KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI.

COLLEGE OF SCIENCE,

DEPARTMENT OF CHEMISTRY

METHOD DEVELOPMENT FOR THE SIMULTANEOUS DETERMINATION AND QUANTITATION OF ARTEMETHER AND LUMEFANTRINE IN ANTI MALARIAL TABLET FORMULATION USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH UV DETECTION

BY

LATIFATU ALHASSAN ABUBAKAR,

APR 2015

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Chemistry, KNUST. Any assistance obtained has been duly acknowledged.

This work has not been submitted for any other degree.

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ABSTRACT

Artemether lumefantrine has been used as a first line treatment for uncomplicated *Plasmodium falciparum* malaria in Ghana since 2004. In this study, a High Performance Liquid Chromatography(HPLC) method was developed and validated. The developed method was used to simultaneously determine the quantity of artemether and lumefantrine in various fixed dose tablets obtained from pharmaceutical and chemical shops in the Kintampo North Municipality. The optimized chromatographic conditions were a Jasco HPLC system equipped with C18 reverse phase column (Ultracarb 3µ ODS (20)) with methanol and phosphate buffer (72:28) pH 2.8 as the mobile phase. The flow rate was 2.7ml/min and detection was by means of a UV detector set to 222nm. The isocratic mode of elution was employed. The retention time of lumefantrine was 5.22 ± 0.19 minutes. And that for artemether was 4.19 ± 0.22. The method was validated by evaluation of different parameters such as accuracy, precision, linearity, ruggedness and robustness. The percentage recovery for artemether and lumefantrine ranged between 99.18-100.19 and 99.96-100.07, respectively. Six brands of artemether-lumefantrine fixed-dose combination tablets (two local and four foreign) from selected chemical shops and pharmaceutical shops in the Kintampo-North Municipality were analyzed. Of the six brands of artemether lumefantrine fixed-dose combination tablets analyzed, all passed with respect to their artemether and lumefantrine content using the developed HPLC method. The percent recovery for the local brands ranges from 93.5 to 99.2% and from 91.3 to 97.2% for artemether and lumefantrine respectively. And from 92.05 to 105.0% and 95.8 99.9% for the foreign brands for artemether and lumefantrine respectively, which complies with the International Pharmacopoeia range of (90-110).

DEDICATION

I dedicate this work to my dear husband, Basala Alhassan, my parents, Alhaji Alhassan and Hajia Salamatu, my uncle, Anas Abdul Rahman and my sisters, Shetu Dauda and Rafia Alhassan

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I would like to thank the Almighty Allah for seeing me through this course successfully.

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My gratitude also extends to all my colleagues, the teaching and non-teaching staff of the Department of Chemistry at the Kwame Nkrumah University of Science and Technology.

May God bless all those who supported and encouraged me throughout my programme of study.

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ABBREVIATIONS

ACT: Artemisinin-based Combination Therapy

AM – LU: Artemether-Lumefantrine

AM: Artemether

API: Active Pharmaceutical Ingredient

BP: British Pharmacopoeia

DHA: Dihydroartemisinin

HPLC: High Performance Liquid Chromatography

ICH: International Conference on Harmonization

IP: International Pharmacopoeia

LU: Lumefantrine

LOD: Limit of Detection

LOQ: Limit of Quantitation

ODS: Octadecylsilane

RP-HPLC: Reverse Phase HPLC

RSD: Relative Standard Deviation

SALMOUS: Standards for Articles Legally Marketed Outside the U.S

SD: Standard Deviation

TLC: Thin Layer Chromatography

USP: United States Pharmacopoeia

UV: Ultra-Violet

WHO: World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

Malaria continues to be one of the major public health problems in Africa, Asia and Latin America.In 2010, 219 million cases of malaria and an estimated 660 000 deaths were recorded, out of which 90 percent occurred in Africa (World Malaria Factsheet, 2012).

Plasmodium falciparum malaria is estimated to be the direct cause of 500 million cases and over one million deaths per year, mostly in women and children under the age of 5 years(Guerra et al., 2008; Lewiston *et al*, 2008). Africa has the highest endemicity of malaria where 0.35 billion people are at a high risk of getting malaria(Hay et al., 2009).

