

a) Acid Hydrolysis : Weighed powder equivalent to 125 mg artesunate in 500 ml volumetric flask, added 10 ml acetonitrile, shaken for 2 minutes, added about 300 ml diluent and sonicated for 15 minutes with intermediate shaking, added 50 ml 0.1N HCl and heated for 60 minutes, cooled at room temperature and neutralized with 0.1N NaOH and then diluted up to the mark with diluent, filtered through GF/C paper. Further pipetted out 5ml of filtrate to 20ml diluent.

(Acid Blank : Added 10 ml acetonitrile and 10ml diluent to 500 ml volumetric flask, added 50 ml 0.1N HCl and heated for 60minutes, cooled at room temperature and neutralized with 0.1N NaOH and then diluted up to the mark with diluent, filter through GF/C paper)

a) Alkali Hydrolysis : Weighed powder equivalent to 125 mg artesunate in 500 ml volumetric flask, added 10 ml acetonitrile, shaken for 2minutes, added about 300 ml diluent and sonicated for 15minutes with intermediate shaking, added 50 ml 0.1N NaOH and heat for 60 minutes, cooled at room temperature and neutralized with 0.1N HCl and then diluted up to the mark with diluent, filter through GF/C paper. Further pipetted out 5ml of filtrate to 20 ml diluent. (Alkali Blank : Added 10ml acetonitrile and 10ml diluent to 500 ml, added 50ml 0.1N NaOH and heat for 60minutes, cooled at room temperature and neutralized with 0.1N HCl and then diluted up to the mark with diluent, filter through GF/C paper)

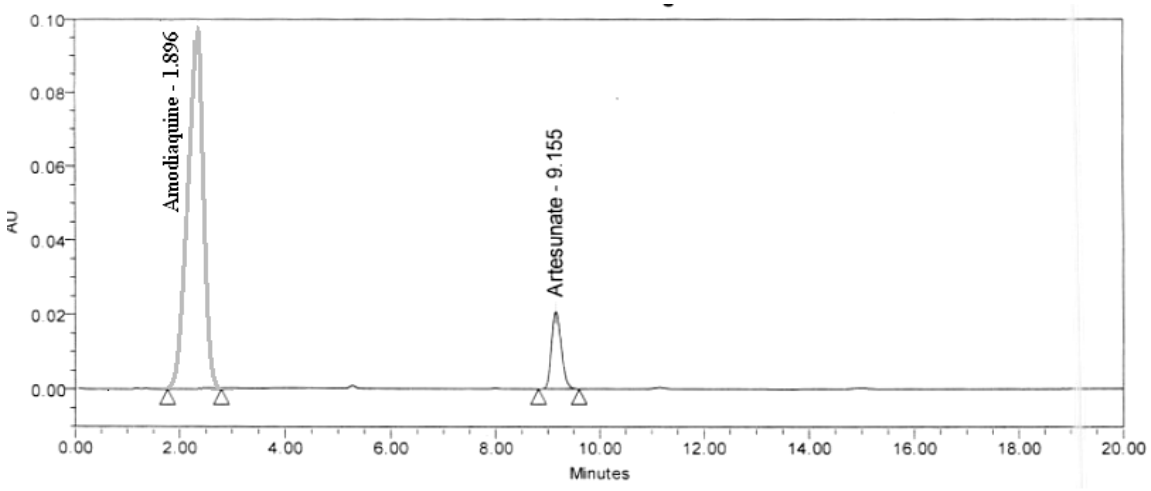
a) Oxidation : Weighed powder equivalent to 125 mg artesunate in 500 ml volumetric flask, added 5ml of 10% H_2O_2, mixed and injected as 0 min. The sample aliquot was taken out after 30 mins, one hour and injected.

b) Heat Treatment: Active artesunate and amodiaquine was exposed to heat at 60°C for 1week, sample equivalent to 125 mg artesunate was weighed and solution was prepared and injected.

c) Photo-stability: active artesunate and amodiaquine was exposed in Photo-stability chamber for stipulated period, sample powder equivalent to 125 mg artesunate was weighed and solution was prepared and injected.

As no peak from placebo was eluted at the Retention Time (R.T.) of artesunate and its impurity and amodiaquine hydrochloride, indicates that placebo interference is Nil and the method is very specific to simultaneous estimation of artesunate and amodiaquine combined dosage forms. From the forced degradation study it was established that no degradant was found to interfere with the retention time of artesunate and amodiaquine hydrochloride and its impurities. Acid Hydrolysis showed complete degradation of artesunate into a new unknown degradant and increase in the peak response of dihydroartemisinin and amodiaquine hydrochloride showed slight decrease in peak area response resulting in the decrease in the assay value. Alkali hydrolysis also showed

drastic decrease in the assay value of both artesunate and amodiaquine hydrochloride and formation of known impurity dihydroartemisinin and artemisinin and unknown impurity at RRT about 2.9 with respect to artesunate.Oxidation method of degradation showed decrease in peak area response of artesunate and amodiaquine hydrochloride and at the same time increase in the dihydroartemisinin, artesmisinin and unknown impurity at RRT(relative retention time) about 2.9 is observed. No major degradation was observed in the active drugs and combination dosage form of artesunate and amodiaquine when exposed to photostability and elevated temperature. From the studies it was also observed and confirmed that no other formulation components and potential degradation products of known and unknown identity do produce any chromatographic responses that would interfere with the main peak area response of artesunate and amodiaquine hydrochloride and its impurities dihydroartemisinin, artemisinin. Hence, this method is said to be selective and specific for the stability indicating of artesunate and amodiaquine combination dosage form on table 3.16 indicates results of specificity study. For all the degraded samples,the artesunate and amodiaquine hydrochloride peak passed the peak purity testing, leading to a conclusion that the peak is spectrally homogeneous. In the other words none of the degradants formed during the stress study co-elute with the artesunate, amodiaquine and their impurities. Figurtes 3.17 and 3.18 represent the peak puirity of standards and degraded sample.



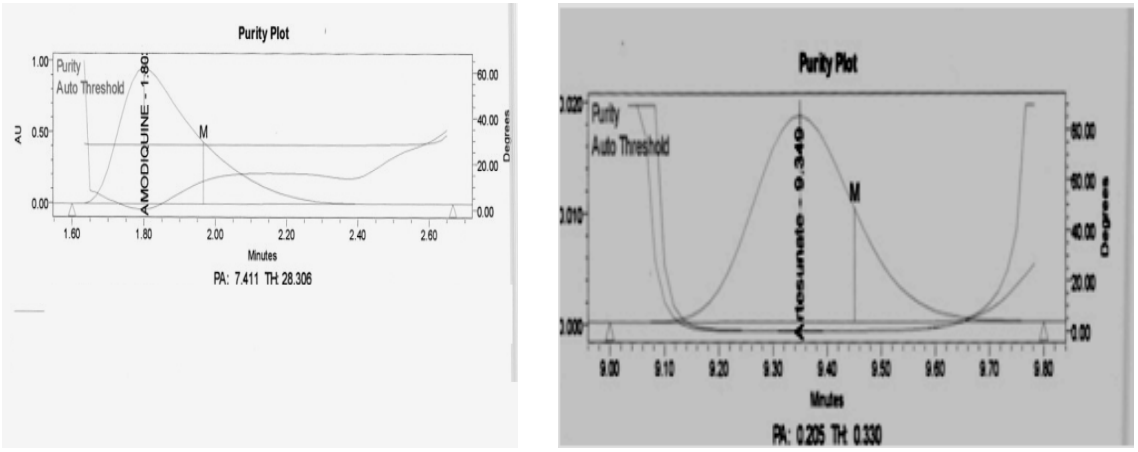


Figure 3.17 – Typical chromatogram and peak purity graph of standard amodiaquine and artesunate.

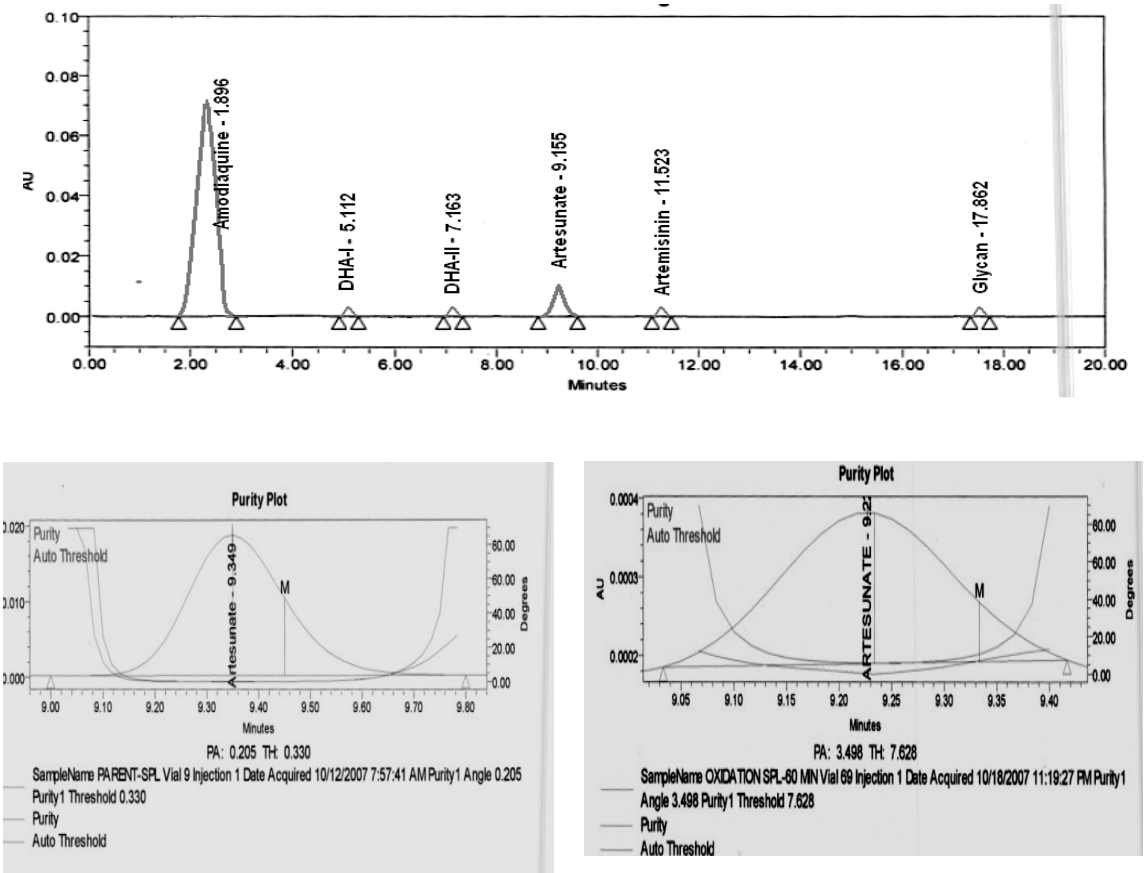


Figure 3.18 – Typical chromatogram and peak purity graph of amodiaquine and artesunate in alkaline stressed condition.

Table 3.16 - Forced Degradation Study Of Drug Substance And Drug Product

Degradation Condition	**% Assay**		**Purity angle is < than purity threshold**
	Artemether	Lumefantrine	
Parent (API)- individual	99.82%	98.77 %	Pure
Parent Artemether & Lumefantrine (APIs in combination)	99.48%	99.11%	Pure
Parent sample (20+120)	98.22 %	97.87 %	Pure
ACID HYDROLYSIS			
Artemether and Lumefantrine(API) individual	95.99%	95.96%	Pure
Artemether & Lumefantrine (APIS in combination)	95.62 %	94.50 %	Pure
sample	86.62%	85.16 %	Pure
ALKALI HYDROLYSIS			
Artemether and Lumefantrine (API) Individual	90.72%	89.68 %	Pure
Artemether & Lumefantrine (APIS in combination)	90.82 %	88.18 %	Pure
sample	90.23 %	89.63 %	Pure
OXIDATION			
Artemether and Lumefantrine (API) individual	95.99%	98.23 %	Pure
Artemether & Lumefantrine (APIS in combination)	99.07 %	99.83 %	Pure
Sample	95.15 %	95.98 %	Pure
HEAT			
Artemether and Lumefantrine(API) Individual	99.82%	98.87 %	Pure
Artemether & Lumefantrine (APIS in combination)	98.79 %	98.65 %	Pure
sample	97.10 %	99.30 %	Pure
PHOTO STABILITY			
0Artemether and Lumefantrine(API) Individual	99.23%	98.57 %	Pure
Artemether & Lumefantrine (APIS in Combination)	98.50 %	98.45 %	Pure
Photo stability sample (20+120)	97.82 %	98.23 %	Pure

3.3.4.3 Accuracy

To study accuracy of the method, recovery experiment was determined by applying the standard addition method in which known quantities of each drug substance corresponding to 80%, 100%, and 120% of the assay concentration of each drug were added to the placebo. Placebo consists of mixture of excipients like manifold, lactose monohydrate, croscarmellose sodium, hydroxypropyl cellulose, colloidal silicon dioxide, magnesium stearate, maize starch and pregelatinsed starch. Each set of additions was repeated three times. The accuracy was expressed as the percentage of analytes recovered by the assay. Table 3.17 lists the recoveries of the two drugs from a series of spiked concentrations. The results indicate the method is highly accurate for simultaneous determination of the two drugs.

Table 3.17 Accuracy of The Method

Level	Amount of placebo (mg)	Amount of drug added in (mg)	Amount of drug recovered in (mg)	%RSD	% Recovery= mg recovered ----------x 100 mg added	% Bias
Artesunate :						
80%	1715	398.0	394.44	0.92	99.24	-0.76
100%	1225	500.9	502.85	0.20	100.39	+0.39
120%	732	601.27	605.93	1.23	100.77	+0.77
Amodiaquine :						
80%	1715	1567.17	1565.26	0.19	99.87	-0.13
100%	1225	1959.80	1963.11	0.41	100.17	+0.17
120%	732	2350.70	2350.73	0.90	100.00	0.00

3.3.4.4 Precision

Precision study was assessed by injection repeatability and sample repeatability. Injection repeatability was confirmed by performing replicate injection of the standard solution and calculating the % RSD of the peak area responses for both the content. The data show good precision of the system with the RSD $\leq$ 2.0% Table 3.18. The sample

repeatability was studied by analyzing the same sample for six times and calculating the % assay value and %RSD for artesunate and amodiaquine were 0.56 and 0.12 respectively, which are well within the limit of 2.0%. The results are listed in Table 3.19, indicate that the amount of each drug in the tablets is within the requirements of 95 -105% of the label claim. Under similar experimental conditions, precision refers to the extent of variability of a group of measurements observed. Observations, which are relatively close in magnitude, are considered to be precise as reflected by small values of standard deviation and relative standard deviation.In the present assay experiment, six samples were weighed separately and analysed. Tables 3.19 shows that the mean amount of artesunate and amodiaquine found per tablet were 99.23 mg and 299.88 mg respectively. The standard deviation and relative standard deviation were found to be 0.56, 0.12 and 0.59, 0.64 for artesunate and amodiaquine respectively. Low values of standard deviation and relative standard deviation indicate high precision of the method.

Table 3.18- System Precision Study

Sr. No.	Mean Peak Area Response of Artesunate Standard	Mean Peak Area Response of Amodiaquine Standard
1	297963	13954863
2	295904	13951569
a	294925	13937074
4	294394	13949961
5	294021	13978209
6	293273	13976873
Mean	295080	13958092
SD	1665.65	16237.4
%RSD	0.56	0.12

Sr.No.	Weight of Sample mg	Mean Peak Area Response of Sample		% Assay of Component	
		Artesunate	Amodiaquine	Artesunate	Amodiaquine
1	3715.4	285515	13614205	98.57	100.01
2	3743.5	291937.5	13839759	100.03	100.90
3	3724.6	289588	13579043	99.73	99.50
4	3735.3	289089	13761110	99.27	100.55
5	3705.4	286553	13494086	99.19	99.39
6	3702.8	284686	13486898	98.62	99.41
Mean				99.23	99.96
SD				0.58	0.64
%RSD				0.59	0.64

Table 3.19 Method Precision Study

3.3.4.5 Linearity of The Detector Response

The linearity of a method is a measure of how well a calibration plot of detector response vs. concentration approximates a straight line. The following experiment was carried out to determine the working concentration range for artesunate and amodiaquine.Linearity was evaluated by analysis of working standard solutions of artesunate and amodiaquine of five different concentrations. The range of linearity was from 150 – 350 µg mL^{-1} for artesunate, 600 – 1400 µg mL^{-1} for amodiaquine. Curves were obtained by plotting the peak areas of respective drugs against concentrations. The slope, Y-intercept and Correlation coefficient (r) were obtained from linear regression analysis. The regression data obtained for the two pharmaceuticals actives are represented in Table 3.20 and 3.21. The result shows that within the concentration range mentioned above, there was an excellent correlation between peak area and

concentration of each drug. Figures 3.19 and 3.20 represtive linearity plots with linearity equation.

Table 3.20 Linearity Of Artesunate

Conc. In µg mL-^{1}	Mean Area
150	169483
200	225040
250	281272
300	339196
350	386425

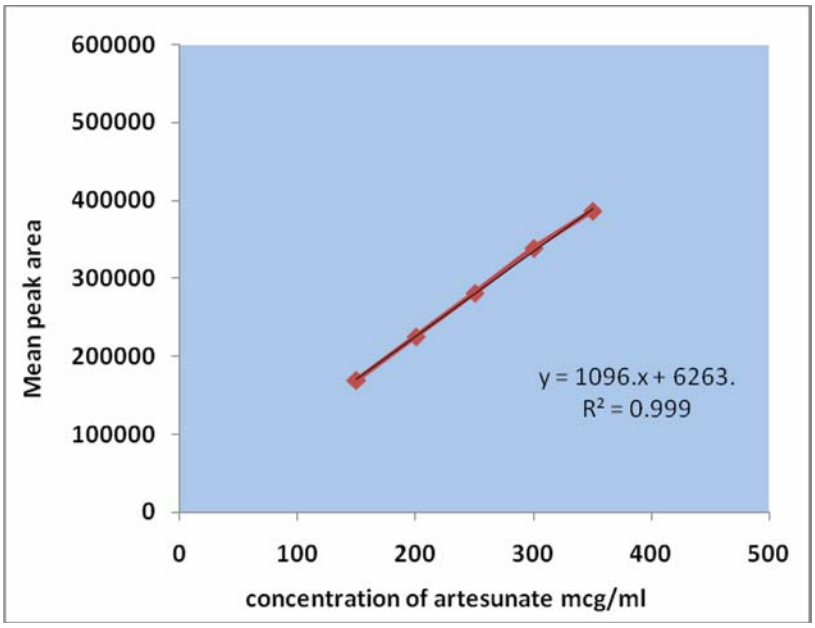


Figure 3.19 Linearity Curve of Artesunate

Table 3.21 Linearity For Amodiaquine

Concentration. in µg mL^{-1}	Mean Area
600	8682013
800	11200194
1000	13836266
1200	16588162
1400	18954108

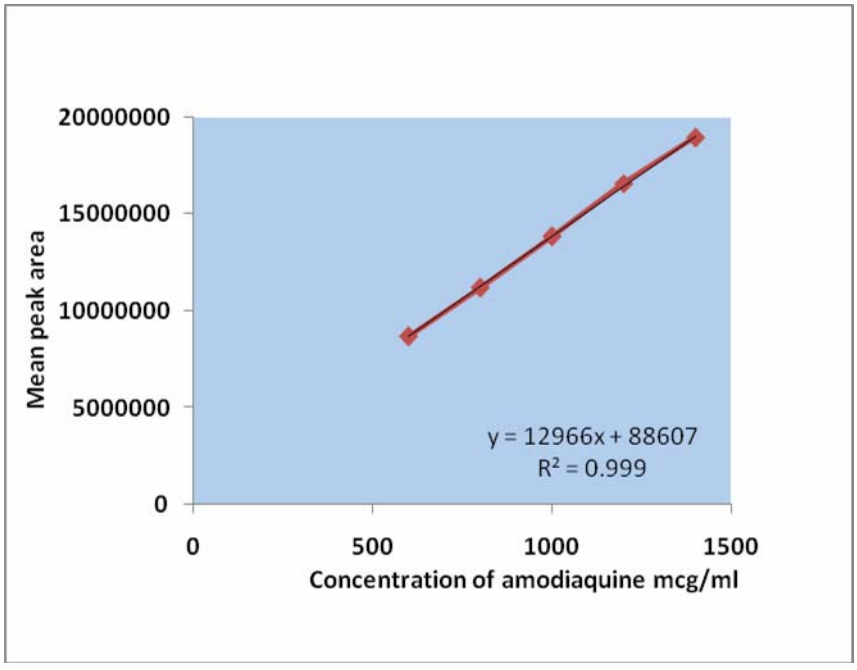


Figure 3.20 Linearity Curve of Amodiaquine

3.3.4.6 Limit of Detection And Limits Of Quantitation

The Limit of Detection was determined by making serial dilutions of standard solutions of artesunate and amodiaquine followed by HPLC analysis. The Limit of Detection data was evaluated from the calibration curve using the formula i.e. DL = [3.3∗ SyX/Slope]. The Limit of Detection of artesunate and amodiaquine were found to be 3.460 µg mL^{-1} and 1.159 µg mL^{-1} respectively. The Limit of Detection of artesunate and amodiaquine were found to be 3.460 µg mL^{-1} and 1.159 µg mL^{-1} respectively. The Limit of Quantitation was determined by making serial dilutions of standard solution artesunate and amodiaquine followed by HPLC analysis. The Limit of Quantitation data was evaluated from the calibration curve using the formula i.e. DL = [10∗ SyX/Slope].The Limit of Quantitation of Artesunate and Amodiaquine were found to be 10.485 µg mL^{-1} and 3.511µg mL^{-1}respectively. Figure 3.21 represents the typical chromatogram of LOQ of artesunate and amodiaquine.