In Ghana malaria accounts for more than 60% of under-five hospital admissions, and 8% of under-five mortality and 9.2% of maternal deaths (Malaria Case Management in Ghana: Training Manual for Pharmacists, 2010).

Malaria control requires an integrated approach made up of prevention including vector control and treatment with effective antimalarial drugs (Reyburn, 2010).

Plasmodium falciparum has become resistant to many commonly used antimalarial drugs such as chloroquine and sulfadoxine–pyrimethamine. In light of this, the WHO has recommended that all antimalarials should consist of a combination of an Artemisinin derivative with a co-drug, such as lumefantrine, amodiaquine, piperaquine or mefloquine (Artemisinin-based combination therapy (ACT)for use as first-line treatment against malaria. This class of antimalarial drugs is now first-line policy inmost malaria-endemic countries (WHO, 2006). ACTs are the most effective antimalarial medicines available today. These therapies combine two active ingredients with different mechanisms of action (World Malaria Report, 2012).

Artemisinins form the most important class of antimalarials currently available, particularly because they are effective against parasites resistant to almost all the other classes (Krishna et al., 2004).

The recent emergence of ACT-resistant *P. falciparum,* on the Thailand/Cambodia border (Dondorp et al., 2009),is of very great concern, especially as there is evidence that drug-resistant falciparum malaria has spread from Asia to Africa (Roper *et a*l., 2004).

Artemisinin derivatives are extremely important antimalarial medicines and their rapid action and lack of side effects have created significant demand in endemic areas. These characteristics, along with a relatively high cost, make them particularly attractive to counterfeiters and spurious manufacturers, who have gone to great lengths to deceive patients, using small amounts of ineffectual bitter chloroquine, copying the blister pack design, and even providing fake holograms on the package (Muchoh et al., 2001).

Furthermore the presence of counterfeit/substandard medicines in the market undermines public confidence in pharmaceutical products and may result in a reduced uptake of potentially lifesaving medicines (Dondorp *et al*., 2009).

There are two main categories of poor quality medicine. Counterfeit, which are deliberately and fraudulently mislabeled with respect to identity and/or source (WHO, 2008), and substandard medicines which are genuine medicines produced by manufacturers authority which do not meet quality specifications set by national standard (Ochola*et al.*, 2006). Substandard medicines frequently, and counterfeits occasionally, contain sub-therapeutic amounts of active pharmaceutical ingredients (API) and/or may show suboptimal release of API (dissolution), exposing parasites to sub-lethal concentrations of API(s)(Caudron *et al*., 2008). However, the percentage API in genuine medicines may also be reduced after manufacture if they are degraded by extremes of temperature and humidity(Keoluangkhot *et al*., 2008).

Antimalarial drug resistance is a major concern for the global effort to control malaria. *P. falciparum* resistance to Artemisinins has been detected in four countries in South East Asia: in Cambodia, Myanmar, Thailand and Viet Nam. There is an urgent need to expand containment efforts in affected countries. For now, ACTs remain highly effective in almost all settings, so long as the partner drug in the combination is locally effective (World Malaria Report, 2012).

The prevention and treatment of malaria has been investigated for hundreds of years; and continues up to the present day, since no effective malaria vaccine has yet been developed and many of the existing antimalarial drugs including amodiaquine and sulphadoxine pyrimethamine are becoming less sensitive to the *Plasmodium* parasite (Willcox et al., 2011). Methods for the assay of Artemether-lumefantrine formulation employ various analytical techniques utilizing the properties of the two APIs. One of such technique is the HPLC utilizing Photo diode array detector, mass spectrometer etc.

1.2 Problem Statement

Universal access to affordable healthcare and quality medication is a fundamental human right that remains elusive to large segments of the population in developing countries (Macunda et al., 2012).There is concern about increasing infiltration into the markets by substandard and fake medications against malaria and other life-threatening diseases in developing countries. This is particularly worrying with regard to the increasing resistance of *Plasmodium falciparum* to affordable anti-malarial medications, which has led to a change to more expensive drugs in most endemic countries(Tipke et al., 2008).

It is estimated that more than 10% of the globally traded medicines are counterfeits (WHO, 2006). In developing countries, where regulatory and control mechanisms are weak, people are at highest risk of purchasing substandard medications (Cockburn et al., 2005).

Pharmaceutical products are an attraction to illegal trade, especially in developing countries. They are easily transportable, have high value per unit, and most importantly, their quality cannot be assessed readily by lay persons or even experts without the aid of a quality testing laboratory (The World Bank: Pharmaceuticals, 2005).

1.3 Justification

Currently, there are HPLC methods for the assay of Artemether in finished pharmaceutical products (FPP) (Cesar and Pianetti, 2009) as well as for lumefantrine analysis (Cesar et al., 2008).

Only a few HPLC methods were reported for the quantitative determination of Artemether and lumefantrine in fixed combination anti-malarial products(Cesar et al., 2008)(Phadke et al., 2009);(Narayankar et al., 2010); Sunil et al., 2010;(Kalyankar and Kakde, 2011).

Existing methods are time consuming, not readily available, not sufficiently reliable and not cost effective. There is therefore the need for a new analytical methods are needed that will eliminate the shortfalls listed above and also assist in the easy determination and quantitation of APIs.

Hence this study seeks to add to the existing methods, a method that is rapid, economical, precise and accurate for the assay of Artemether and Lumefantrine..

1.4 Aims and Objectives

1.4.1 Main Objectives

The main objective of the study is to:

- Develop an HPLC method that can simultaneously determine and quantify the APIs in Artemether Lumefantrine anti-malarial formulation.

1.4.2 Specific Objectives

- To analyze Artemether and Lumefantrine reference standards for purity
- To develop an HPLC method for simultaneous determination of the Artemether and Lumefantrine
- To determine the quantities of the APIs using the developed method
- Apply the method developed to various Artemether Lumefantrine anti-malarial formulations.

CHAPTER TWO

2.0 Review of Literature

2.1 Malaria

Malaria is caused by a parasite called Plasmodium, which is transmitted via the bites of infected mosquitoes. In the human body, the parasites multiply in the liver, and then infect red blood cells(World malaria Fact Sheet, 2012). Human malaria results from infection with *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale* or *Plasmodium malariae*. Occasional infections with monkey malaria parasites, such as *Plasmodium knowlesi,* also occur (WHO guidelines for treatment of malaria, 2010). Plasmodium falciparum is among the most prevalent species infecting humans killing more than two million people every year (Shelty, 2012).

Key interventions to control malaria include prompt and effective treatment with artemisinin-based combination therapies, use of insecticidal nets by people at risk and indoor residual spraying with insecticide to control the vector mosquitoes (World malaria Fact Sheet, 2012).

2.2 Anti Malarials

In many parts of the malaria endemic areas, particularly the African region, the only effective method of preventing the mortality and reducing the morbidity caused by the disease is through the use of antimalarial drugs (Olaniyi, 2005).

2.2.1 Classification of Anti-malarials

Antimalarial drugs are designed to prevent or cure malaria. In other words they are drugs which are used for prophylaxis, treatment & in the prevention of malaria.

Anti-malarial are categorized according to either their therapeutic action against the different life cycle stages of the parasites in human hosts or the chemical structure. The parasite stages

that the anti-malaria targets include the sporozoites (8-aminoquinolines) schizonts (quinine) and gametes (artemisinin).

Based on chemical structure, anti-malarial are classified as:

- Aryl Amino Alcohols – Quinine, Quinidine, Mefloquine, Halofantrine, Lumefantrine.
- 4-aminoquinolines – Chloroquine, Amodiaquine
- Folate synthesis inhibitors – Sulphonamides, Biguanides like Proguanil and Chloroproguanil
- 8-aminoquinolines – Primaquine
- Peroxides – Artemisinin derivatives and analogues
- Antimicrobials – Tetracyclines, Clindamycin, Azithromycin, Fluoroquinolones
- Naphthoquinones – Atovaquone
- Iron chelating agents – Desferrioxamine

Currently, the anti-malarial available include the Quinoline based antimalarial agents, which are structural derivatives of quinine, egs are mefloquine, amodiaquine, halofantrine, Folates antagonists, which inhibit the synthesis of parasitic pyrimidines, and thus parasitic DNA. Eg.Sulfadoxine and the Artemisinin and its derivative, which include arthemeter, artheether, dihydroartenisinin, artesunate.