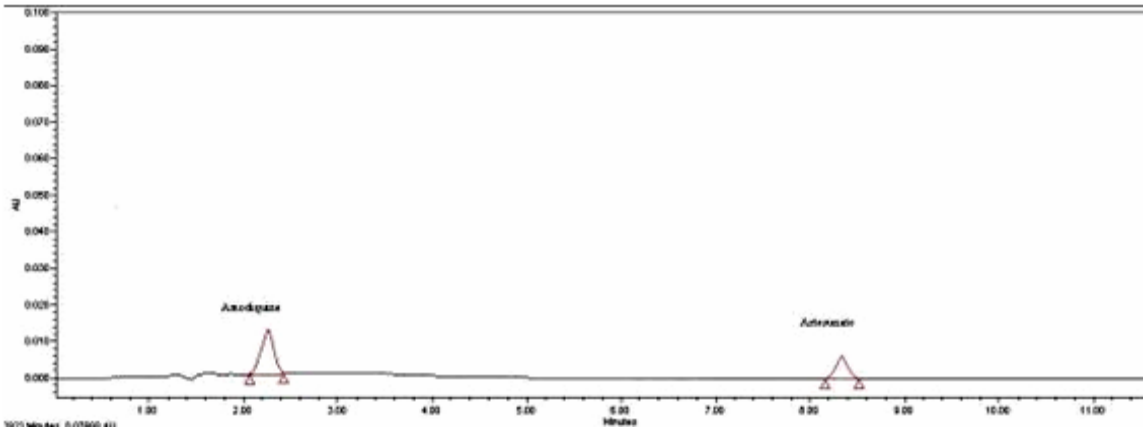


Figure. 3.21 Chromatogram of LOQ of Artesunate and Amodiaquine.

3.3.4.7 Filter Paper Evaluation

The possible interference from different filter papers used during manual sampling was checked. An assay value obtained for the filtered solution was checked against assay value of the solution filtered through acrodisc syringe filter 0.45µm. The difference was calculated and reported. (NMT 2.0%). On filteration through Whatman filter paper #41 and #42 the solution remained turbid, hence used Whatman filter paper #GFC and

acrodisc micron syringe filter 0.45µm. The sample preparation was centrifuged for 20 minutes, but the solution being not clear it is filtered through acrodisc syringe filter 0.45µm. The results showed no significant change in the assay value, when the sample preparation is filtered through Whatman filter paper #GFC and acrodisc syringe filter 0.45µm.

Table 3.22 - Filter Paper Evaluation Study of Sample

Type of filter paper	Artesunate content		Amodiaquine content	
	Area	% Assay	Area	% Assay
Acrodisc syringe filter 0.45µm	284686	98.62	13486898	99.41
Whatman # GFC	286834.5	99.36	13583639	100.12

3.3.4.8 Solution Stability

The stability of the standard and sample solutions was determined at the time periods representative for storage. Standard solution stability was done by injecting replicate injections of standard preparation as per the method at initial time point or 0 hrs. Then at other intervals and compared against freshly prepared standard, the % difference of area within the mean of each interval should not be more than ±2.0%. Sample solution stability was determined by comparing to its initial value. Sample solution stability was studied by storing the sample preparation at ambient temperature as well as in the fridge at 2°C to 8°C. Absolute difference was calculated and it should be not more than ± 2.0. The results for standard and sample solutions are presented in Tables. The stability of sample was determined to be 2hrs when kept at ambient temperature and 8hrs. When kept in fridge at 2°C to 8°C.

The study of stability of sample Solution concluded that the sample solution was stable for 2 hrs when kept at ambient temperature and 8hrs when kept in fridge at 2°C the results for standard and sample solutions are presented by a graph in figures. 3.22 and 3.23. From the data, it is clear that the solution is not stable for more than 2 hrs at

ambient temperature, hence a note is added in the method of analysis that the solution should be injected freshly or within 8 hrs if stored at 2°C to 8°C.

Table 3.23 Solution stability at Ambient temperature

Time in hours	% Assay	
	Artesunate	Amodiaquine
0	99.16	99.63
0.5	98.69	99.77
1	98.43	100.04
2	97.69	100.52
4	96.14	100.64
6	94.73	100.94
8	92.98	101.42

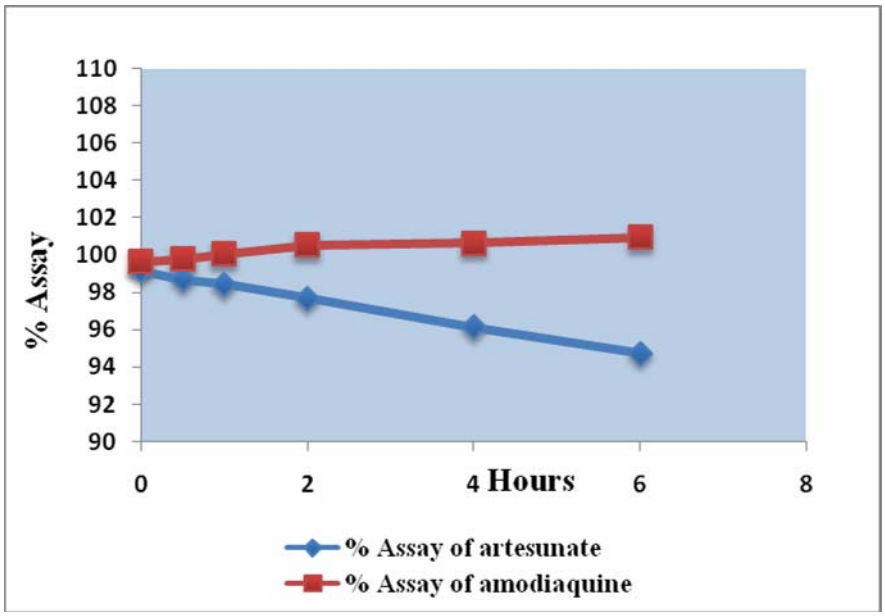


Figure 3.22-Solution Stability Curve for Artesunate and Amodiaquine at Ambient Temperature.

Table 3.24 Solution stability at 2°C TO 8°C

Time in hours	% Assay	
	Artesunate	Amodiaquine
0	99.16	99.63
2	98.52	99.99
4	98.62	100.19
6	98.12	100.69
8	97.94	100.73
16	96.11	101.97

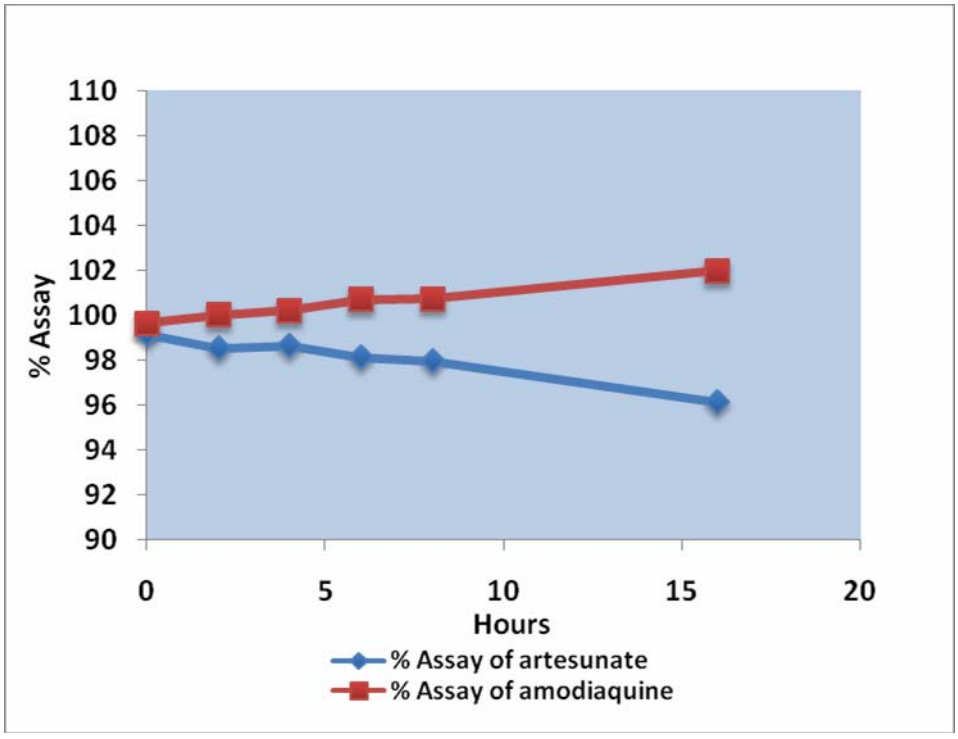


Figure. -3.23 Solution Stability Curve for Artesunate and Amodiaquine at 2°C to 8°C

3.3.4.9 Ruggedness or Intermediate precision

The ruggedness or the Intermediate precision of stability indicating assay was carried out by two different analytical persons on two different instruments on different dates to know the degree of reproducibility of results obtained by the analysis of the same sample. % RSD for the set of samples (n =12) was observed to be 0.47 for artesunate and 0.82 for amodiaquine hydrochloride. The results are presented in table 3.25 and table 3.26.

Table 3.25 - Intermediate Precision or Ruggedness Study for Artesunate in artesunate and amodiaquine tablets

Sr. No.	Day- I , Analyst -I Instrument & Model: Waters X Location: ADL,Mumbai			Day- II, Analyst- II Instrument & Model: Dionex II Location: ADL,Mumbai		
	Wt. of sample	Area	% Artesunate	Wt. of sample	Area	% Artesunate
1	3715.4	285515	98.57	3739.7	276938	99.24
2	3743.5	291937	100.03	3742.1	279521	100.10
3	3724.6	289588	99.73	3720.7	275346	99.17
4	3735.3	289089	99.27	3762.5	278330	99.13
5	3705.4	286553	99.19	3719.1	275168	99.15
6	3702.8	284686	98.62	3710	274733	99.24

Table 3.26- Intermediate Precision or Ruggedness Study for Amodiaquine in Artesunate and Amodiaquine Tablets.

Sr. No.	Day- I , Analyst -I Instrument & Model: Waters X Location: ADL,Mumbai			Day- II, Analyst- II Instrument & Model: Dionex II Location: ADL,Mumbai		
	Wt. of sample	Area	Assay % Amodiaquine	Wt. of sample	Area	Assay % Amodiaquine
1	3715.4	13614205	100.01	3739.7	14072185	100.19
2	3743.5	13839759	100.90	3742.1	14085099	100.21
3	3724.6	13579043	99.50	3720.7	14103531	100.92
4	3735.3	13761110	100.55	3762.5	14106380	99.82
5	3705.4	13494086	99.39	3719.1	13918022	99.64
6	3702.8	13486898	99.41	3710	14241999	102.21

3.3.4.10 Robustness

The robustness of the method was studied by deliberately changing the composition of mobile phase (± 20%), flow rate (± 0.2ml) and pH of the mobile phase (preferable ± 0.1unit). The system suitability parameters are studied for these changes. All system suitability parameters are found to be within the acceptance criteria. The method was found to be robust for above changes. Table 3.27, represents results of robustness study.

Table 3.27-Robustness Study

Parameters	Changes	Artesunate		Amodiaquine	
		% RSD (NMT 2.0%)	% Assay (Within 2.0% of the actual parameters)	% RSD (NMT 2.0%)	% Assay (Within 2% of the actual parameter)
A - Flow Rate (ml/min)					
0.6	-0.2	0.24	99.55	0.46	99.75
0.8	0	0.54	98.57	0.07	100.01
1.0	0.2	0.16	100.84	0.42	100.16
B - Composition of mobile phase (Buffer: ACN)					
480:420	-0.20%	0.15	100.12	0.22	100.40
500:400	0%	0.54	98.57	0.07	100.01
520:380	0.20%	0.2	99.83	0.15	99.77
C - pH of Buffer					
2.9	-0.1	0.07	99.13	0.20	99.72
3.0	0	0.54	98.57	0.07	100.01
3.1	0.1	0.68	98.66	0.44	99.91

3.3.5 Application of the validated method

The validated method was applied in the QC lab for product release and for stability testing. Stability batches at different conditions (25°±2 ° C,60% ± 5% RH, 30° C, ±2 °

C, 65% ± 5% RH 40° C±2 ° C 75% ± 5% RH) were withdrawn at definite time intervals (1,3,6 months) by formulator R & D (Formulation), and analysed. Stability batches were analysed by validated stability indicating method and results showed the assay of artemether decreases at 40° C±2 ° C 75% ± 5% RH with increase in DHA hence it is also concluded that product is stable only at or below 30° C, ±2 ° C, 65% ± 5% RH. Figure 3.24 a,b represent typical chromatogram of one of the stability batches at all conditions.

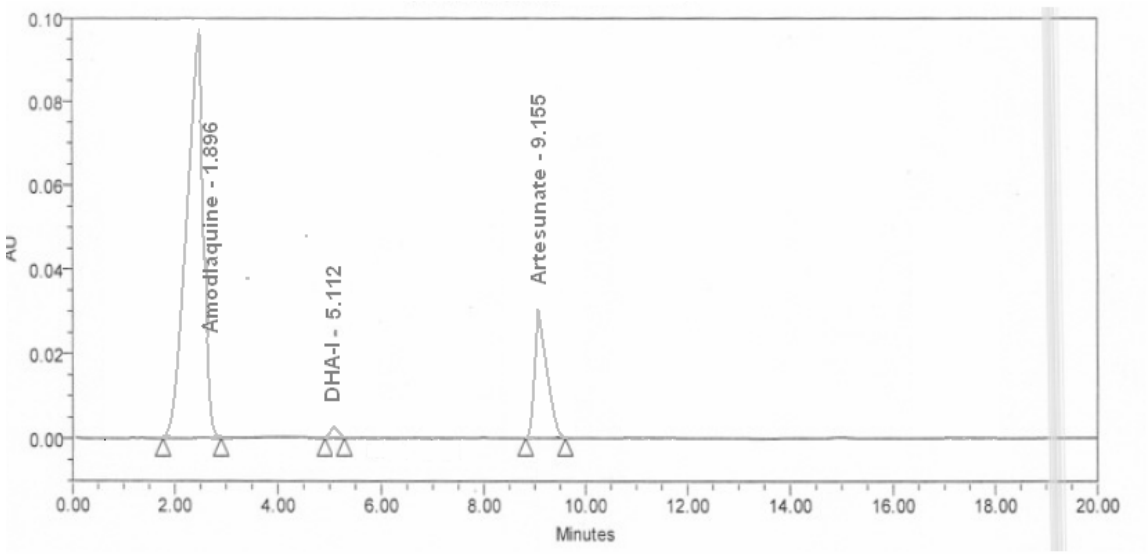


Figure 3.24 (a **) :** Chromatogram of Sample at 25^0C 60% RH at 3 Months.

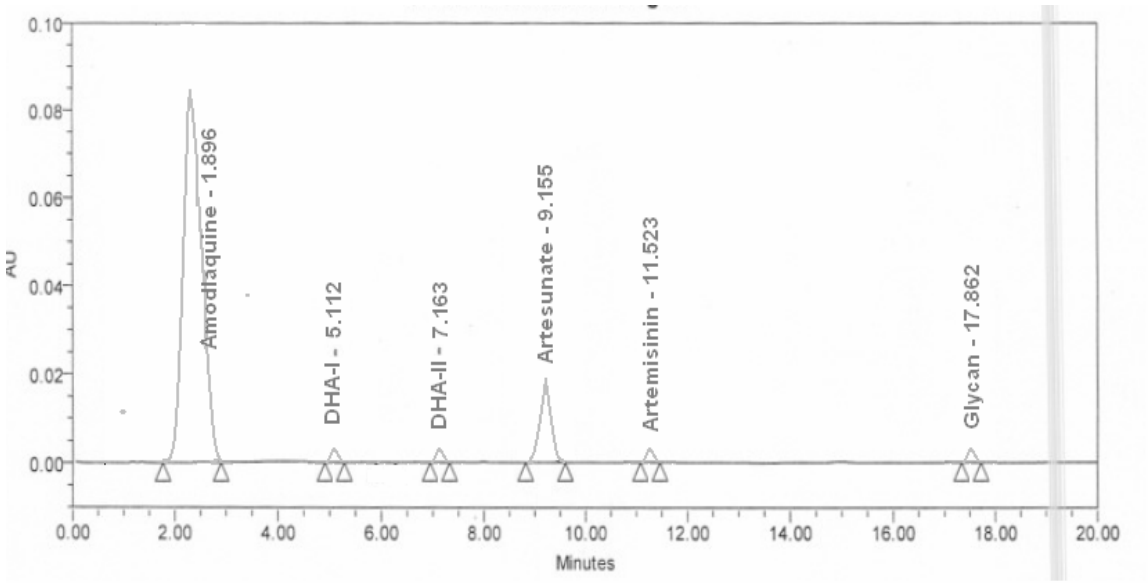


Figure 3.24 (b**) :** Chromatogram of Sample at 40^0C 75% RH at 3 Months

3.3.6 Conclusion

A new high performance liquid chromatographic method is developed for simultaneous determination of artesunate and amodiaquine from their fixed dosage form. The developed method has been validated as per ICH guideline Q2R and regulatory guideline and it can be conveniently used for simultaneous estimation of artesunate and amodiaquine in combination dosage form using dual wavelength. The method was found to be specific, accurate and stability indicating thus, the proposed HPLC method can be successfully applied for the routine quality control analysis of artesunate and amodiaquine from their fixed dosage form. Also the proposed method is precise and can be used for routine analysis as well as to monitor the stability studies successfully applied for all the dosage form like 100+270 mg , 50+135 mg , and 25+67.5 mg of artesunate and amodiaquine tablets ,as well as for all type of formulations like artesunate and amodiaquine dry powder suspension , artesunate amodiaquine bilayer tablets and artesunate and amodiaquine dispersible tablets.

4.1 Rational for Development of Stability Indicating Related Substances Methods By HPLC.

4.1.1 Introduction

Worldwide, impurity profiling (characterization and determination of impurities associated with drugs or drug products) is increasingly viewed as a valuable and essential part of quality requirements. Various regulatory authorities like United States Food and Drug Authority[210] (USFDA), European Directorate of Quality Medicine[211] (EDQM), Therapeutic Goods Administration [212], World Health Organization (WHO)[213] and other health agencies [214-216] are emphasizing on the purity requirements and the identification of impurities in drug substance and products. A key component of the overall quality of a pharmaceutical is control of impurities, as their presence, even in small amounts, may affect drug safety and efficacy. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines [217-224] are developed with the joint efforts of regulators and industry representative from the European Union, Japan and the United States. The guidelines helped to ensure that the different regions have consistent requirements for the data that should be submitted to the various regulatory agencies. As per ICH Q3A(R) [220] and ICH Q3B(R) [221] guidelines, unknown impurities associated with bulk drug and dosage form or drug product, greater than the identification threshold should be identified.