An ideal antimalarial drug should have the following characteristic;

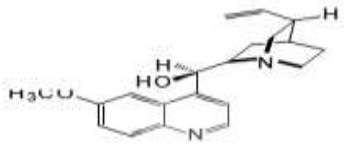
1. Rapidly relieve symptoms of the disease.

2. It should be harmless to the patient and have no unpleasant side-effects

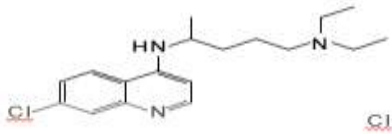
3. It should preferably destroy all the stages of development of plasmodium species including the gametocytes

4. It should be economically cheap and easy to administer.

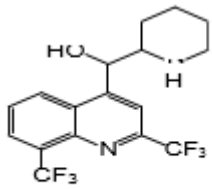
Figure1Structure of some Quinoline based Antimalarials



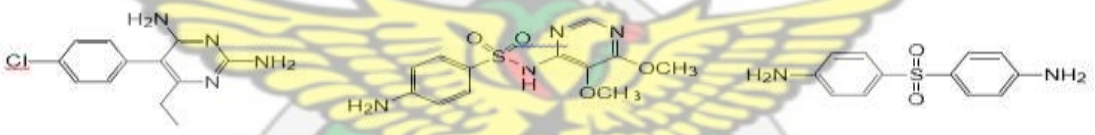
Quinine



ChloroquineAmodiaquine

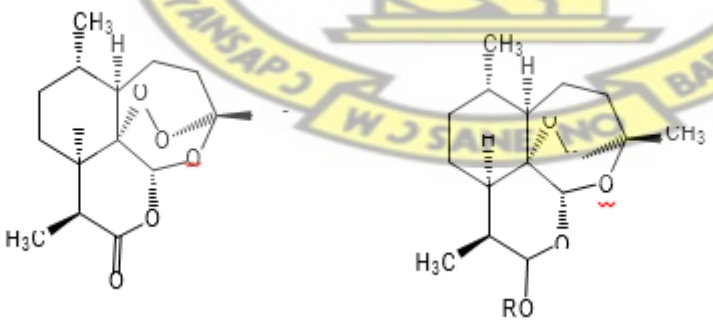


Mefloquine



Pyrimethamine Sulfadoxine Dapsone

Figure 2 Structure of Artemisinin and its derivatives



R=H, Dihydroartemisinin

R=Me, arthemethe

R=Et, artheether

R=OCO(CH2)2COONa, Artesunate.

R=OCH2 (*p*-C6H4)COOH, Artelinic acid

2.3 Artemisinin based Combination Therapies

The Artemisnin-based Combination Therapies (ACTs), which are recommended by the WHO, have become the main-stay of malaria treatment. Artemether-lumefantrine is the first fixed-dose ACT regimen to be manufactured under Good Manufacturing Practice conditions, and is the most widely adopted ACT regimen used in malaria control programs (Adjei *et al.*, 2009).Artemisinin-based combinations offer a new and potentially effective way to counter drug resistance (Atemnkeng, De Cock *et al*., 2007).In spite of increasing popularity in the use of artemisinin based therapies, the mechanism of action of these sesquiterpene lactone endoperoxides has eluded researchers due to its controversial nature(Posner *et al.,* 2004**).**

2.3.1 Artemisinins

Artemisinins are isolated from the plant *Artemisia annua,* or sweet wormwood. Artemisinin and its derivatives are powerful medicines known for their ability to swiftly reduce the number of *Plasmodium* parasites in the blood of patients with malaria(WHO, 2012).Artemisinin extracted from the plant can be chemically converted into several active derivatives. Artemisinin derivatives such as artesunate, artemether, and dihydro-artemisinin (DHA) are extremely potent antimalarial that act rapidly against both the parasite‘s asexual and sexual stages, which could potentially help to reduce the rate of malaria transmission (Sutherland *et al*., 2005).

In addition, artemisinin-derived drugs have been shown to be highly efficacious against parasites resistant to other antimalarial drugs (Olumese, 2006).

Artemisinins have several advantages over existing antimalarial drugs which are mentioned below. Firstly, the presence of endoperoxides clear the peripheral blood of parasites more rapidly than other available drugs do. Moreover, resistance to the endoperoxides has not yet been developed despite widespread clinical use (Meshnick*et al.*, 1996). Finally there is little or no cross-resistance with other antimalarial drugs.