The impurity profiles for pharmaceutical products require a basis for well reasoned and rational argument that seeks to limit both the number and amount of impurities on safety grounds. The efficacy, safety and the dosage of drug product will determine the level of impurities to be present in drug product. The daily dose of active substance administered will vary considerably between products. It is obvious, but frequently ignored, that this will influence the amount of impurity administered when impurities are controlled on a percentage or parts per unit basis [225,226].

The toxicological assessor in a regulatory agency cannot necessarily be reassured on safety, simply by the limitation of an impurity to a low percentage level. Some drugs

are administered by mouth at doses of >30 g/day, so that even 0.01% of an uncharacterized impurity gives a potential patient exposure of >3 mg daily. Conversely, the analyst may not need to struggle to achieve a low (e.g. 0.1%) limit for detection, quantification, validation or reporting, if the daily dose to humans will only be in the microgram or low milligram range. As a rough guide, the limitation to 1 mg daily oral intake of an uncharacterized or poorly characterized impurity will probably satisfy a safety assessment for regulatory purposes [220,221].

It is, therefore, necessary on safety point of view, to know enough about the impurity profile (both qualitative and quantitative) to allow a judgment that the impurity will not pose any concern over safety or will be an acceptable risk factor for treating serious diseases where there is no other therapy suitable for a particular patient.

Impurity profile of any pharmaceutical drug product is a crucial part of product development; it was felt necessary to develop the modern chromatographic and mass spectrometric methods for qualitative and quantitative determination of impurities associated with antimalarial drug product.

4.1.2 Definition and terminology

Definition

According to ICH guidelines, a drug substance impurity is “any component of the new drug substance that is not the chemical entity defined as the new drug substance.”

Or any material that affects the purity of the material of interest like drug substance is termed as drug impurity [189]. An impurity in new drug product is defined as any component of the new drug product that is not the drug substance or excipients in the drug product [220]. Impurities are generally assumed to be inferior to API (active pharmaceutical ingredient) because they may not have the same level of pharmacological activity. Impurities in synthetic drug substance are commonly termed as by-products, degradation products, intermediates, penultimate intermediates, transformation products and related products

United States Pharmacopeia (USP) [227] deals with impurities in several sections

(a) Impurities in official articles (b) Ordinary impurities (c) Organic volatile impurities

Inorganic, organic, biochemical, isomeric, or polymeric components may be considered as impurities. Following terms are described for different type of impurities.

Foreign Substances, concomitants, ordinary impurities, signal impurities, organic volatile impurities and inorganic Impurities.

ICH terminology

According to ICH guidelines, impurities in the drug substance produced by chemical synthesis can be broadly classified into three categories;

(a) Organic impurities (Process and drug related), (b) Inorganic impurities (c) Residual solvents (organic volatile impurities).

Organic impurities: These impurities can arise during the manufacturing process and/or storage of the API. They can be identified or unidentified, volatile or non volatile e.g.: (i) Starting materials (ii) By-products (iii) Enantiomeric impurities (iv) Intermediates (v) Degradation products (vi) Reagents, ligands and catalysts

Inorganic impurities: These impurities can result from the manufacturing process; they are normally known and include: reagents, ligands, catalysts, heavy metals or other residual metals, Inorganic salts, other materials, e.g. filter aids, charcoal

Solvents are inorganic or organic liquids used as vehicles for the preparation of solutions or suspensions in the synthesis of drug substances. Since these are generally of known toxicity, the selection of appropriate controls is easily accomplished.

Impurities in new drug products classified as degradation products of the drug substance or reaction products of the drug substance with an excipients and/or immediate container closure system (collectively referred to as “degradation products” in this guideline). Generally, impurities present in the new drug substance need not be monitored or specified in the new drug product unless they are also degradation products

4.1.3. ICH Guidelines

The ICH Q3A (R2) [220] and ICH Q3B (R3) [221] document is intended to provide guidance for the content and qualification of impurities in new drug substances produced by chemical synthesis. Drug substance or, more appropriately, active pharmaceutical ingredient (APIs), can be obtained from different sources and therefore,

it is very important that the associated impurities should be monitored and controlled [228].

The impurities are classified in three categories as discussed earlier.

a) Organic Impurities

Control of organic impurities in drug substance is based on maximum daily dose and total daily intake (TDI) of the impurities. The table 4.1 provides the ICH threshold for control of organic impurities in new drug substance [220, 221].

According to ICH guideline Q3A (R2) [220] any impurity at a level greater than (>) the identification threshold in the commercially manufactured API should be identified. In addition, any degradation product observed in stability studies at recommended storage conditions at a level greater than (>) the identification threshold should be identified.

According to ICH guideline Q3B (R3) [221].The degradation products observed during manufacture and/or stability studies of the new drug product at a level greater than (>) the identification threshold should be identified. This should be based on sound scientific appraisal of potential degradation pathways in the new drug product and impurities arising from the interaction with excipients and/or the immediate container closure system. In addition, efforts should be taken for laboratory studies conducting to detect degradation products in the new drug product. The test results should be generated test results of batches manufactured during the development process and batches representative of the proposed commercial process. A rationale should be provided for exclusion of those impurities that are not degradation products (e.g., process impurities from the drug substance and impurities arising from excipients). The impurity profiles of the batches representative of the proposed commercial process should be compared with the profiles of batches used in development and any differences discussed.

Any degradation product observed in stability studies conducted at the recommended storage condition should be identified when present at a level greater than (>) the identification thresholds given in figure 4.1[221]. When identification of a degradation product is not feasible, a summary of the laboratory studies demonstrating the unsuccessful efforts to identify it should be included in the registration application. Degradation products present at a level of not more than(≥)the identification threshold

generally would not need to be identified. However, analytical procedures should be developed for those degradation products that are suspected to be unusually potent, producing toxic or significant pharmacological effects at levels not more than (≥) the identification threshold. In unusual circumstances, technical factors (e.g., manufacturing capability, a low drug substance to excipients ratio, or the use of excipients that are crude products of animal or plant origin) can be considered as part of the justification for selection of alternative thresholds based upon manufacturing experience with the proposed commercial process.

Table 4.1 Impurity Thresholds

Maximum Daily Dose[a]	Reporting Threshold[b, c]	Identification Threshold[c]	Qualification Threshold[c]
≤ 2g/day	0.05%	0.10% or 1.0 mg per day intake (whichever is lower)	0.15% or 1.0 mg per day intake (whichever is lower)
> 2g/day	0.03%	0.05%	0.05%

[a] The amount of drug substance administered per day

[b] Higher reporting thresholds should be scientifically justified

[c] Lower thresholds can be appropriate if the impurity is unusually toxic

Adequate data may be available in the scientific literature to qualify an impurity. Additional safety testing becomes necessary when safety data from the literature is not available and decreasing the level of impurity below the threshold is not possible.

An illustration of the decision tree for consideration of safety studies is given in figure 4.1[221]. These thresholds do not apply to the genotoxic impurities. According to EMEA CHMP recommendation [229] genotoxic impurities should be controlled based on compound specific risk assessment.

b) Inorganic Impurities

Inorganic impurities are introduced from the synthetic process of the drug substance (e.g., catalyst). These impurities are normally detected and quantified by pharmacopoeial or other appropriate procedures.

c) Residual Solvents

The control of residual solvents in the new drug substance is described in the ICH Q3C Guideline [222]. The objective of this guideline is to recommend acceptable amounts for residual solvents in pharmaceuticals for the safety of the patient. Residual solvents are divided by a risk assessment approach into three classes.

Class 1 solvents: These are known human carcinogens, strongly suspected human carcinogens, and environmental hazards; therefore, these solvents should be avoided in the production of drug substance, excipients, or drug products, unless their use can be strongly justified in a risk-benefit assessment. If unavoidable, the level of an individual Class 1 residual solvent should be strictly controlled below the concentration limits (for example the limit for benzene is 2 μg/ml).

Class 2 solvents: These are non-genotoxic animal carcinogens or possible causative agents of other irreversible toxicity such as neurotoxicity or teratogenicity. Class 2 solvents are controlled according to the PDEs (Permitted Daily Exposure) and Maximum Daily Dose (Option 1 and Option 2). ICHQ3C provides PDEs of all Class 2 solvents.

Class 3 solvents: These are the solvents with low toxic potential to man. It is recommended that amounts of these residual solvents of 50 mg per day or less would be acceptable without justification.

4.1.4. Sources of Impurities

The different sources of impurities are given below

a) Crystallization-related impurities

There are crystal systems where substances can exist in different crystal packing arrangements, all of which have the same elemental composition. Some crystal systems also exists in different packing arrangements, each of which has a different elemental composition this phenomenon is known as solvatomorphism. Thus a nature of structure

adopted by compound upon re-crystallization is accounted towards crystallization-related impurities.

b) Stereochemistry-related impurities

The compounds that have similar chemical structure but different spatial orientation are called stereo-chemical isomers. Enantiomers (chiral molecules) have similar chemical structure but different spatial arrangements. The undesired isomer is considered as enantiomeric impurity in drug substance.

c) Impurities originating from synthetic processes of the drug substance

Starting materials and intermediates are the chemical building blocks used to construct the final form of a drug substance molecule. Un-reacted starting materials and intermediates, particularly those involved in the last a few steps of the synthesis, can potentially survive the synthetic and purification process and appear in the final product as impurities. Impurities present in the staring material could follow the same reaction pathways as the starting material itself, and the reaction products could carry over to the final product as process impurities.The selectivity of a chemical reaction is 100%, and side-reactions are common during synthesis of drug.

By-products from the side-reactions are among the most common process impurities in drugs. By-products can be formed through a variety of side reactions, such as incomplete reaction, overreaction, isomerization, dimerization, rearrangement, or unwanted reactions between starting

materials or intermediates with chemical reagents. Chemical reagents, ligands, and catalysts used used in the synthesis of a drug substance can be carried over to the final products as trace level impurities.

d) Residual solvents

The source of solvent residues is the solvents of crystallization of the bulk drug substance. Residual solvents are defined as organic volatile chemicals that are used or produced in the manufacture of drug substances (e.g., solvents for chemical reaction, separation, and crystallization) or excipients (e.g., solvents for wet granulation and coating), or in the preparation of drug products. Residual solvents are expected

impurities, therefore, identification of residual solvents is relatively straightforward. The solvents are not completely removed by practical manufacturing techniques. Appropriate selection of the solvent for the synthesis of drug substance may enhance the yield, or determine characteristics such as crystal form, purity, and solubility. Therefore, the solvent may sometimes be a critical parameter in the synthetic process. Since there is no therapeutic benefit from residual solvents, all residual solvents should be removed to the extent possible to meet product specifications, good manufacturing practices, or other quality-based requirements.

e) Impurities arising during storage or degradation impurity

Degradation of the drug substance is one of the main sources of impurities in both bulk drug and formulated product. Degradation of the drug substance is caused by chemical instability of the drug substance under the conditions (e.g., heat, humidity, solvent, pH, light, etc.) of manufacturing, isolation, purification, drying, storage, transportation, and interactions with other chemical entities in the formulation (e.g., excipients and coating materials). Chemical stability is an inherent property of a drug substance and is a reflection of the chemical properties of all functional groups in the drug molecule.

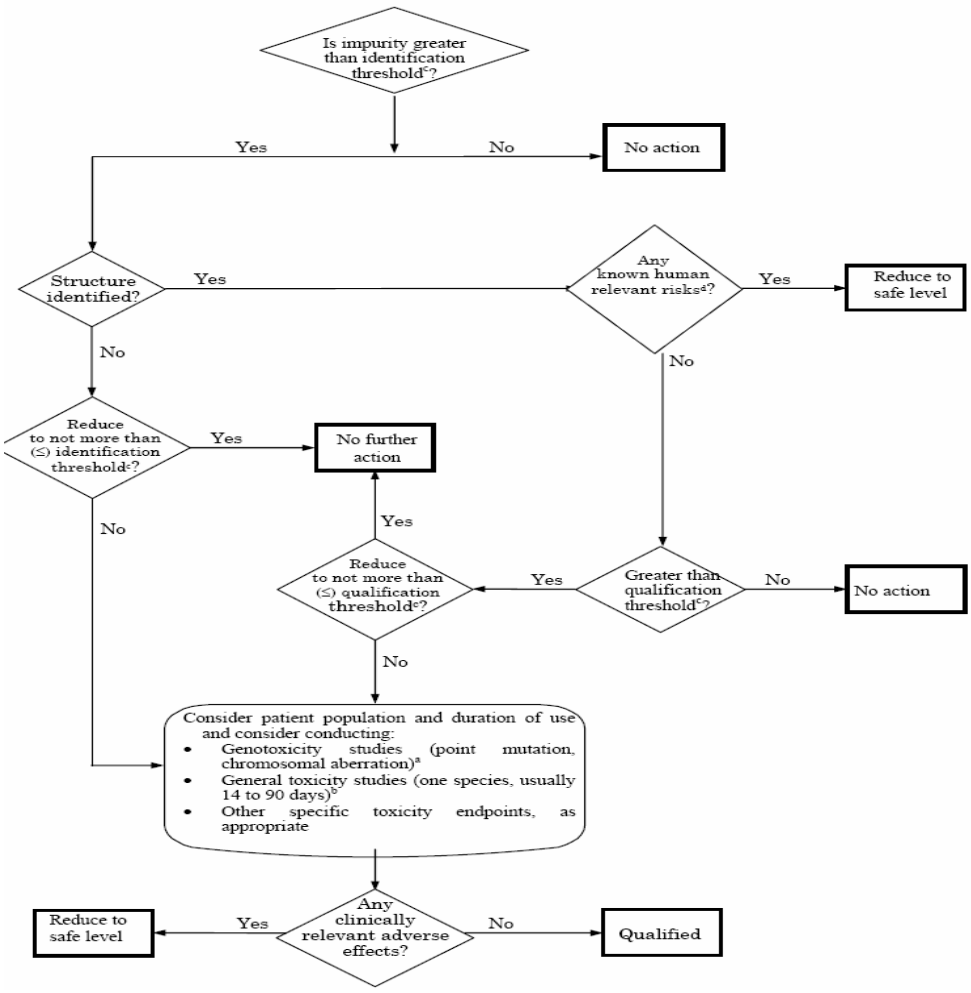


Figure 4.1 Impurity identification and qualification

4.1.5 Identification of Impurities

Identification of impurities is an analytical activity aiming to elucidate the chemical structures and the possible mechanisms of formation of unknown impurities. Because of the complexity and diversity of the impurities in both their origins and properties, the identification strategies are determined by the specific situations.

A general strategy can be set for the estimation of the impurity of bulk drug substances or drug products by the rational use of analytical techniques. The schematic use of the methods [230] for impurity profiling is shown in fig.4 .1. Analytical procedures should be validated and should be suitable for the detection and quantitation of degradation products (see ICH Q2A and Q2B guidelines on analytical validation). In particular, analytical procedures should be validated to demonstrate specificity for the specified

and unspecified degradation products. As appropriate, this validation should include samples stored under relevant stress conditions: light, heat, humidity, acid/base hydrolysis, and oxidation. When an analytical procedure reveals the presence of other peaks in addition to those of the degradation products (e.g., the drug substance, from the impurities arising synthesis of the drug substance, excipients and impurities arising from the excipients), these peaks should be labeled in the chromatograms and their origin(s) discussed in the validation documentation. The quantitation limit for the analytical procedure should be not more than (≤) the reporting threshold.Detection of an unknown impurity is the first step in impurity identification. Typically, unknown impurities are observed during analysis of intermediates or drug substances for process control or at release or drug product at release. Once the decision for identification is made, the nature and origin of the impurity can be assessed, based on when, where, and how the unknown is initially observed. Depending on the structure of the drug substance, the synthetic scheme, impurities in the starting material, known process impurities, degradant due to formulation ingredients, and the analytical method that is used for the initial detection of the unknown impurities, it is possible to evaluate the impurities. The impurities can be identified predominantly by different methods like reference standard method, separation methods and isolation methods. The characterization of impurities can be carried out using spectroscopic, spectrometric and hyphenated techniques.

Reference standard method

This method can be adapted by making use of the available standard of the impurity. If the initial analysis indicates that the observed impurity falls into this category, the impurity identification turns into practice. This can be achieved by three experiments. First, analysis of sample followed by analysis of standard and then spiked sample with standard by applying any of the chromatographic or spectroscopic techniques.

Separation methods

The following methods are being regularly used for the separation of impurities and degradation products: high performance liquid chromatography (HPLC), gas chromatography (GC), thin layer chromatography (TLC), high performance thin layer chromatography (HPTLC), capillary electrophoresis (CE), super critical fluid

chromatography (SFC), and Gel permeation chromatography (GPC). Recently UPLC is emerging as a fast separation liquid chromatographic technique.

High Performance Liquid Chromatography

HPLC is a chemistry based tool for separation, quantification and analysis of mixtures of chemical compounds. As a consequence of the enormous development of the analytical technology in the last two decades entirely new possibilities have been created for the determination of the purity of drug materials. Nearly all organic impurities are determined by chromatographic or related methods of which HPLC has been the most important for over a decade.HPLC is regarded as the most important analytical method in pharmaceutical analysis as it provides a number of highly selective variants to resolve almost every type of separation problem. Derivatization of the drugs prior to analysis is normally not required. HPLC can be operated in both modes i.e. reverse phase and normal phase mode.

Ultra Performance Liquid Chromatography (UPLC).

Ultra-performance Liquid Chromatography (UPLC) is a new category of separation technique based upon well established principles of liquid chromatography, which utilizes sub- 2 μm particles for stationary phase. With the objective of reducing analysis time and maintaining good efficiency, there has been substantial focus on high-speed chromatographic separations. UPLC has proven to be one of the most promising developments in the area of fast chromatographic separations. The sub- 2 μm particles operate at elevated mobile phase linear velocities to affect dramatic increase in resolution (Figure 4.2 A,B,C), sensitivity and speed of analysis [231]. Because of its speed and sensitivity this technique is gaining considerable attention in recent years for pharmaceutical and biomedical analysis [232,233].

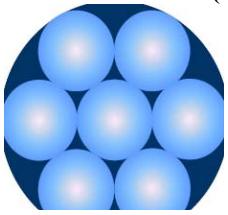
Gas chromatography (GC)

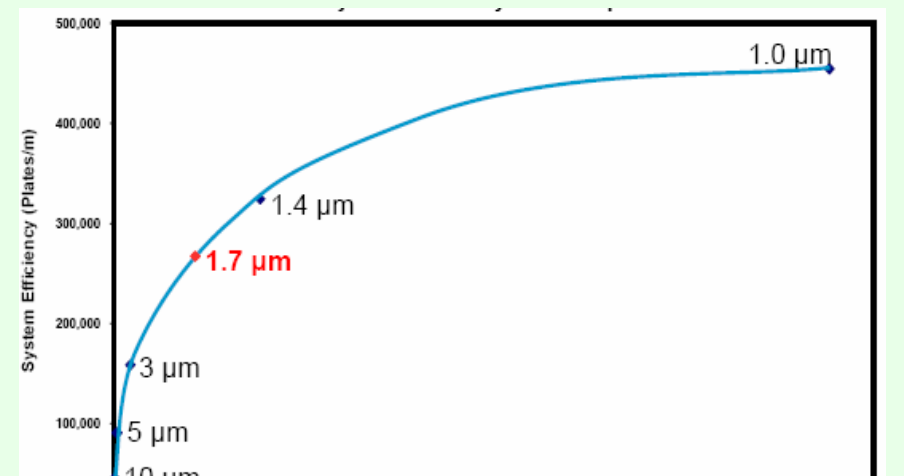
GC is a dynamic method for separation and detection of volatile organic compounds in a mixture [30]. It involves the partitioning of gaseous solutes between an inert gas mobile phase and stationary solid or liquid phase. GC especially when combined with head space sampler is the most popular and selective analytical technique for residual solvent analysis in drug substances. Modern capillary-column gas chromatographs can

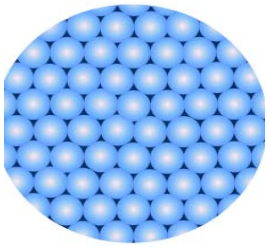
separate a large number of volatile components, permitting identification through retention characteristics and detection at µg/ml levels using a broad range of detectors.
The most popular detectors are; flame ionization detector (FID) and electron capture detector (ECD). FID is an universal detector for carbonaceous material (especially organic volatile compounds), with the exception of a limited number of small molecular compounds such as carbon disulfide formic acid, formaldehyde, carbon tetrachloride, etc [234].This is a very important feature from the impurity profiling point of view. ECD is especially suited to detection of halogenated compounds. However, FID is by far the most preferred for release-related tasks because of its low detection limits, wide linear dynamic range, and robustness, ease of operation, and general reliability and utility, especially for trace organic compound.