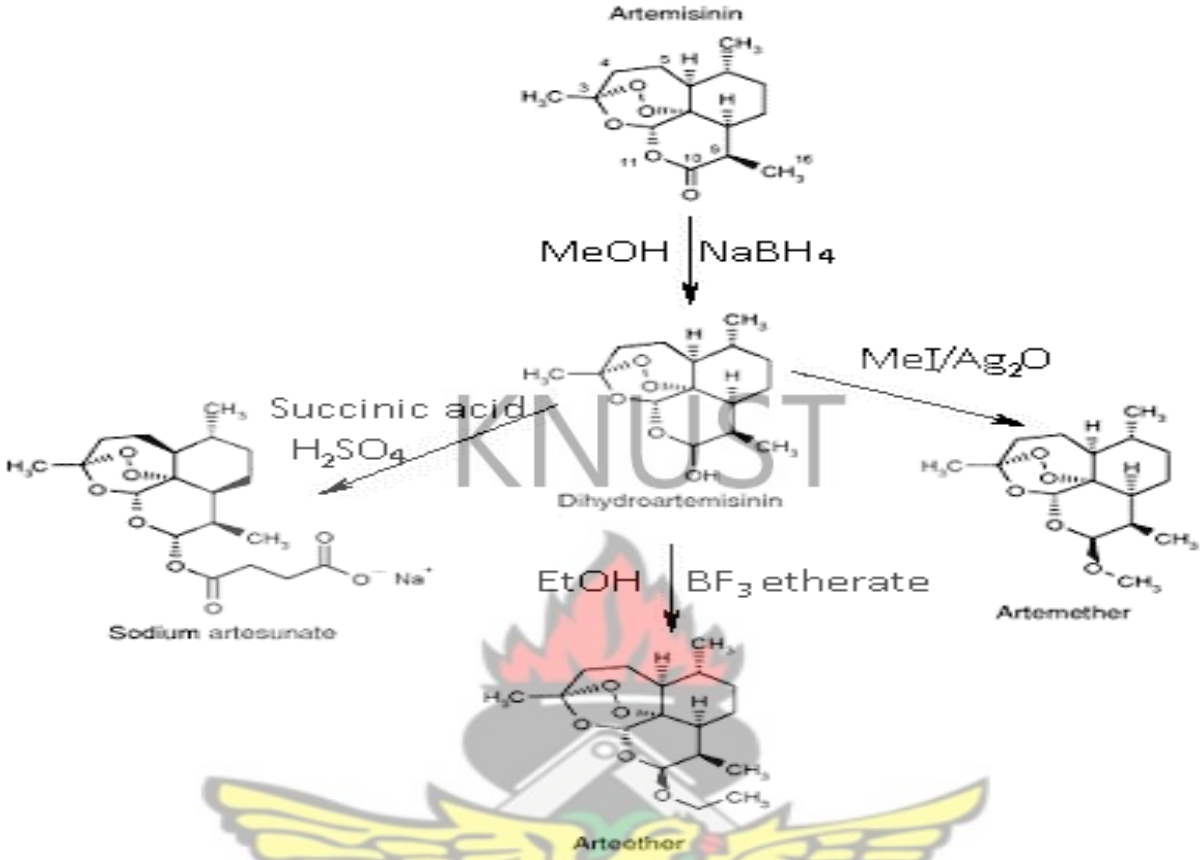
Therefore, the WHO recommends their use in combination with long acting antimalarial drugs such as lumefantrine or mefloquine to manage drug resistance, recrudescence, and noncompliance (Gautam*et al.*, 2009).

2.3.2 Synthesis of Artemisinin Derivatives

Resistance of malaria parasites to the traditional treatments has led to extensive work in discovering newer artemisinin analogs and derivative (Vennestrom*et al.*, 2004). Artemisinin itself is a highly crystalline compound that does not dissolve in oil or water and so can only be given by the enteral route (Haynes *et al*., 2005). This resulted in the need to synthesize derivatives with better properties for administration. The parent compound has been chemically modified at the C10 position to produce artesunate, artemether, arteether, dihydroartemisinin, and artelinic acid. These compounds have variously been formulated for oral, rectal, and parenteral administration. Artemisinin structure, which includes an endoperoxide bridge (C-O-O-C), is unique among antimalarial drugs. Dihydroartemisinin is the reduced lactol derivative of artemisinin, and the semi-synthetic derivatives artemether, arteether, artesunate and artelinate are ethers or esters of the lactol (Meshnick*et al*., 1996).