For a given degradation product, its acceptance criterion should be established by taking into account its acceptance criterion in the drug substance (if applicable), its qualified level, its increase during stability studies, and the proposed shelf life and recommended storage conditions for the new drug product.Furthermore, each acceptance criterion should be set no higher than the qualified level of the given degradation product.
Where there is no safety concern, degradation product acceptance criteria should be based on data generated from batches of the new drug product manufactured by the proposed commercial process, allowing sufficient latitude to deal with normal manufacturing and analytical variation and the stability characteristics of the new drug product. Although normal manufacturing variations are expected, significant variation in batch-to-batch degradation product levels can indicate that the manufacturing process of the new drug product is not adequately controlled and validated (see ICH Q6A guideline on specifications, decision tree #2, for establishing an acceptance criterion for

(A)







(C)

Figure 4.2(A) 5 µm particle size in HPLC (B) 1.7 µm particle size in UPLC (C) Backpressure of system increases for lower particle size while system performance (measured in terms of Plates/m) is higher for smaller particles

a specified degradation product in a new drug product). In summary, the new drug product specification should include, where applicable, the following list of degradation products

- Each specified identified degradation product
- Each specified unidentified degradation product
- Any unspecified degradation product with an acceptance criterion of not more than (≤) the identification threshold
- Total degradation products.

Abiding by the regulatory for, ICH guidelines the present research work focuses on development of impurity profile of antimalarial fixed dose dosage form or ACTs artemisinin combination therapy.

The World Health Organization has endorsed ACT as first-line treatment where the potentially life-threatening parasite Plasmodium falciparum is the predominant infecting species. The particular features of ACT relate to the unique mode of action of the artemisinin component, which includes the following:

• Rapid and substantial reduction of the parasite biomass,

• Rapid parasite clearance,

• Rapid resolution of clinical symptoms,

Therefore, it is especially important to ensure the quality of anti-malarial drugs and drug product. A fixed dose tablet formulation is beneficial in terms of its convenience and patient compliance.

Selected fixed dose drug products are

1. Artemether and lumefantrine tablets
2. Artesunate and amodiaquine tablets

4.2 Development and validation of RP HPLC Method for Impurity Profiling of Artemether and Lumefantrine Fixed Dose Pharmaceutical Preparation

4.2.1 Introduction

Artemether,3R,5aS,6R,8aS,9R,10S,12R,12aR)-Decahydro-10-methoxy-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin., Its molecular formula is $C_{16}H_{26}O_5$ and its molecular weight is 298.4. is used in the treatment of malaria(Fig 2.1) and Lumefantrine chemically (1R,S)-2-Dibutylamino-1-{2,7-dichloro-9-[(Z)(4-chlorobenzylidene)-9H-fluoren-4-yl}-ethanol and its molecular weight is 528.4 (Fig 2.2), is also antimalarial active substance.

The relevant related substances are separated into two groups:

1) Significant related substances (degradent): Linearity, accuracy and response factor should be established for significant related substance during method validation. In this case dihydroartemisinin, artemisinin and alpha artemether and impurity A are the degradedness.

2) Other related substances: These are not significant or potential degradation products. The developed HPLC conditions only need to provide good resolution of these related substances to show that they are not interfering with degradedness and the method is specific. Here in this combination impurities of lumefantrine, para chloro benzaldehyde (PCB) and ά – (dibutylamine methyl)-2, 7-dichloro-4-fluromethanol, (DBA) are process related. Lumefantrine is listed as a stable molecule in the WHO list. Hence, in the present work the separation of these two was shown to prove the specificity of the method.

The Dihydroartemisinin, α-artemether, impurityA and artemisinin are impurities arises from artemether. Impurities para chloro benzaldehyde(PCB), and DBA are the process related impurities of lumefantrine. All these impurities were considered for development Figure 4.3 repersent the structures of artemether impurities.

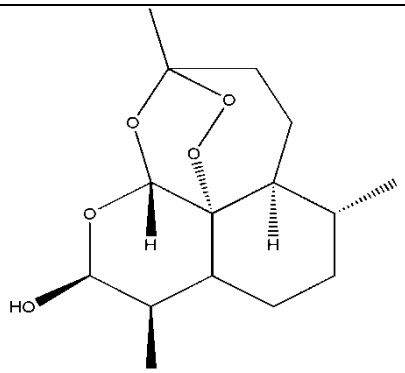
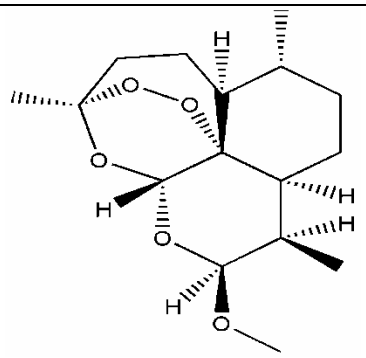
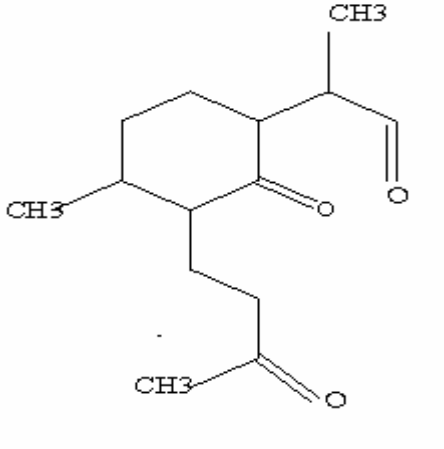
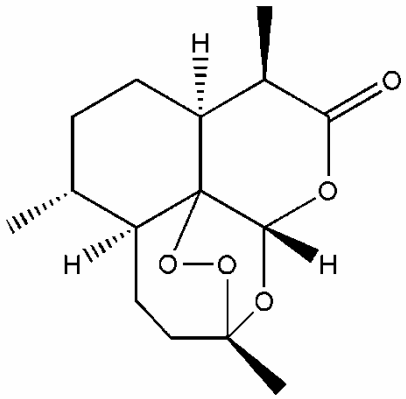
 Dihydroartemisinin (DHA) (3R,5aS,6R,8aS,9R,12S,12aR)- octahydro-3,6,9-trimethyl-3,12-epoxy- 12H-pyrano[4.3-j]-1,2-benzodioxepin-10(3H)-one	 α-artemether 3,6,9-trimethyldecahydro-3,12-epoxy-12H-pyrano[4,3]1,2-benzodioxepine,
 Impurity A 2-[4-methyl-2-oxo-3-(3-oxobutyl)cyclohexyl}propranal	 Artemisinin (3R,5aS,6R,9R,12S,12aR)-octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-/]-1,2-benzodioxepin-10(3H)-1

Figure 4.3 Structures of impurities in artemether and lumefantrine tablets

Thin layer chromatographic methods have been reported for the determination of dihydroartemisinin , artemisinin ,alpha artemether , impurity A in artemether active substance and artemether and lumefantrine tablets in International Pharmacopoeia [130]. Literature search revealed that several analytical methods are available of determination of artemether separately in formulations, in biological fluids and in presence of other anti-malarial combinations agents. Recently published method for the determination of artemether along with its impurities by TLC [157] and lumefantrine by liquid chromatography [157] limits its application by separately carrying out the determination. A HPLC method is also reported for identification and determination of lumefantrine and its impurities in active drug substance [198]. If the reported individual methods are applied for the related substances analysis of tablets containing artemether and lumefantrine, it would require double time of analysis, method would not be rapid, less expensive or economical, whereas simultaneous determination of related substances would save analysis time and also economical. So far, to our present knowledge, there is no HPLC method for concomitant determination of impurities of artemether and lumefantrine in the combination product using single chromatographic conditions. In the work, discussed in this chapter, we therefore, focused on finding optimum HPLC conditions with flow and wave length gradient elution for separation and quantization of their potential impurities in artemether and lumefantrine in fixed dosage form and validation as per ICH guidance documents.

4.2.2 Materials And Methods

Chemical and Reagents

All working standards of artemether, lumefantrine and impurities like dihydroartemisinin, alpha artemether , artemisinin , impurity A of artemether , PCB and DBA of lumefantrine were procured from Ipca laboratories Ltd, Mumbai, India .Combination product of artemether and lumefantrine (label claim :artemether 80 mg and lumefantrine 480 mg) of Ipca Laboratories Ltd, were used for the development and validation. Acetonitrile of HPLC grades, decane sulphonic acid sodium salt, sodium dihydrogen phosphate monohydrate and triethylamine were procured from Merck

(India). Milli-Q water was used. Poly vinidene fluoride (PVDF),0.45µ filter paper was obtained from whatmann. All dilutions were prepared in standard volumetric flasks.

4.2.3 Instrumentation and Chromatographic conditions

Chromatography was performed with Waters Alliance system, Waters 2695 separation module and Waters 2996 photo diode array detector. The output signal was monitored and processed using chromeleon software. A column Waters symmetry C_{18} column, (250 × 4.6mm dimensions) having particle size 5µm was used for the separation as a stationary phase. The buffer was prepared by dissolving 0.56% of decane sulphonic acid sodium salt , 2.8 g sodium dihydrogen phosphate monohydrate and 5ml of triethylamine in 1000 mL water, pH adjusted to 2.3 with ortho-phophoric acid AR grade (88%) and diluting to 1000 mL with water, filtered through 0.45 µm membrane filter and degassed in ultrasonic bath prior to use as mobile phase A . Acetonitrile was used as mobile phase B. The flow rate was 1.0 mL min^{-1} . The injection volume amounted to 50 µl. The analysis was carried out under gradient condition as mentioned in table 4.2 and 4.3

Table 4.2 Gradient Program

Time	Buffer	Acetonitrile	Flow Rate
0.0 min.	40	60	1.0 ml / min.
25.0 min.	40	60	1.0 ml / min.
26.0 min.	40	60	2.0 ml / min.
45.0 min.	40	60	2.0 ml / min.
46.0 min.	40	60	1.0 ml / min.
50.0 min.	40	60	1.0 ml / min.

Table 4.3 Wavelength Programming

Time In minutes	Wavelength Program
0 – 4 min	380nm
4 – 25 min	210nm
25 – 50 min	380nm

detection was monitored at a wavelength of 210nm and 380 nm. A mixture of buffer (Buffer Preparation for Diluent : Dissolve 2.8 g of sodium dihydrogen phosphate monohydrate, in 1000 ml water. Adjust the pH to 2.3 with Orthophosphoric Acid.) and acetonitrile in the ratio of (25:75 *v/v*) was used as diluent in the preparation of analytical solutions.

System Suitability solution

Resolution solution of 24 µg mL^{-1} of artemether and 12 µg mL^{-1} of α-artemether was used as system suitability solution.

Diluted Standard solution: Standard stock solution of artemether (2400 µg mL^{-1}), lumefantrine (1440 µg $mL^{-1,}$) ,dihydroartemisinin (960 µg mL^{-1}) are prepared in diluents. Further stocks are mixed and diluted with diluent in such way that it has concentration of each of artemether , lumefantrine and dihydroartemisinin (DHA) as 24 µg mL^{-1}(equivalent to 0.5% with respect to artemether) , 72 µg mL^{-1} (equivalent to 0.25% of lumefantrine w.r.t lumefantrine in test sample and 96 µg mL^{-1}(equivalent to 2.0% of dihydroartemisinin w.r.t artemether in test sample

Sample preparation

Twenty tablets were weighed and crushed to homogenous powder using a mortar and pestle. An accurately weighed portion of the powder, equivalent to 480 mg of artemether into a 100 ml volumetric flask, added 50 ml diluent, and shaken for about 15 minutes. Then dispersed with the aid of ultrasound for 10 minutes with intermittent swirling The flask was further shaken with the means of mechanical shaker for 15 minutes and allowed to reach the ambient room temperature The volume was made up to 100 mL with diluent and mixed. Filtered the solution through 0.45µ PVDF filter.

4.2.4 Results and Discussion

The challenge was to separate seven impurities and two actives. Here the selection of column , wavelength and mode of separation was kept same as developed for stability indicating method (SIM) (3.2.2). The main target of the chromatographic method is to detect and quantify the known degradent impurities dihydroartemisinin, artemisinin, alpha artemether and impurity A of artemether and separate other related impurities like PCB and DBA of lumefantrine in fixed dose tablets by utilizing same chromatographic setup in single run. Optimization of conditions for simple, accurate and reproducible analysis involves analyzing system suitability solution on varying stationary phase, strength of aqueous phase, pH, and proportion of acetonitrile – aqueous phase, flow rate and column temperature. Our preliminary experiments indicated that using different concentration of acetonitrile and even different pH of the buffers did not produce suitable separation of dihydroartemisinin I and PCB and artemether and α. Artemether. Mobile phase strength or solvent strength is optimized to get the optimum α and K'> 1. Hence, ion pair reagent (oppositely charged ion) in the mobile phase was used which reacts with them to form neutral ion pair enabling to retain on non –polar stationary phase. When different ion pairs were used like pentane salt , hexane salt, heptane salt and octane salt of sulphonic acid showed no proper resolution of dihydroartemeisinin and PCB , lumefantrine elution was not satisfactory where K'> 30 is observed. Addition of higher strength ionic salt like Sodium salt of decane sulfonic acid helps to retain basic lumefantrine for longer time till all other peaks elute and helps lumefantrine to elute at later stage. Further lumefantrine being strongly basic in nature, it becomes important to select suitable pH for simultaneously retaining and separating artemether and lumefantrine from its impurities. Due to the ionization capacity of these charged analytes, pH played an important role. Being acidic in nature (pH of 10% solution is 3.3) artemether in alkaline pH will elute much faster affecting K'. At the same time dihydroartemisinin and lumefantrine being alkaline and having pka >8.0 elution helps pH around 6.5. The trials are carried out at pH 2.5 ± 0.2 and pH 6.5± 0.2. Artemether found to elute at much faster t0 and dihydroartemisinin I and dihydroartemisinin II almost merged where resolution (Rs) < 1.5 at pH 6.5. At pH 2.3 which is less than pKa of artemether, it retains artemether well. And due to non ionized conditions

dihydroartemisinin I and dihydroartemisinin II elutes with Rs >2.0 and low retention K'>1. Lumefantrine has counter ion effect and elutes comparatively late but addition of salt takes care of the elution of lumefantrine faster rate . The choice of pH 2.3 for the mobile phase was made for excellent separation and reasonable retention time, also for longer column life. Sample loading was another challenge to achieve required LOQ. In the desired method LOQ is related to the ICH reporting limits. If the corresponding ICH reporting limit is 0.1% , the method LOQ should be less to ensure the results are accurate up to one decimal place.Because of the high dependence on the mobile phase composition, the attempts to improve the selectivity and peak shapes by altering buffer and acetonitrile composition in isocratic mode were successful. Further to reduce the total run time or to reduce the retention time flow gradient mode of separation was chosen by altering the flow to twice after elution of artemether which reduces total run time to 50 mins. K' is well fitted as 1> k'< 20 (k' for lumefantrine was 12) which leads to isocratic separation [235]. Investigation of column selectivity of the method [152] showed improvement in the peak profile of artemether, dihydroartemisinin, artemisinin and its impurities more significantly on C_{18} column than on C_8 column while studying the different concentration of ion pair reagent, keeping pH constant (2.3). Finally, Waters Symmetry C_{18} column was utilized as separation unit. Waters Symmetry C18 was selected since this being packed with particles of silica gel, surface of which has been modified with chemically bonded octadecylsilyl groups was well fitted to the two studied drugs which are cationic species in the acidic mobile phase. Further, column with 5µ particle size gave better resolution between dihydroartemisinin a degradant of artemether, and artemether compared to 10µ particle size column where there was merging of the both the peaks observed. Challenge for selection of wavelength was due to the six fold concentration difference in the dosage form that is 80 mg Artemether and 480 mg of Lumefantrine. UV absorption spectra of artemether, lumefantrine and its impurities recorded in HPLC system using photodiode array detection it is observed that UV absorption maxima of artemether showed optimum UV absorption at 210 nm and lumefantrine shows UV absorption in a wide range i.e.210-400nm. The response of lumefantrine decreases with increase in wavelength. Hence it is concluded to use dual wavelength for simultaneous detection of artemether and lumefantrine. Wavelength 210

nm for artemether content determination and 380 nm for lumefantrine content determination in the combined dosage form. The wave length programming is adjusted in such way that all impurities of artemether were separated at 210nm at the same time, to avoid the response of placebo, the wave length was kept at 380 nm for initial 4 minutes then it is switched over to 210 nm till 25 minutes and artemether and its impurities are well separated at flow rate of 1 mL min $^{-1}$ then after from 26 mins to 45 mins wavelength is switched to again 380 nm and flow rate is increased to 2.0 mL min $^{-1}$. During this time interval lumefantrine and its impurities are well separated. System is set back to 1mL min $^{-1}$ flow 46 to 50 mins. Column oven temperature is maintained at 50 ^{o} C .The high column temperature in the procedure evidenced the impurities and actives were stable throughout the column separation process. A typical chromatogram showing the separation of the impurities in the sample was given in figure 4.4.

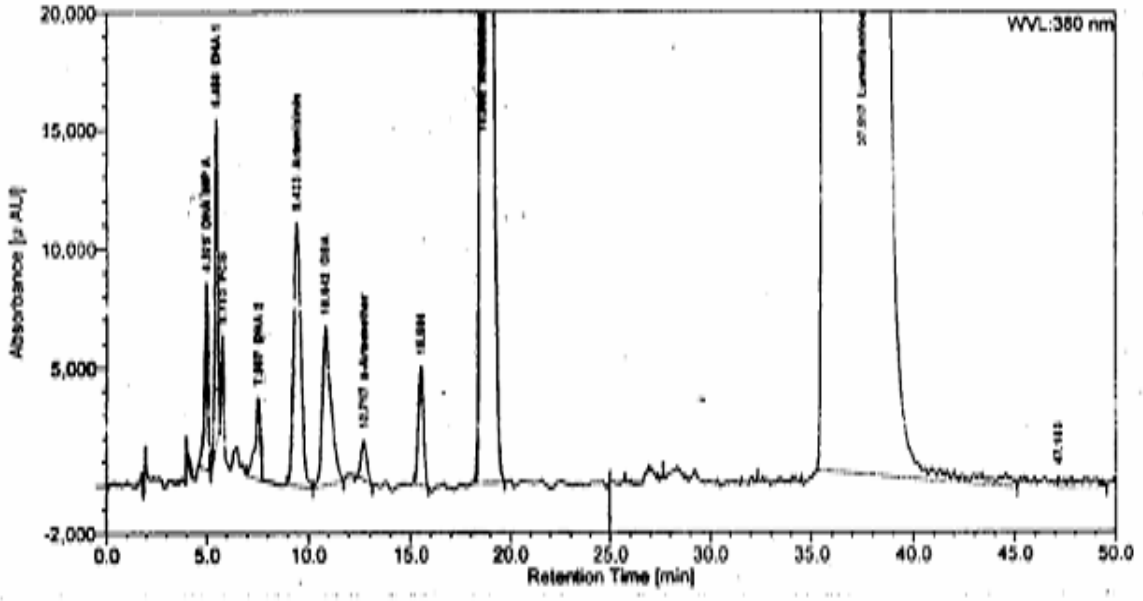


Figure 4.4 Typical Chromatogram of Artemether Lumefantrine and Its Impurities in Tablets.

4.2.5 Method Validation

The method was validated for Specificity, linearity, accuracy, precision, range, robustness, system suitability and reproducibility according to the International Conference on Harmonization (ICH) guidelines [47-49,159].

4.2.5.1 System Suitability

System suitability tests were performed in accordance with ICH guidelines to confirm the reproducibility of the equipment was adequate for the analysis to be performed. The test was performed on chromatogram obtained from system suitability solution to check the resolution between closely eluted peaks of artemisinin and artemether , theoretical plates for peak due to artemether in resolution solution and %RSD of peak area responses for artemether and lumefantrine and dihydroartemisinin in diluted standard solution. The values obtained are listed in table 4.4.

Table 4.4 Table For System Suitability Study

PARAMETER	LIMITS	VALUE
Resolution between α-Artemether and Artemether	NLT 5.0	11.46
Theoretical plates for peak due to Artemether in resolution solution	NLT 5000	16430

4.2.5.2 Specificity:

The specificity of the method was demonstrated by comparing chromatograms of diluent, actives, the related impurities and the placebo (containing all the ingredients of the formulation except the analytes) of the tablets as per the procedure applied to sample solution. No peak was detected at the retention time of artemether and lumefantrine and their respective impurities hence proving the specificity of the method.Table 4.5 indicate the relative retention times (RRT) of all the impurities.

Table 4.5 Identification Study

Sr. No	Components		Retention Time in mins	Relative retention time (RRT)		Placebo peak RT
1	α-Artemether		12.771	0.69		ND
2	DHA I		5.396	0.34		ND
3	DHA II		7.571	0.41		ND
4	Artemisinin		8.642	0.47		ND
5	PCB		5.783	0.29		ND
6	DBA		15.60	0.85		ND

ND- Not detected.

4.2.5.3 Accuracy

The accuracy was expressed as percent recovered at each level of concentration and as overall accuracy when the data are normalized to percent theoretical. The accuracy study of the known impurities Impurity A, dihydroartemisinin, and artemisinin, α artemether was determined for the following levels, 50%, 100% and 150% of the specified limit (i.e. 2% for dihydroartemisinin, 1.5% for impurity A and 1.0% for artemisinin and 0.5% of α artemether). 100% accuracy at loq level of each impurity is studied by spiking the loq concentration of each impurity in the sample and calculated by difference method. All the impurities showed the recovery >80% concluding the accuracy of the method. The test solution with known impurity concentration was injected first and this was treated as a blank. Each sample solution was prepared by spiking the test solution with known amount of impurity. The difference in peak area response before and after spiking impurities in the test sample at the three levels was calculated and the accuracy of the results was compared to the actual amount of impurity added and the amount of the same recovered. The accuracy was expressed as percent recovered. Also average, standard deviation and relative standard deviation was

reported. The results for all the impurities are presented in table 4.6 and table 4.7 which have met the acceptance criteria.

Table 4.6 Accuracy. Of The Method

Level	Amount of placebo (mg)	Amount of impurity added in (µg)	Amount of drug recovered in (µg)	% Recovery= µg recovered/ µg added*100	% Bias
Impurity A (Specification limit NMT 1.5%)					
50%	5909.12	36.02	36.15	100.36	-0.36
100%	5909.74	7 2.2	71.9	99.58	+0.42
150%	5909.24	108.2	108.6	100.35	+0.35
Dihydroartemisinin (Specification limit NMT 2.0%)					
50%	5909.12	47.98	48.76	101.62	-1.62
100%	5909.74	95.97	94.31	98.27	1.73
150%	5909.24	143.95	136.57	94.88	5.12
Artemisinin (Specification limit NMT 0.5%)					
50%	5909.12	12.1	11.6	95.86	4.14
100%	5909.74	24.3	24.0	98.76	1.24
150%	5909.24	36.5	35.0	95.89	4.11
α- artemether (Specification limit NMT (1.0%)					
50%	5909.12	23.9	24.1	100.83	-0.83
100%	5909.74	48.2	47.9	99.37	+0.63
150%	5909.24	72.5	73.1	100.83	-0.83

Table 4.7 Accuracy at LOQ Level of Each Impurity

LOQ Level 100%	Amount of placebo (mg)	Amount of drug added in (µg)	Amount of drug recovered in (µg)	% Recovery (85-115%)=µg recovered/ µg added*100
Impurity A	5909.43	5.1	4.89	95.88
DHA	5909.43	12.9	11.8	91.47
Artemisinin	5909.43	9.1	8.9	97.80
α artemether	5909.43	9.0	8.9	98.88

4.2.5.4 Precision

System precision was verified using diluted standard solution, which was analyzed for six times and RSD of artemether, and lumefantrine peak areas were 7.76% and 1.91% respectively and found to be within 10.0%. Results of system precision are presented in table 4.8. Precision of the method was studied for repeatability and intermediate precision. Repeatability was demonstrated by analyzing six separate artemether and lumefantrine tablets sample solutions that were prepared by spiking of the known impurities at 100% level of the specification limit. The RSD for each related substance was evaluated and % RSD for impurity A, dihydroartemisinin, artemisinin, α-artemether, unknown max impurity and total impurities were 2.98, 2.50, 5.18, 4.03, 6.44 and 2.16 respectively which were well within 10% confirming the precision of the method. Table 4.9 represents the method precision results.

4.2.5.5 Correction Factor:

The response factor for each impurity was determined by injecting solution containing mixture of all known impurities and actives at same concentration 100 µg ml^{-1}. The correction factor is the inverse of response factor. Correction Factor (C.F.) is used while estimating the levels of known related substances and all other unknown impurities using diluted standard solution. C.F. for impurity A is 0.85, dihydroartemisinin 1.8, for

artemisinin 0.91 and for α –artemether 0.96. The C.F. is for unknown impurity so considered as 1. For all calculation purpose C.F. is used in multiplication.For impurities having C.F. between 0.8-1.2 for all practical purpose, it is considered as one. In this case except for dihydroartemisinin for all other impurities C.F. is taken as one.

Table 4 .8 - System Precision

Sr. No.	Diluted artemether	Diluted lumefantrine
1.	870	12351
2.	736	12246
3.	876	11993
4.	767	11810
5.	744	12191
6.	787	12433
MEAN	797	12171
RSD	7.76	1.91

Table 4.9 Method Precision

Sr. No	**Weight of the Sample**	**%Impurity A (NMT 1.5%)**	**%DHA (NMT 2.0%**	**% Artemisinin (NMT 0.5%)**	**% α-Artemether(NMT 1.0%)**	**% Max. unknown impurity (NMT 0.2%)**	**% Total impurity (NMT 2.0% excluding DHA)**
1	5909.91	1.45	2.13	0.45	0.99	0.08	2.97
2	5909.55	1.38	2.16	0.44	0.90	0.08	2.8
3	5909.33	1.49	2.04	0.43	0.92	0.09	2.93
4	5909.28	1.42	2.18	0.48	0.98	0.08	2.96
5	5909.17	1.46	2.11	0.42	0.97	0.09	2.94
6	5909.27	1.39	2.18	0.42	0.99	0.09	2.89
Mean		1.43	2.13	0.44	0.96	0.085	2.91
%RSD		2.98	2.50	5.18	4.03	6.44	2.16

4.2.5.6 Linearity and Range:

Linearity of the method for artemether, lumefantrine, impurity A, dihydroartemisinin, artemisinin, and α-artemether was determined using concentration from LOQ to 150 % of the concentration of the each solution. Linear regression analysis on data was performed using concentration as the independent (X) variable and peak area response as the dependent (Y) variable. Linear regression of the data included a correlation coefficient, residual square(r^2), slope, and y- intercept .The range was decided to be LOQ to 150% of each of respectively lumefantrine, artemether, impurity A, dihydroartemisinin, artemisinin and α-artemether are presented in tables 4.10 table 4.15, respectively. Similarly, figure 4.5 to figure 4.10 represent linearity graphically.The regression data obtained met the acceptance criteria.

4.2.5.7 Limits of quantitation (LOQ)

The limits of quantitation were determined for artemether, lumefantrine, impurity A, dihydroartemisinin, artemisinin and α –artemether as per ICH guidelines from the standard deviation of the peak areas and slope of linearity data. The calculated and LOQ concentrations of all the components were verified for precision by calculating % RSD .The limit of quantitation were 0.45µg ,0.252 µg ,0.24 µg ,0.6 µg ,0.45 µg and 0.43µg respectively.Figure 4.11 represent the typical chromatogram of same.

4.2.5.8 The intermediate precision or ruggedness

It was determined on six separate sample solutions prepared from same batch by spiking the related substances at the specification level by a different analyst using different mobile phase and diluents preparation and instrument on a different day with different lot of same brand column. The overall RSD was evaluated and were within the acceptance criterion of NMT 10.0% .The results were presented in table 4.16.

Table 4.10 Linearity Study of Lumefantrine

Concentration in % w.r.t. test concentration of lumefantrine In µg mL $^{-1}$	Concentration in In µg mL $^{-1}$	Mean peak area
0.035	5	2204
0.5	72	13107
0.8	115.2	20451
1.0	144	25815
1.2	172.8	31178
1.5	216	37722

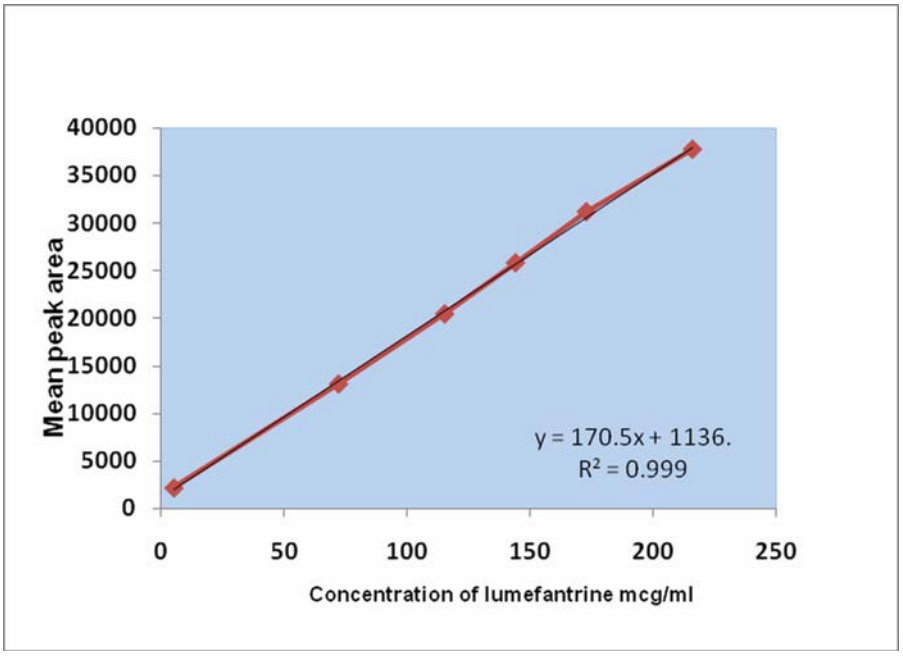


Figure 4.5 Linearity Curve of Lumefantrine.

Table-4.11 Linearity Study of Artemether

Concentration in % ,W.r.t. test concentration i.e. 4800 In µg mL $^{-1}$	Concentration in In µg mL $^{-1}$	Mean peak area
0.19	9.3	322
0.25	12	444
0.4	19.2	665
0.5	24	833
0.6	28.8	998
0.75	36	1301

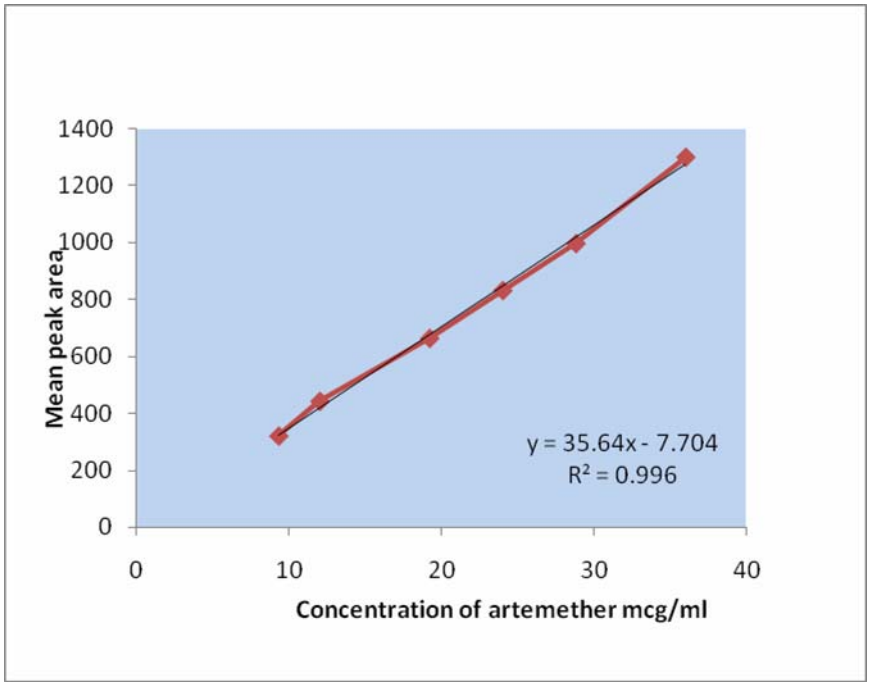


Figure 4.6 Linearity Curve of Artemether.

Table 4.12 Linearity of Impurity A

Concentration in %,w.r.t. test concentration of artemether i.e. 4800 In µg mL $^{-1}$	Concentration in In µg mL $^{-1}$	Mean Peak Area
0.10	5.04	269
0.20	7.2	384
0.75	36	1922
1.2	57.6	3072
1.5	72	3854
1.8	86.4	4597
2.25	108	5677

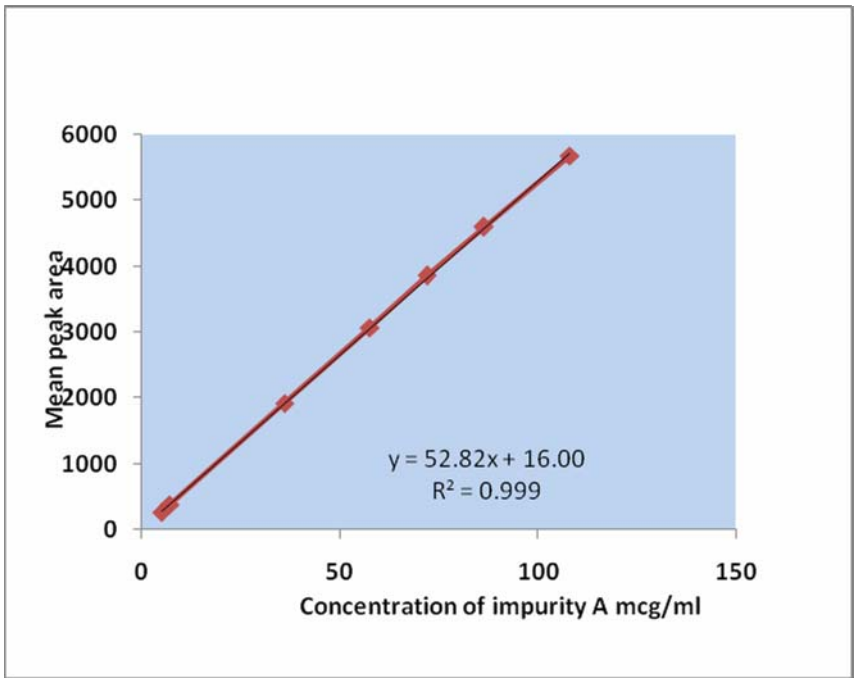


Figure 4.7 Linearity Curve of Impurity A

Table 4.13 for Linearity Study of Dihydroartemisinin

Concentration In % w.r.t. Test Concentration Of Artemether i.e.. 4800 In µg mL $^{-1}$	Conc. In µg mL $^{-1}$	Mean Area
0.25	12	284
1.0	48	981
1.6	76.8	1605
2.0	96	1952
2.4	115.2	2395
3.0	144	3050

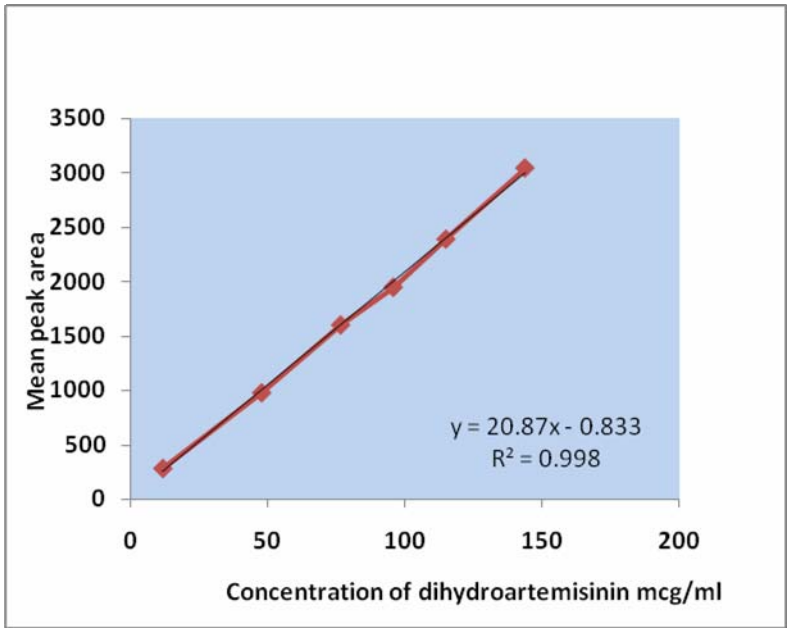


Figure 4.8 Linearity Curve of Dihydroartemisinin

Table 4.14 Linearity of Artemisinin

Conc. In % w.r.t. Test Concentration Of Artemether i.e. 4800 mcg/ml	Conc. In mcg/ml	Mean Area
0.18	8.8	289
0.25	12	398
0.4	19.2	545
0.5	24	684
0.6	28	782
0.75	36	966

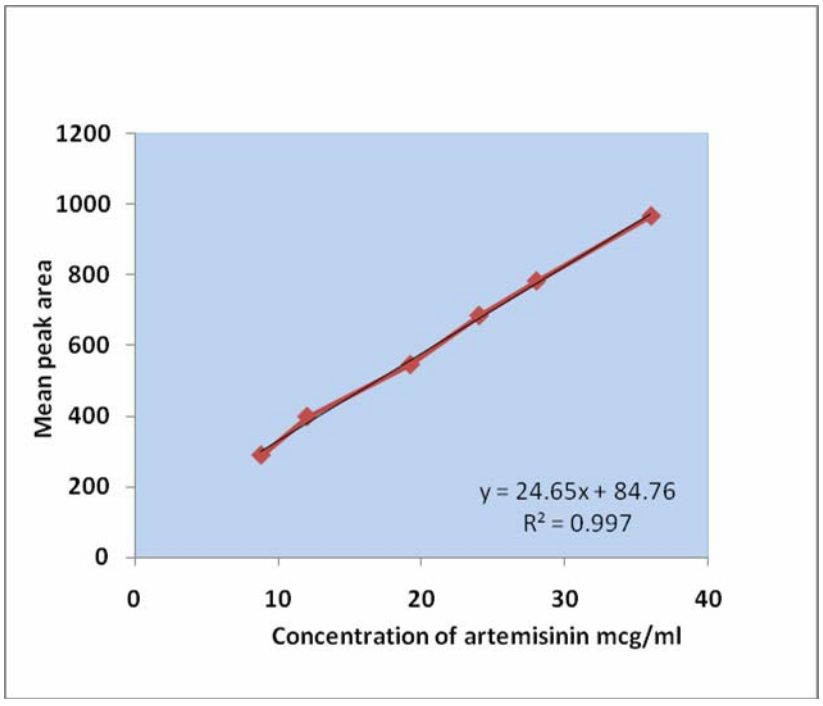


Figure 4. 9 Linearity Curve of Artemisinin

Table 4.15 Linearity Study Of ά-Artemether

Conc. In % w.r.t Test Concentration Of Artemether i.e 4800 mcg/ml	Conc. In mcg/ml	Mean Area
0.19	9.1	324
0.5	24	870
0.8	38.4	1378
1.0	48	1722
1.2	57.6	2056
1.5	72	2600