Artemisinin is reduced with sodium borohydride to produce dihydroartemisinin as a mixture of epimers (Olaniyi, 2005). The mixture is treated with methanol in acidic medium to produce Artemether. Artesunate is the esterification product of the dihydroartemisinin produced (Chekem and Wieruck,i 2006).

Figure 3 Synthesis of Artemisinin derivatives



2.3.3 Artemether

Artemether is chemically [3R-(3R,5aS,6S,8aS,9R,10R,12S,12aR)]decahydro-10-methoxy-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin with its molecular formula $C_{16}H_{26}O_5$ and molecular weight of 298.4g/mol. It is practically insoluble in water; very soluble in dichloromethane and acetone. It is freely soluble in ethyl acetate and dehydrated ethanol.

Artemether is a methyl ether derivative of Artemisinin, which is a peroxide lactone isolated from the Chinese anti-malarial plant *Artemisia annua* (Martindale, 2009).

2.3.3.1 Mechanism of action of Artemether

Representing a new class of antimalarial agents, Artemether, like other artemisinin derivative is a sesquiterpenetrioxane lactonewhose endoperoxide bridge is essential for the antimalarial activity. The endoperoxidepharmacophore alone stimulates the development of several different classes of totally synthetic endoperoxides (Vennerstrom*et al.*, 2004). Artemether, a semisynthetic chiral acetal derivative of Artemisinin, interferes with parasitic transport proteins, produces disruption of mitochondrial functions, inhibits angiogenesis and modulate host immune function. By so doing artemether reduces parasite biomass by 10, 000-fold per reproductive cycle in two days (Byakika*et al.*, 2010).

2.4 Aryl Amino Alcohol Antimalarials

The aryl amino alcohol group of antimalarial include, quinine, quinidine, mefloquine, halofantrine, lumefantrine and chloroquine. Their use as antimalarials has been of great importance. Biochemical studies suggest that their antimalarial effect involves lysosomal trapping of the drug in the intra-erythrocytic parasite, followed by binding to toxic haemin that is produced in the course of haemoglobin digestion. This binding prevents the polymerization of haem to non-toxic malaria pigment (WHO, 2001).

2.4.1 Lumefantrine

Lumefantrine is a dichlorobenzylidine derivative effective for the treatment of various types of malaria. The antimalarial agent is active against multi-drug resistant strains of *Plasmodium falciparum*. In combination with artemether, the drug is also used for the treatment of uncomplicated *falciparum* malaria (Ezzet *et al.*, 2000).

Lumefantrine is chemically 2-Dibutylamino-1-[02,7-dichloro-9-(4- chlorobenzylidene)-9H-fluoren-4-yl]-ethanol with molecular formula $C_{30}H_{32}Cl_3NO$.It is practicallyinsoluble in water; freely soluble in dimethylformamide and ethyl acetate. It is soluble in dichloromethane and

slightly soluble in ethanol and methanol. Lumefantrine (benflumetol) is a 2,4,7,9-substituted fluorine (2,3-benzindene) (The Merck Index, 2001). It is a highly lipophilic flourene derivative and a Biopharmaceutical

Classification System Class II drug which is an important agent in the treatment of falciparum malaria (Ashley *et al.*, 2007)

Lumefantrine is proved to possess marked blood schizontocidal activity against a wide range of *Plasmodium*, among them chloroquine-resistant *Plasmodium falciparum* (Faladee*t al*,2005).