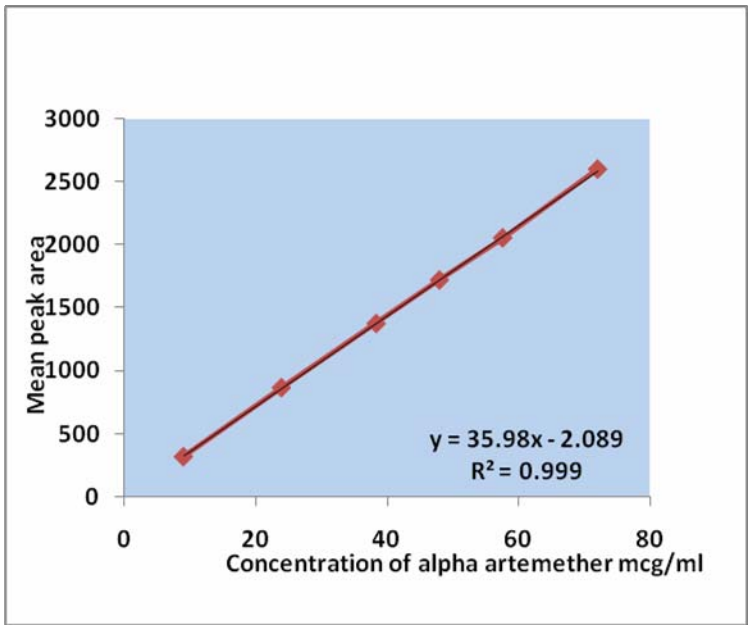


Figure 4.10 Linearity Curve of α- Artemether

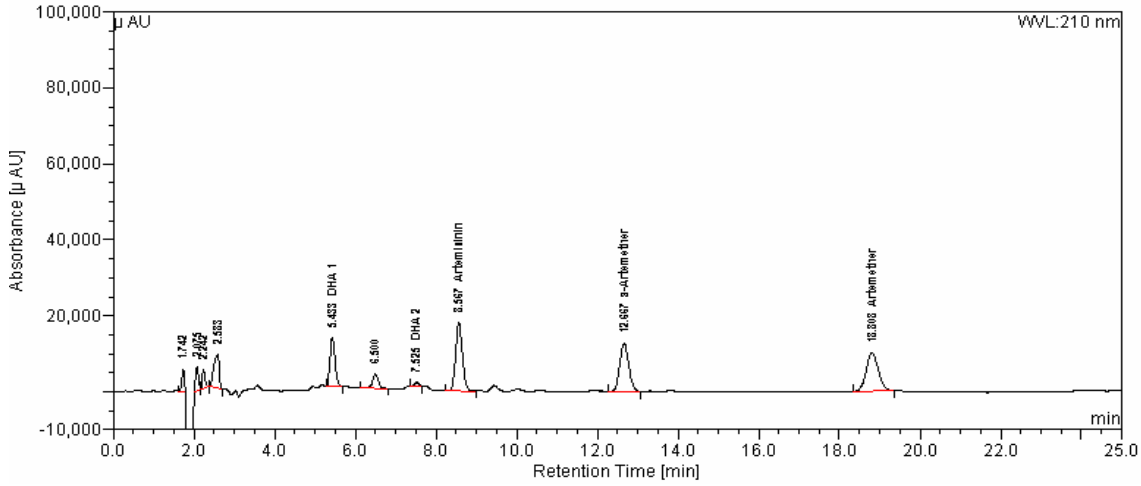


Figure 4.11 Chromatogram of LOQ of All Impurities.

Table 4.16 Intermediate Precision Study

Instrument	Waters XII								Dionex II			
Analyst	A								B			
	% Impurity A		DHA		Artesmisinin		% α-Artem ether		%Ink. Imp. Max		% Total Unknown impurities	
Sr. No.	(A)	(B)	(A)	(B)	(A)	(B)	(A)	(B)	(A)	(B)	(A)	(B)
1.	1.45	1.44	2.13	2.15	0.45	0.44	0.99	0.97	0.08	0.08	2.97	2.95
2.	1.38	1.40	2.16	2.11	0.44	0.43	0.90	0.95	0.08	0.07	2.8	2.9
3.	1.49	1.47	2.04	2.05	0.43	0.42	0.92	0.94	0.09	0.08	2.93	2.87
4.	1.42	1.44	2.18	2.15	0.48	0.46	0.98	0.98	0.08	0.07	2.96	2.96
5	1.46	1.45	2.11	2.10	0.42	0.48	0.97	0.98	0.09	0.07	2.94	2.84
6	1.39	1.42	2.18	2.17	0.42	0.43	0.99	0.97	0.09	0.08	2.89	2.94
MEAN	1.43		2.12		0.44		0.96		0.08		2.91	
SD	0.03		0.05		0.03		0.028		0.007		0.053	
%RSD	2.31		2.21		4.90		2.96		9.23		1.83	

4.2.5.9 Stability of analytical solution

To determine the stability of sample solution, the combination sample solution spiked with impurities at specified level were prepared and analyzed immediately after preparation and after different time intervals up to 24 hrs, while maintaining the sample solution at ambient temperature. The results from these studies indicated that the sample solution and standard solution was stable till 8 hrs after which dihydroartemisinin was found to increase as presented in figure 4.12 and figure 4.13. Table 4.17 and table 4.18 represent the solution stability data of standard and sample solution respectively.

Table – 4.17 Solution Stability Data Of Standard Solution

Time in hours	Diluted standard area	
	Artemether 0.5% solution	Lumefantrine (1.0%) Solution
0 hr.	13223	778
8 hrs.	13046	778
10 hrs.	13204	768
12 hrs	13386	781
24 hrs	13055	770

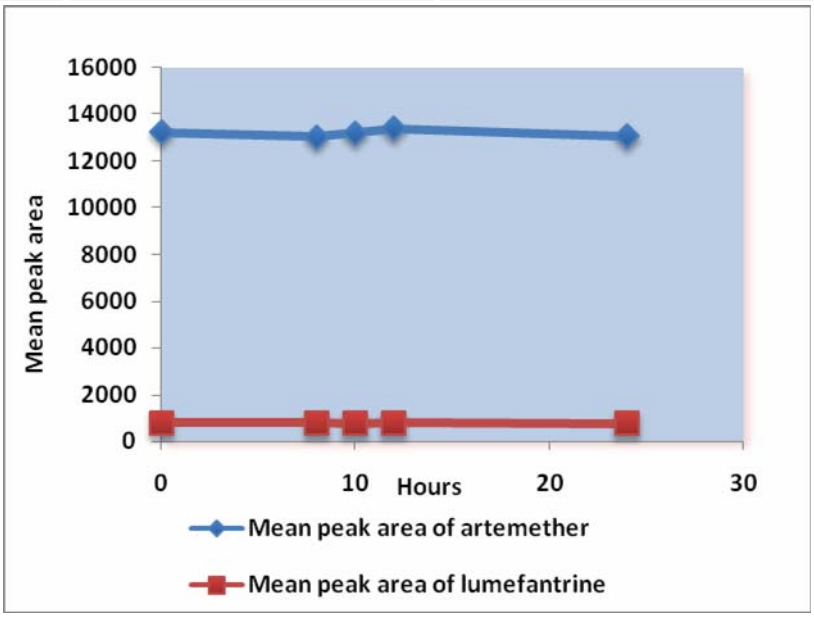


Figure 4.12 Solution Stability Curve for Standard Solution

Table 4.18 Solution Stability Data Sample Solution

Time in hrs.	Impurity A	DHA	artemisinin	A-artemether	Unknown Maximum	Total impurities
	%	%	%	%	%	%
0 hr.	1.45	2.13	0.45	0.99	0.08	2.97
8 hrs.	1.47	2.16	0.45	0.98	0.081	2.98
10 hrs.	1.64	2.4	0.46	0.96	0.087	3.14
24 hrs	1.84	3.2	0.46	0.95	0.091	3.34

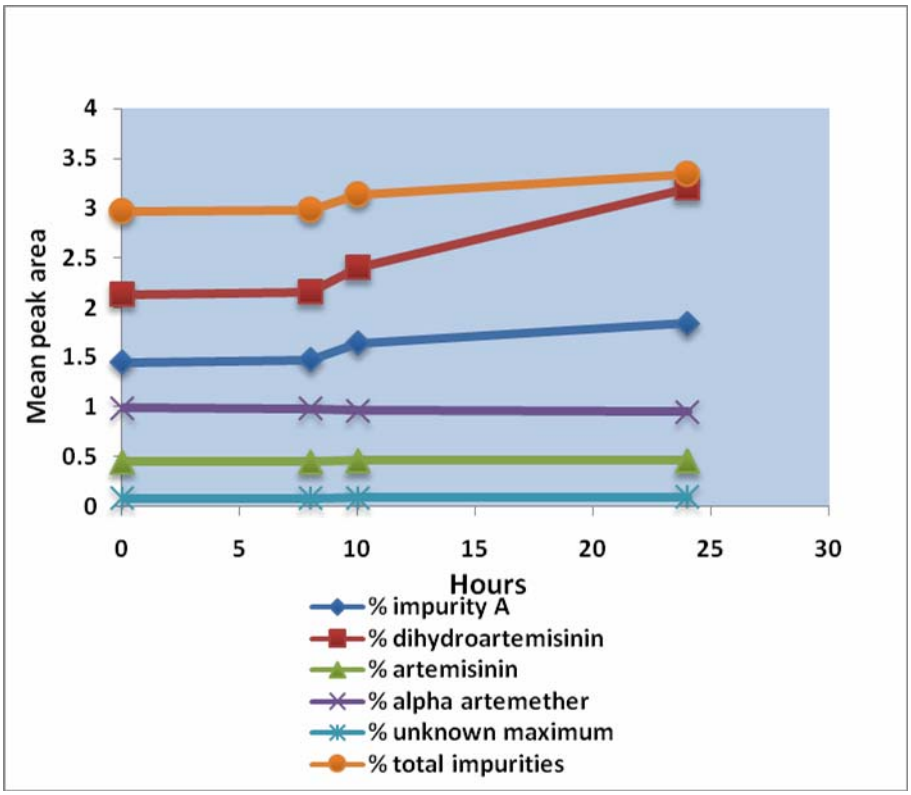


Figure 4.13 Solution Stability Curve of Sample Solution

4.2.5.10 Robustness

In all the deliberate varied chromatographic conditions carried out (flow rate, pH of the mobile phase and column temperature), the resolution between closely eluting impurities, namely artemisinin and artemether was greater than 1.5, table 4.19 illustrates the robustness of the method.

Table 4.19 Robustness Study

Parameters	**Changes**	**Artemether**			**Lumefantrine**
		% RSD (NMT 2.0)	Theoretical plates of artemether in resolution solution	Resolution between α-artemether and artemether	% RSD (NMT 2.0)
A - Flow Rate (ml/min)					
2.1	-0.2	9.1	20263	13.44	1.47
2.3	0	7.76	16430	11.46	1.91
2.5	+0.2	9.39	20770	13.51	1.53
B - Composition of mobile phase (Buffer: ACN)					
36:64	-10%	8.62	19232	11.41	2.15
40:60	0	7.76	16430	11.46	1.91
44:56	+10%	5.23	19885	11.44	0.68
Column Oven temperature					
45^0c	-5^0c	9.21	17545	10.81	3.21
50^0c	0	7.76	16430	11.46	1.91
55^0c	$+5^0$c	5.25	18210	11.64	0.95

4.2.6 Conclusion

The proposed flow gradient RP-HPLC method for the simultaneous detection and quantitation of impurity A, dihydroartemisinin, artemisinin, α-artemether and unspecified impurities in artemether and lumefantrine tablets is highly sensitive, accurate and precise. This procedure can be easily adopted for the routine quality control analysis of tablet dosage form without any interference from the excipients or each other. Method was validated for its performance parameters such as specificity (placebo interference), linearity and range, recovery, LOD, LOQ, precision and ruggedness. The investigated validation elements showed the method has acceptable specificity, accuracy, linearity, precision, robustness and high sensitivity. The method is carried out with commercially available and conventional HPLC equipment with easy sample preparation .It is simple, accurate and reproducible for the quantization of the impurities from the formulation. It was concluded that the developed method offers several advantages such as single chromatographic condition for the determination of impurities of two drugs , simple mobile phase and sample preparation steps, improved sensitivity makes it specific and reliable for its intended use. Additionally, the method is applicable to all the strengths like 20+120, 40+240 and 60+360 mg and all types of formulations such as dispersible tablets and dry syrup.

4.3 Development and Validation of Stability Indicating UPLC Method for Determination of Related Impurities in Artesunate And Amodiaquine Fixed Dose Tablets.

4.3.1 Development of HPLC/UPLC method

4.3.1.1 Streamlining Development

HPLC is ubiquitous in the pharmaceutical industry and is employed throughout the whole drug analysis process, including drug discovery screening, raw material analysis, impurity profiling, stability studies, pharmacokinetic studies and final product testing.

However, despite HPLC's popularity, by the end of the 1990s it had reached its technical performance limitations with existing technology. In an increasingly competitive environment, pharmaceutical scientists were looking for a next-generation solution that would not only increase productivity and efficiency, but also improve the quality of the information output. Whether employed in a standalone manner in high volumes to address the complicated task of method development, in conjunction with capital-intense mass spectrometers to improve assay sensitivity and maximize performance, or in process analytical technology (PAT) analysis to facilitate real-time quality testing, UPLC has become the technique – and investment – of choice.

The process of developing a reversed-phase HPLC method can take anywhere from weeks to months, incurring significant expense. Scientists have found that by utilizing UPLC technology for methods development, a six-fold improvement in throughput can be realized. This, in turn, reduces cost per sample and time of analysis considerably, while maintaining or improving separation integrity.

By developing rapid, high-resolution analytical methods, products can be brought to market faster, therefore, improving the overall profitability of the assay. In one case, a leading pharmaceutical company was able to reduce its regulatory filing time by 50 percent by adopting this technology.

New method development calls for a well thought-out experimental design. A systematic screening protocol that explores selectivity factors such as pH, organic modifier and column chemistry allows chromatographers to quickly determine which experimental parameters are most effective in manipulating the selectivity of a

separation. By employing this type of strategy, the total number of steps necessary to develop a method is reduced, providing an efficient and cost-effective approach.

Productivity improvements associated with employing UPLC technology for methods development are shown in the table. When we compare the UPLC methods development strategy to one directly scaled to conventional HPLC, we see a five-fold improvement in time. This significantly reduces the overall instrument time required to develop chromatographic methods to one work day, as opposed to one work week with conventional HPLC.

4.3.2 Extending performance

UPLC has been instrumental in facilitating unprecedented improvements in all fields of analytical chemistry; none more so than in the area of liquid chromatography/mass spectrometry (LC/MS). In countless examples, novel particle technology, low carryover and reduced cycle times have enabled extremely high-efficiency separations for improved resolution and sensitivity and increased throughput. To the best of our knowledge, no reports are available on stability indicating analytical method for artesunate and amodiaquine tablets and each of pharmaceutical ingredient (API). It is, therefore, felt necessary to develop a new stability indicating method for the related substance determination and quantitative estimation of known and unknown impurities. As artesunate is known for low solution stability, it was necessary to develop a faster chromatographic technique and hence ultra-performance liquid chromatography (UPLC) was selected for the present study. Ultra performance LC (UPLC) is a new category of separation science which builds upon well established principles of liquid chromatography, using sub 2 µm porous particles. These particles operate at elevated mobile phase velocities to produce rapid separations with increased sensitivity and increased resolution. Thus, UPLC technology allows analysts time to be drastically reduced while still meeting assay acceptance criteria based on plate count, resolution and analyze retention.

Sub 2 µm packed columns offer advantages over the more traditional columns packed with 3 & 5 µm particles through shorter analysis time, improvement in resolving power, sensitivity and peak capacity when transferring methods from HPLC to UPLC [236].

Several approaches can be taken depending on the analytical needs. If column dimensions are maintained and only particle size is reduced then improvement in efficiency, resolution and peak capacity is obtained. 2nd approach is reducing not only particle size but also column dimension, which has a benefit of reducing analysis time. In both cases, care must be taken to ensure operating flow rate, gradient profiles and injection volumes are scaled approximately to obtain an equivalent or superior separation [237]. Literature survey showed that amodiaquine hydrochloride is very stable drug and artesunate is highly unstable drug component [238]. Hence focus of this study was to separate prominently the major degradents of artesunate. Hence initially the isocratic and gradient HPLC conditions were optimized for artesunate and its impurities in artesunate and amodiaquine tablet sample. The main target of the chromatographic method was to achieve separation of impurities and main component artesunate and amodiquine. Dihydroartemisinin–I and dihydroartemisinin –II are major degradant of artesunate. Glycan, and artemisinin are some more potential degradant [239] impurities in artesunate and amodiquine dosage form. Srtucture and name of the impurities represented in figure.4.14. During LC development study it was observed that dihydroartemisinin II and artesunate were eluting with a meager resolution while glycan impurity was eluting at higher retention time. The response of all these impurities and artesunate was found to be maximum at 210 nm.The chromatographic separation was achieved on a Base Deactivated column C_{18}, 100mm×4.6 mm, 3µm column. In an isocratic mode using a mobile phase consisting of buffer (0.01M KH_2PO4 pH adjusted to 3.0 with orthophosphoric acid) and acetonitrile in the ratio of 56:44 (v/v), there was a good separation between dihydroartemisinin-II and artesunate while glycan impurity was found to be eluting at higher retention time. Sample loading another challenge to achieve required LOQ, The desired method LOQ is related to the ICH reporting limits. If the corresponding ICH reporting limit is 0.1% , the method LOQ should be less to ensure the results are accurate up to one decimal place. This requires looking further modification for in the developed SIM (section 3.3). To achieve the required LOQ the test concentration needs to increase almost 20 times. Due to increased column loading the k' for amodiaquine reduces to less than 1 and k' for glycan impurity goes to >20 which is not the good sign for good chromatography. Also

the Rs between artemisinin and artesunate < 1.2 which needs to relook in the method. The column, wavelength , and mobile phase is kept same as developed for stability indicating method (chapter 3.3). To reduce the run time and achieve better peak shape it was decided to switchover to gradient HPLC mode. To start with initial linear gradient of 5-100% ACN was employed and observed late eluting glycan elutes at 8-10 minutes where as all other peaks elutes at near t0. The same gradient is run with 5-100% buffer where glycan impurity does not resolve and artesunate elutes very later stage. Peaks of the sample eluting during the gradient are not adequately separated. If the difference between the retention times of the first (t_1) peak and the last peak (t_t) of chromatogram divided by the gradient time t_G >0.25 a gradient is needed [235] . In above case it is 0.425. The first step is to optimize gradient range by adjusting initial % of ACN for minimum separation time for initial peaks t_1 and adequate spacing early eluting peaks dihydroartemisinin I and amodiquine. Next final % of ACN is selected to elute artesunate, artemisinin and glycan near to the end of gradient. Initial gradient was tried as presented in table 4.20. In this gradient all other peaks separated but artemisinin and artesunate peaks elutes at the same RT at about 22.0 mins and with Rs 0. hence minor change in the gradient I to separate artemisinin is decided as described in table 4.22.

Table 4.20 Gradient I

Time	**Buffer**	**Acetonitrile**	**Flow Rate**
0.0 min.	88	12	1.0 ml/min
12 min	88	12	1.0 ml/min
15 min	70	30	1.0 ml/min
15.1min.	50	50	1.0 ml/min
35.0 min.	50	50	1.0 ml/min
36.0 min.	88	12	1.0 ml/min
40.0 min.	88	12	1.0 ml/min

Table 4.21 Gradient II

Time	Buffer	Acetonitrile	Flow Rate
0.0 min.	90	10	0.8 ml/min
20 min	50	50	0.8 ml/min
30 min	50	50	0.8 ml/min
40 min.	30	70	0.8 ml/min
40.1 min.	90	10	0.8 ml/min
45.0 min.	90	10	0.8 ml/min

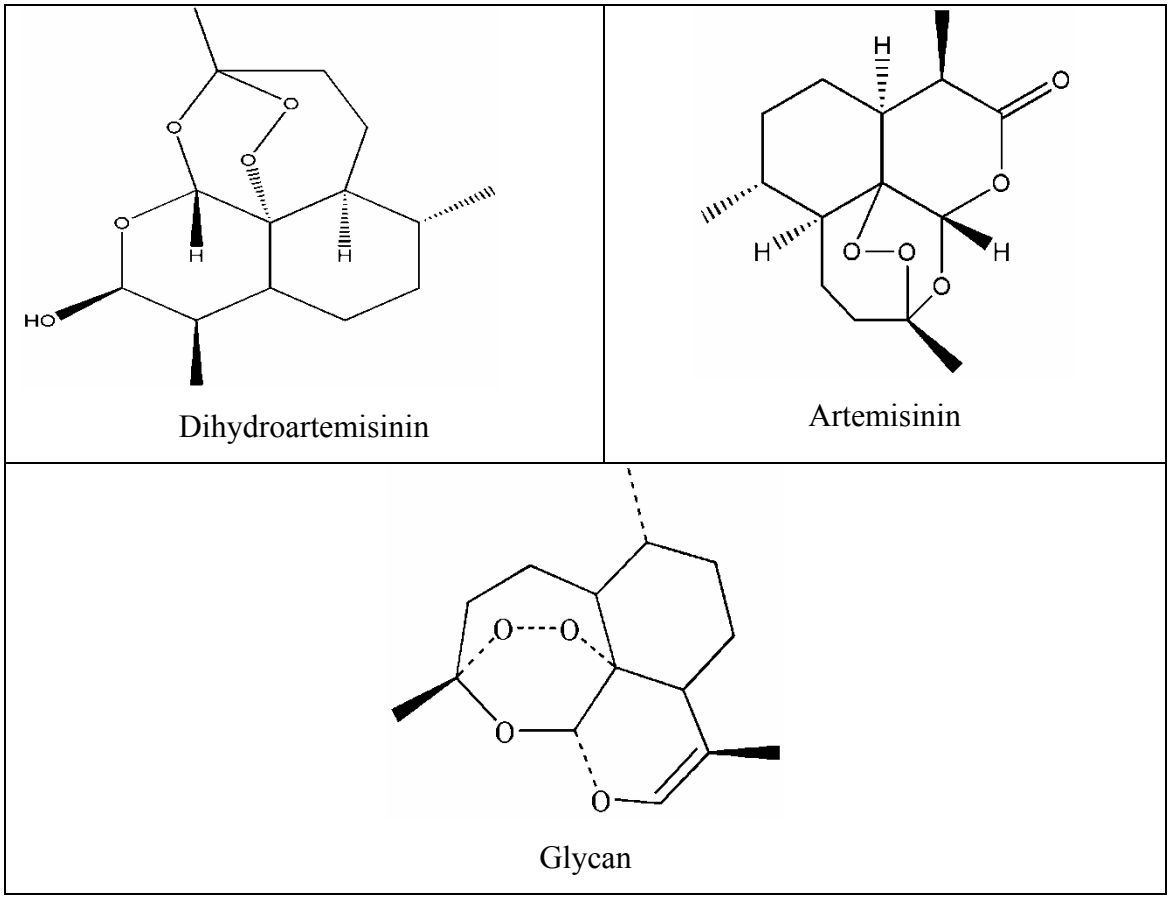


Figure 4.14 Structure Impurities of Artesunate and Amodiaquine Tablets.

This gradient gave very good resolution between artesunate and artemisinin (3.20) and glycan resolved at around 40 mins. Total run time is 50 mins. Artesunate is highly unstable at room temperature and immediately gets converted to dihydroartemisinin and hence the solution need to be prepared freshly and injected immediately. This limits number of analysis and hence needs switching over to UPLC arise.

4.3.3 Method Transfer to UPLC

The basic chromatographic conditions like stationary phase, solvents and UV detection, maintained in HPLC were taken into account while developing the new UPLC method. The parameters detection wavelength, column temperature, nature of buffer and solvent used in HPLC were maintained as such. The stationary phase of C_{18} was chosen in order to have similar chemistry to that used with the HPLC. A BEH C_{18}, 50 mm x 2.1mm, 1.7 µm column was employed for the separation [240-242].

To transfer the method geometrically to the smaller column geometrics packed with 1.7 µm particles and therefore, ensure equivalent chromatography, it is necessary to scale the flow rate, injection volume and gradient.

Step 1 : Adjust the flow rate

Calculate the flow rate by following equation

$F2= F1 \times (dc2^2/dc1^2) \times (dp1/dp2)$

Where F1- Original flow rate

F2-New flow rate

dc1- Original column id (mm)

dc2- New column id (mm)

dp1- Original particle size

dp2- new particle size.

Solving this equation we got the new flow rate as 0.208ml/min approximately 200 ul .

Step 2. Adjustment of injection volume

$Vi2= Vi1x(dc2^2 xL2/ dc1^2 xL1)$

Vi2-New injection volume

Vi1- Original injection volume

dc1- Original column id (mm)

dc2- New column id (mm)

L1- Original column length (mm)

L2- new column length (mm)

This equation gives 10 µL injection volume

Step 3 Adjustment of gradient profile

Expected flow and injection volume shows scaling down of 5 times

At the beginning an isocratic mode was chosen with the same ratio of buffer to acetonitrile as used in isocratic HPLC mode (56: 44 v/v). The flow rate was scaled to 200 µL/min by above equation. Though by calculation 10 µL was the injection volume for better back pressure and sharp peaks 5 µL injection volume is selected. Using these conditions a satisfactory separation was achieved between artesunate and artemisinin while glycan impurity was eluting around 9-10 min giving a total run time of 12 minutes. Further increase in the flow rate increases the back pressure of the system to more than 13000 psi which will make very difficult for system to run for long term hence 200 µL was selected as the final flow.

4.3.4. Comparison Study of Chromatographic Performance

A comparative data on chromatographic performance of HPLC gradient and UPLC (gradient) has been obtained by injecting a solution of artesunate and impurities. The performance parameters of both the systems are shown in Table 4.23.

It is observed that elution time of glycan in UPLC was reduced by four-fold to that of gradient mode HPLC .The resolution and asymmetry obtained for artesunate and DHA-II in UPLC showed comparatively better separation efficiency than HPLC. Theoretical plates obtained for all impurities in gradient mode HPLC were obviously higher than UPLC. The typical chromatograms of final HPLC and UPLC conditions are depicted in figure 4.15.

Table 4.22 Optimized Gradient

Time	Buffer	Acetonitrile	Flow Rate ml/min
0.0 min.	95	5	0.2
2.40 min	75	25	0.2
2.5 min	56	44	0.2
7.5min.	56	44	0.2
10 min.	20	80	0.2
10.10 min.	95	5	0.2
12.0 min	95	5	0.2

Table 4.23 A Comparison of system performance of HPLC and UPLC

Component	Elution Time (min)		Resolution		Tailing Factor		LOQ (µg)	
	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC
Amodiaquine	5.45	2.4	NA	NA	1.52	1.20	0.01	0.005
DHA-I	18.7	5.0	NA	NA	1.01	0.66	0.25	0.022
DHA-II	21.0	6.2	NA	NA	0.77	0.65		
Artesunate	23.64	6.8	3.6	4.8	0.93	0.54	0.14	0.013
Artemisinin	24.4	7.6	2.20	5.4	1.17	0.55	0.13	0.012
Glycan	39.8	10.1	NA	- NA	1.04	0.52	0.05	0.0065

4.3.5. UPLC Method validation

The method validation was carried out as per ICH guidelines [243].

4.3.5.1 System Suitability

The validation study allowed the evaluation of method suitability for routine analysis. The tablets of artesunate and amodiaquine tablets showed presence of impurities like dihydroartemisinin and glycan up to level of 0.2% the system suitability parameters obtained for related substance method are given in table 4.24.

Table 4.24 System suitability report for UPLC related substance method

Component	Resolution (USP)	USP Tailing Factor	USP Plate count
Artesunate	1.0	0.54	6500
Artemisinin	3.20	0.55	5980

4.3.5.2 Specificity

It is the ability of analytical method to measure the analyte response in the presence of its potential impurities and degradents. The specificity of the UPLC method was determined by injecting individual impurity samples, wherein no interference was observed for any of the components. Samples obtained in forced degradation experiment (section 3.3.5.) were analysed by UPLC. Major degradation was observed in acid and base hydrolysis , oxidation of artesunate. The chromatogram of parent sample (tablet) is shown in figure 4.15(a) ,4.15(b). The reduction of artesunate and amodiaquine content resulted in the formation of a major degradent dihydroartemisinin and glycan. Peak purity of artesunate and amodiaquine in the degradent sample was verified by PDA. The peak purity spectra of artesunate and amodiaquine obtained in reductive and oxidative degradation are pure and no co-elusion is observed confirming the peak purity. Forced degradation studies were also performed for artesunate and amodiaquine bulk drug samples to demonstrate the stability indicating power of the newly developed UPLC method. During the forced degradation study a considerable

degradation of drug substance was observed in oxidative and reductive conditions.The chromatograms were checked for the appearance of any extra peak. Peak purity of these samples under stressed condition was verified using a photodiode array (PDA) detector. The purity of the principle and other chromatographic peaks was found to be satisfactory. This study confirmed the stability indicating power of the UPLC method.

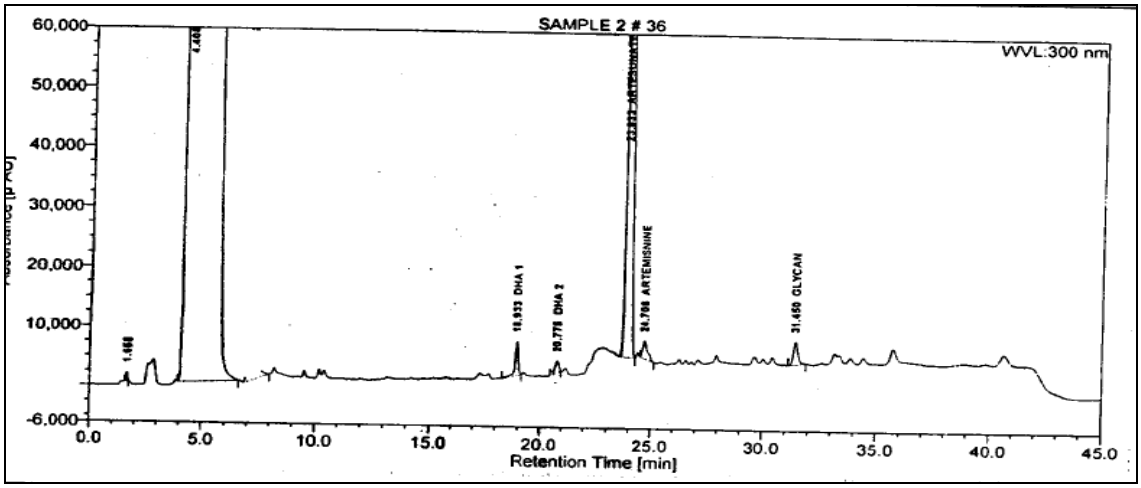


Figure 4.15 (a) Typical chromatogram of HPLC Gradient Method.

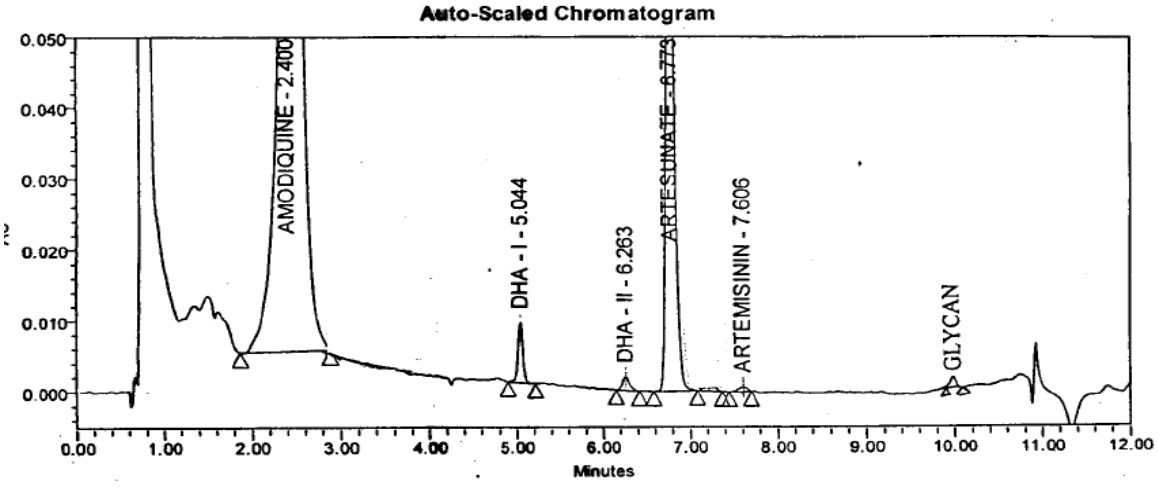


Figure 4.15 (b) Typical Chromatogram of UPLC Gradient Method.

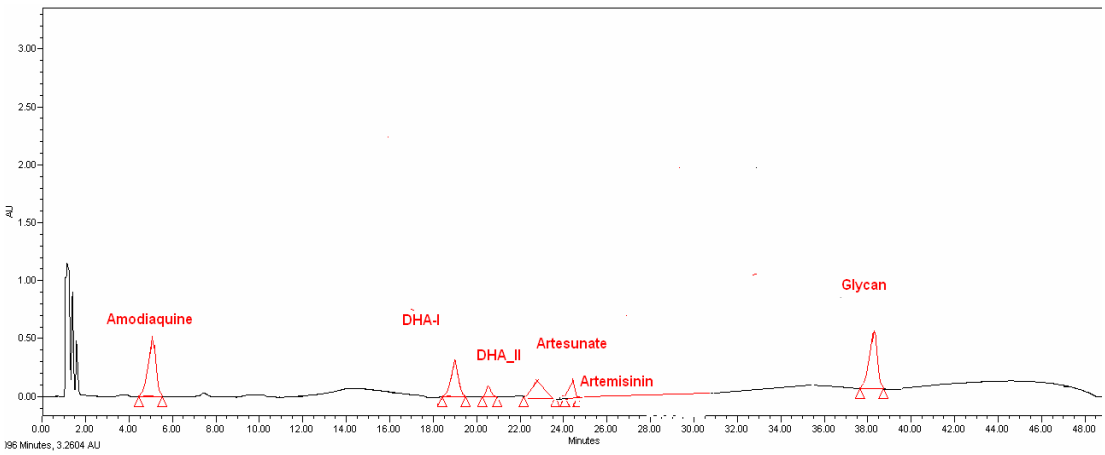


Figure 4.16 (a) Typical Chromatograms of LOQ of artesunate and amodiaquine and its impirities Tablets using HPLC.

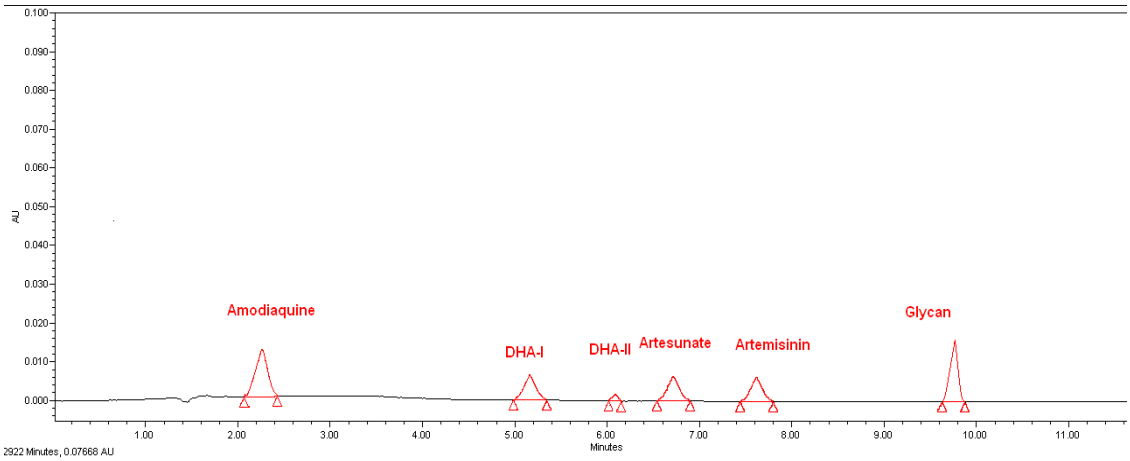


Figure 4.16 (b) Typical Chromatograms of LOQ of artesunate and amodiaquine and its impirities Tablets using UPLC.

4.3.5.3 Precision

The precision of related substance method was examined using six replicate injections of standard impurity solution. The RSDs for diluted standard were found to be 1.98 %and 1.35% for amodiaquine and artesunate respectively. These values are well within the generally acceptable limit of 5%. RSD of responses for impurities dihydroartemisinin ,artemisinin and glycan were well within 10%, confirming good precision of the related substance method.

Table 4.25 System precision

Sr. No.	Diluted artesunate	Diluted amodiaquine
1.	148370	8662
2.	149848	8589
3.	151105	8717
4.	153905	8337
5.	153204	8440
6.	151206	8784
MEAN	151273	8588
SD	2056	170.
RSD	1.35	1.98

Table 4.26 Method Precision

Sr. No	**%Dihydro Artemisinin (NMT 2.0%**	**% Artemisinin (NMT 0.5%)**	**% Glycan (NMT 1.0%)**	**% Max. unknown impurity (NMT 0.2%)**	**% Total impurity (NMT 2.0% excluding Dihydro artemisinin)**
1	2.02	0.49	0.99	0.11	1.59
2	2.02	0.46	0.98	0.13	1.57
3	2.02	0.47	0.98	0.13	1.58
4	2.02	0.48	0.99	0.13	1.6
5	2.02	0.48	0.97	0.13	1.58
6	2.02	0.49	0.99	0.13	1.61
Mean	2.02	0.478	0.98	0.126	1.58
%RSD	2.02	2.44	0.83	6.44	0.92

4.5.5.4 Accuracy

The accuracy of the method was determined for the related substance by spiking known amount of impurities in artesunate and amodiaquine tablet (test preparation) in triplicate at levels 80%, 100% and 120% of the specified limit. The recoveries of impurities were calculated and given in Table 4.27. The percentage recovery of dihydroartemisinin-I , dihydroartemisinin -II ,artemisinin and glycan drug product samples ranged from 98.78 to 102.95 % . Table 4.27 represents the accuracy results.

Table 4.27 Accuracy of Impurities

Level	Amount Added (µg/ml)	Amount Recovered (µg/ml)	Recovery %
At 80% (n=3)			
DHA	40	39.998	99.99
Artemisinin	8	7.592	94.9
Glycan	8	7.712	96.40
At 100% (n=3)			
DHA	50	49.421	98.84
Artemisinin	10	9.931	99.31
Glycan	10	10.591	105.91
At 120% (n=3)			
DHA	60	63.991	106.65
Artemisinin	12	11.981	99.84
Glycan	12	12.288	102.40

4.3.5.5 Limit of Quantification

The limit of quantification (LOQ) values for , amodiaquine ,dihydroartemisinin , artesunate ,artemisinin and glycan were found to be 0.005 µg, 0.022 µg , 0.013 µg, 0.012 µg and 0.0065 µg respectively each of analyte concentration(1000µg/mL) .Figures 4.16 (a) and 4.16 (b) represent the LOQ of all impurities.

4.3.5.6 Linearity

Linear calibration plots for the related substance method were obtained over the calibration range (LOQ to 150%) at six concentration levels in triplicate. For amodiaquine dihydroartemisinin , artesunate, artemisinin and glycan corresponding correlation coefficient (R) are 0.999,0.9991,0.9990, 0.999 and 0.9991 greater than 0.998. respectively with the regression coefficient (R) greater than 0.999. The results showed excellent correlation between the peak area and concentration of impurities Figures 4.17 to 4.21 represent linearity graphs and tables 4.28 to 4.32 represent linearity data of all impuirites and artesuante and amodiaquine.