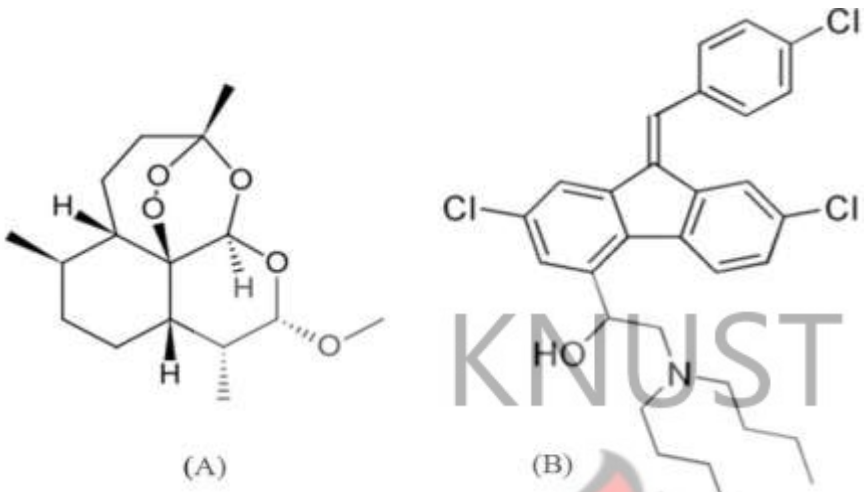
2.4.2 Mode of Action of Lumefantrine

Lumefantrine has primary action as blood schizontocidal and secondary action as inhibition of nucleic acid and protein synthesis within the malaria parasite, thus having a longer duration of antimalarial drug action (Ferreira *et al.*, 2008).

Investigations involving aryl-methanol compounds have suggested the coordination of the Iron centre of Haem (Fe(III)PPIX) and related porphyrins by the alcohol functionality, indicating the structural activity relationship of the anti-malarial drug lumefantrine (Villiers and Egan, 2009). Hence, structural analogues of lumefantrine also possess marked anti-malarial effects. Halofantrine, an aryl amino alcohol analogue of lumefantrine, is also an anti-malarial drug, but is known to be potentially cardiotoxic (Traebert *et al.*, 2004).

Lumefantrine, a racemic mixture of synthetic fluorine, interferes with the conversion of heme, (a toxic intermediate produced during haemoglobin break down), to a non-toxic hemozoin. Accumulation of the heme and free radicals results in the parasite's death (Mwesigwa*et al.*,2010).

Figure 4 Chemical structure of AM (A) and LU (B)



2.5 General Methods for the Assay of Artemether and Lumefantrine

Methods for the assay of Artemether-lumefantrine formulation employ various analytical techniques utilizing the properties of the two APIs.

2.5.1 HPLC

In the modern pharmaceutical industry, high-performance liquid chromatography (HPLC) is now the most widely used of all the analytical separation techniques and an integral analytical tool applied in all stages of drug discovery, method development and production.

Chromatographic separation are based on a forced transport of the liquid (mobile phase) carrying the analyte mixture through the porous media and the differences in the interaction at analytes with the surface of this porous media resulting in different migrations at analyte with the surface of this porous media resulting in different migration times for a mixture of components (Kazakevich and Lorutto., 2007).

HPLC is an analytical technique used to separate, identify and quantify specific compounds. Separation is achieved by molecular interactions with a particular matrix, while identification and quantification is achieved by the retention times and spectrophotometric properties of the selected compound. HPLC can be coupled to a variety of detectors, such as the single wavelength ultraviolet/ visible absorbance, fluorescence, photodiode array, electrochemical or refractive index. HPLC also allows coupling to a mass spectrometer (liquid chromatography-mass spectrometry [LC-MS]).These techniques necessitate a reference standard to determine the amount of API present in a sample.

HPLC can be used for both qualitative and quantitative analysis. In Qualitative analysis, the retention time of the compounds are made use of. In quantitative analysis on the other hand, the area under the peak, which is proportional to the concentration of the compound is used.

Within the past 8 years, HPLC has become the predominant method for separation and quantitation of Artemether and Lumefantrine. Of course a number of factors are responsible for this popularity:

1. Ready availability of rugged commercial equipment
2. Considerable standardization of equipment and techniques
3. Appropriate lower limits of detection and simple quantitation
4. High accuracy
5. High specificity
6. High precision

2.5.1.1 HPLC Chromatograph

A Classical HPLC Chromatograph consists of the following main components:

Solvent Reservoirs**:** Storage of sufficient amount of HPLC solvents for continuous operation of the system. Could be equipped with an online degassing system and special filters to isolate the solvent from the influence of the environment (Rasmussen and Ahujah, 2007).