Table 4.28 Linearity of Amodiaquine

% with respect to test concentration of amodiaquine i.e. 3000 µg/ ml	Concentration in µg/ml	Mean Peak area
0.033	1.0	5042
0.50	15	75636
0.8	24	121029
1.0	30	151273
1.2	36	189527
1.5	45	226809

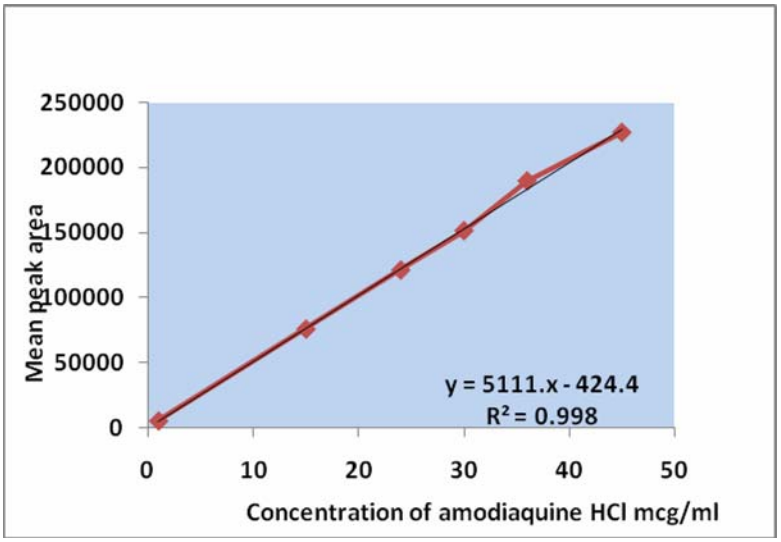


Figure 4.17 Linearity Curve of Amodiaquine.

Table 4.29 Linearity of Artesunate

%With respect to test concentration of artesunate i.e. 1000 µg/ml	Concentration in µg/ml	Mean Peak area
0.23	2.3	2311
05	5	4294
0.8	8	6870
1.0	10	8588
1.2	12	10305
1.5	15	12882

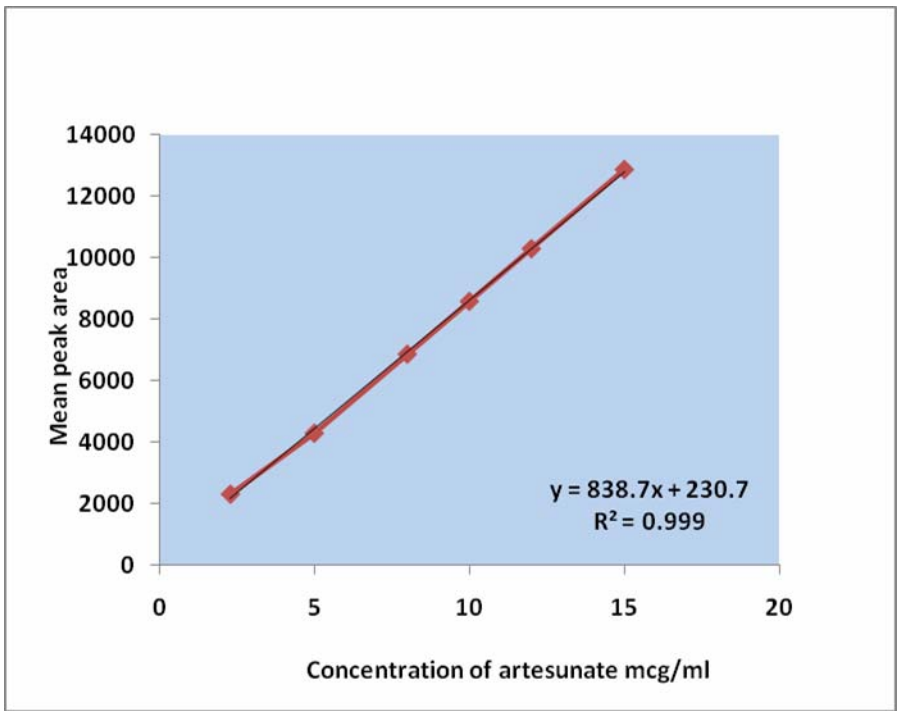


Figure 4.18 Linearity Curve of Artesunate.

Table 4.30 Linearity of artemisinin

% with respect to test concentration of artesunate i.e. 1000 µg/ml	Concentration in µg/ml	Mean Peak area
0.26	2.6	2264
0.5	5	4589
0.8	8	6870
1.0	10	9002
1.2	12	10803
1.5	15	13503

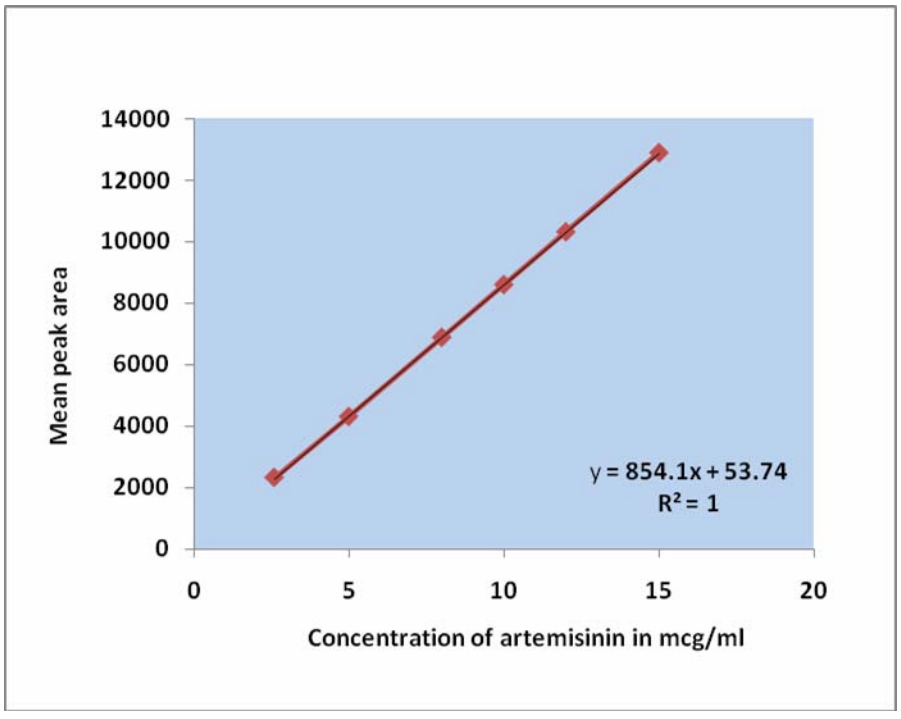


Figure 4.19 Linearity Curve of Artemisinin.

Table 4.31 Linearity of Dihydroartemisinin

% with respect to test concentration of artesunate i.e. 1000 µg/ml	Concentration in µg/ml	Mean Peak area
0.45	4.5	1269
2.5	25	13312
4.0	40	21300
5	50	26626
6.0	60	31951
7.5	75	39939

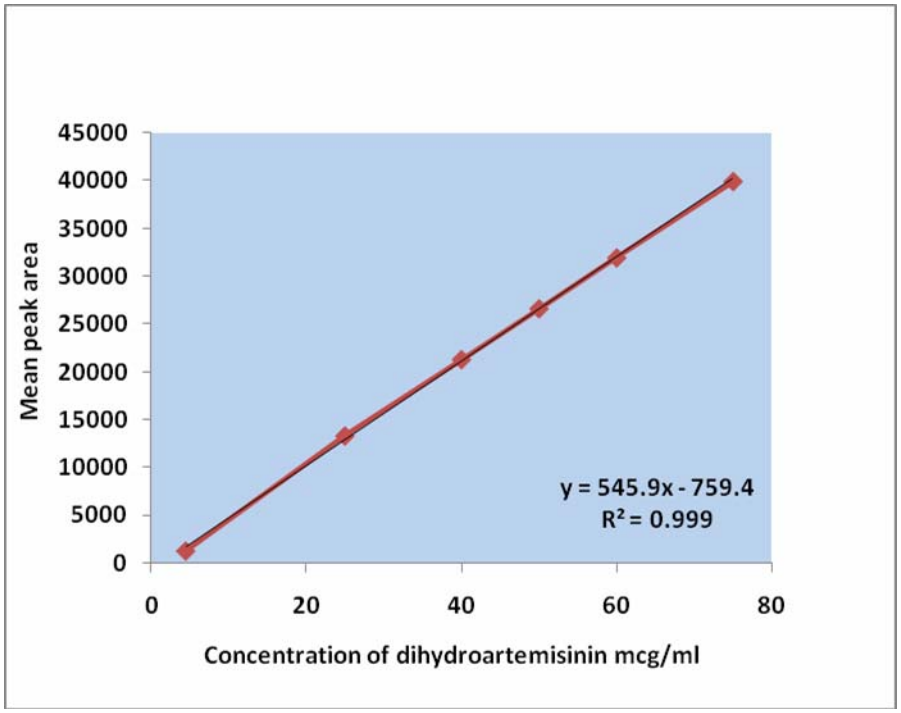


Figure 4.20 Linearity Curve of Dihydroartemisinin.

Table 4.32 Linearity of Glycan

% with respect to test concentration of amodiquine i.e. 1000 µg/ml	Concentration in µg/ml	Mean Peak area
0.13	1.3	1329
0.5	5	58308
0.8	8	84939
1.0	10	116412
1.2	12	139939
1.5	1.5	174924

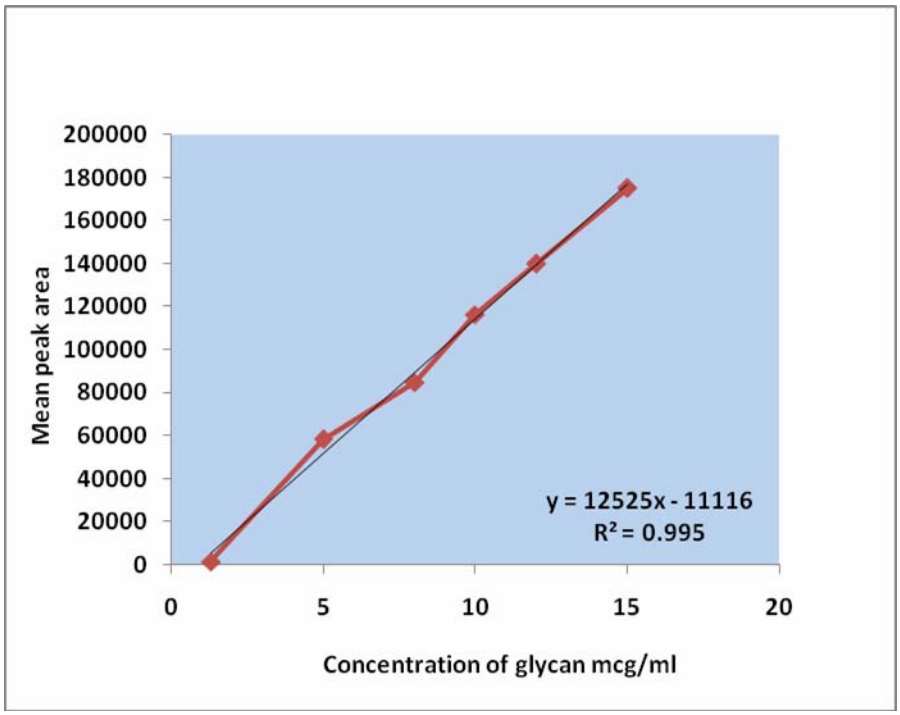


Figure 4.21 Linearity Curve of Glycan

4.3.5.7 The intermediate precision or ruggedness

It was determined on six separate sample solutions prepared from same batch by spiking the related substances at the specification level by a different analyst using different mobile phase and diluents preparation and instrument on a different day with different lot of same brand column. The overall RSD was evaluated and were within the acceptance criterion of NMT 10.0% RSD. The results were presented in table 4.33.

Table 4. 33 Intermediate Precision Study

UPLC –Waters										
A					B					
	%Dihydroartemisinin		%Aretemisinin		% Glycan		%Unk. Imp. Max		% Total Unknown impurities	
Sr. No.	(A)	(B)	(A)	(B)	(A)	(B)	(A)	(B)	A	(B)
1.	2.02	2.15	0.49	0.46	0.99	0.97	0.11	0.11	1.59	1.54
2.	2.03	2.11	0.46	0.48	0.99	0.98	0.13	0.12	1.58	1.55
3.	2.11	2.05	0.47	0.49	0.92	0.97	0.13	0.13	1.52	1.56
4.	1.99	2.15	0.48	0.47	0.98	0.96	0.13	0.11	1.59	1.56
5	1.96	2.1	0.48	0.48	0.97	0.99	0.13	0.12	1.58	1.58
6	2.01	2.17	0.49	0.48	0.99	0.98	0.13	0.13	1.61	1.58
MEAN	2.07		0.48		0.97		0.12		1.57	
SD	0.069		0.10		0.21		0.01		0.02	
%RSD	3.37		2.21		2.26		7.19		1.58	

4.3.5.8 Robustness

In all the deliberately varied chromatographic conditions the chromatogram for system suitability solution for related substance showed satisfactory resolution between artesunate and artemisinin.

4.4 Conclusion

The HPLC gradient method can be succefully transferred on UPLC . Transfer of the method was accomplished by geometrically scaling flow rate, injection volume, and gradient profile. Analysis time was reduced by four fold with an improvement of 64% in the resolution of critical pair of artesunate and artemisinin. Sensitivity of UPLC over HPLC was evaluated by comparison of LOQ values of dihydroartemisinin and glycan obtained in gradient HPLC for both systems. The LOQ concentration for UPLC was found to be 0.005 µg, 0.022 µg , 0.013 µg, 0.012 µg and 0.0065 µg for amodiaquine , dihydroartemisinin ,artesunate ,artemisinin and glycan respectively with RSD 6.89%, 2.71%, 3.98%, 1.36% and 1.2% at injection volume 5 µL. The LOQ concentration for HPLC was found to be 0.05 µg, 0.25 µg , 0.14 µg, 0.12 µg and 0.055 µg for amodiaquine , dihydroartemisinin ,artesunate ,artemisinin and glycan respectively with RSD 6.97.% 7.2% ,5.3% ,and 2.3% at injection volume 50 µL. The almost ten times lower LOQ values with higher precision are attributed towards better sensitivity of UPLC method.

The present work demonstrated the ease of HPLC to UPLC method transferability, and the benefits that can be obtained in any time and resource. It proved that UPLC can significantly increase thoughput with quality results.With variety of column dimensions, scientists have the flexibility to tailer their UPLC separations to the goals at hand.

5.1 Conclusion

Thorough literature survey indicated that some methods have been reported for individual determination or single component determination but there is no work on simultaneous determination of artemether and lumefantrine or artesunate and amodiaquine and their degradants or metabolites like dihydroartemisin in fixed dose combination dosage form. Thus, a new high performance liquid chromatographic method is successfully developed for simultaneous estimation of artesunate and amodiaquine from their fixed dosage form. The method was proved to be stability indicating . The developed method has been validated as per ICH guideline Q2R and regulatory guideline and it can be conveniently used for simultaneous estimation of artesunate and amodiaquine in combination dosage form using dual wavelength. The method was found to be specific, accurate and thus, the proposed HPLC method can be successfully applied for the routine quality control analysis of artesunate and amodiaquine from their fixed dose formulations. Further, the developed method for the determination of artemether and lumefantrine from combined dosage form is simple, fast and accurate. Although there is a difference in the polarity between the two compounds, the simultaneous determination could be achieved successfully. Method was validated for its performance parameters such as specificity (placebo interference), linearity and range, recovery, precision and ruggedness and robustness. .It was concluded that the developed method offers several advantages such as rapid and simple mobile phase, and sample preparation steps, improved sensitivity and comparative short run time makes it specific and reliable reproducible in any quality control setup provided all the parameters are followed accurately for its intended use. The developed flow gradient RP-HPLC method for the simultaneous detection and quantitation of dihydroartemisinin, artemisinin, and unspecified impurities in artemether and lumefantrine tablets is highly sensitive, accurate and precise. This procedure can be easily adopted for the routine quality control analysis of tablet dosage form without any interference from the excipients or each other.

The investigated validation elements showed the method has acceptable specificity, accuracy, linearity, precision, robustness and high sensitivity with the detection limits and quantitation limits ranging from 0.005µg mL^{-1} ,,0.233µg mL^{-1} and 0.016µg mL^{-1} , 0.0065µg mL^{-1} respectively. The method was carried out with commercially available and conventional HPLC equipment with easy sample preparation .It is simple, accurate and reproducible for the quantization of the impurities from the formulation. Developed gradient HPLC method for determination of degradants of artesunate and amodiaquine tablets namely dihydroartemisinin, artemisinin, glycan could be easily transferred to UPLC. That gave four fold reductions in total analysis time . Sensitivity of UPLC over HPLC was evaluated by comparison of LOQ values of dihydroartemisinin and glycan obtained in gradient HPLC for both systems. The almost ten times lower LOQ values with higher precision are attributed towards better sensitivity of UPLC method.

The present work demonstrated the ease of HPLC to UPLC method transferability, and the benefits that can be obtained in any time and resource. It proved that UPLC can significantly increase throughput with quality results. With variety of column dimensions, scientists have the flexibility to tailor their UPLC separations to the goals at hand.

Finally, the developed methods can be utilized for estimation of artemether and lumefantrine , artesunate and amodiaquine and their degradent impurities from different combinations of above drugs in any type of formulations.

Future plan

Further scope of research is that these developed methods can be applied to forensic laboratories, in bioanalytical studies. Concept of two extreme concentration formulations to be analysed by dual wavelength can be applied to other formulations with other antimalarial fixed dose combinations like artesunate and lumefantrine , dihydroartemisinin and piperaquine phosphate and also of other therapeutic categories like anti-hypertensive, anti diabetic , anti inflammatory etc.

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List of Publications

1. M.U.Phadke, V.K.Jadhav, R.K.Jadhav,S.S.Dave, D.S.Patil., Simultaneous RP-LC Determination Of Artesunate and Amodiaquine in Pharmaceutical Preparations. Chromatographia. 2008, 68:1003-1007.

2. M.U.Phadke, V.K.Jadhav, R.K.Jadhav, D.S.Patil., A report on Forced degradation studies of artesunate and amodiaquine tablets. Analytical Chemistry an Indian Journal.2008, 7(12):857-861.

3. M.U. Phadke, V.K.Jadhav, R.K.Jadhav, Y.K.Bansal, D.S.Patil.,Simultaneous RP LC Determination of antimalarial in fixed dose combination pharmaceutical preparations. Separation Science asia pacific.2009,1(5) :18-25.

4. M.U.Phadke, V.K.Jadhav, R.K.Jadhav, Y.K.Bansal, D.S.Patil.HPLC study of related substances in artemether and lumefantrine Tablets . Analytical Chemistry an Indian Journal. 2009, 8(2):179-188.

5. M.U.Phadke, V.K.Jadhav, R.K.Jadhav, Y.K.Bansal, D.S.Patil. Development And Validation Of Stability Indicating UPLC Method For Determination Of Related Impurities In Artesunate And Amodiaquine Fixed Dose Tablets.-Paper accepted by Separation Science asia pacific. – In Press.

Biography of Dr. Vivek Jadhav

Dr. Vivek Jadhav is CEO of VAB pharma Mumbai. He completed his Bachelors of Science (B.Sc.) and Master of Science (M.Sc.) from Mumbai University, and Ph.D. from UDCT Mumbai. He has done his Post Doctoral from University of Manchester Science and Technology (UMIST). He has more than 23 years of research experience. He has published research articles in renowned journals and presented papers in conferences in India. He is an expert member of technical committee of IP and IDMA. He is also a prominent speaker in various Industrial seminars.

Biography of Manisha Phadke

Mrs.Manisha Phadke has completed her Master of Science . (M.Sc.- Chemistry) from S.N.D.T. Mumbai University, in the year 1994. She has been working in Ipca Laboratories Ltd. in Analytical Research since 1998 and perusing her Ph.D. from Bits Pilani since 2006 . She has published research papers in renowned journals and also filed patents. She has more than 15 years of Industrial research experience and excels in leading a team of research scientists.