Pump**:** This provides the constant and continuous flow of the mobile phase through the system. Majority of the modern pumps allow controlled mixing of different solvents from different reservoirs (Rasmussen and Ahujah, 2007).

Injectors**:** This allows an introduction or injection of the analytes mixtures into the stream of the mobile phase before it enters the column. It generally known that the larger the sample size the more difficult it is to effect resolution. Thus the injector type affects column performance.

Commonly used sample injectors include; the syringe, valve, and automated injectors.

I. The syringe injector

With this type of injectors the pump is put off temporarily and the injection done at atmospheric pressure. Even though it is a very simple technique, it is beset by problems practically. Particles from the Teflon disk may plug the column after repeated injections causing high back pressure. It is also difficult to achieve reproductive results.

II. The valve injector

This consist of stainless steel and Teflon block drilled to provide two alternate paths for solvent flow and each selected by rotating a valve. In 'fill' position, the solvent flows through one path directly unto the column and the analyte is loaded into the stainless steel loop through another path in the 'inject' position. However, fixed volume (20 - 1000 µL) of analyte solution is washed by the mobile phase from the loop into the column. Results obtained using this injector type are reproducible and the injector can be automated.

III. Automated injector

This injector type allows the operation of the HPLC without operator assistance. This form of operation becomes handy when attempting to optimize chromatographic conditions for sample analysis and also for the analysis of a large number of samples. It is most effective when coupled with an automated data handling system (Beckett and Stenlake, 1998).

Most modern injectors are auto-samplers; which allow programmed injections of different volumes of samples that are withdrawn from the vials in the auto-sampler tray (Kazakevich and Lorutto, 2007).

Column**:** This is the heart of HPLC systems and it actually produces a separation of the analytes in the mixture. A column is the place where the mobile phase is in contact with the stationary phase, forming an interface with enormous surface (Kazakevich and Lorutto,2007). This is usually made of stainless steel and is normally straight with a length ranging from 10-100 cm and 1-5 mm in internal diameter (ID). Analytical columns are available in standard sizes of 25 cm length and 4.5 cm internal diameter (Gardner, 1977; Dolan, 1991). Also available are micro-bore, semi-preparative and preparative columns. The efficiency of liquid chromatography depends to a large extent on the nature and type of packing materials. Also of importance is the surface chemistry and porosity of the packing material. How well the packing is done and the ability of the packing material to withstand high pressure, also affect column stability and efficiency.

A number of materials are used for packing of columns depending on the type of chromatography being undertaken e.g. rigid solids are the most widely used. These solids are usually silica based. The stationary phase may be the packing material itself or a liquid coated or chemically bonded on the packing material. Generally however the most widely used form

of the packing material are bonded surfaces e.g. Octadecylsilane (ODS) which generally can stand pressure. Other materials available are hard and soft gels used for size exclusion chromatography and gel filtration. These unfortunately cannot withstand high pressure and thus have limited use (Spruce *et al.,* 1998). Pellicular Packing is achieved by coating stainless steel or glass beads with stationary phase. The stationary phase can be either be silica or its derivatives, ion exchange material or alumina. Irrespective of the type of packing material, the particle size is between (5-10 µm). The micro particles have greatly improved the efficiency, reproducibility and life span of columns.

More recent HPLC systems have pre-columns. These, though optional, serve useful purposes. These columns are usually short (5-10 cm). In columns where the stationary phase consist of a liquid coated on solid support, contact with the mobile phase may slowly dissolve the stationary phase. The pre-column contains a high proportion of liquid phase compared with column proper. Thus it saturates the mobile phase and retards dissolution. It also aids in the trapping of particulate matter and retain substances which would be irreversibly absorbed on the analytical column (Spruce *et al.,* 1998). Most of the chromatographic development in recent years favored the design of many different ways to enhance this interface contact (Kazakevich and Lorutto, 2007).

Detector**:** This is a device for continuous registration of specific physical (sometimes chemical) properties of the column effluent (Kazakevich and Lorutto, 2007). That is to say that detectors in liquid chromatography simply monitor the concentrations of solutes in the mobile phase as the mobile phase leaves the column. Most often in liquid chromatography the mobile phase used has similar properties compared with the solute thus making detector selection difficult. The detectors used in liquid chromatography employ one of the following mechanisms:

- Eliminating the solvent before the sensor (flame ionization detector). This ensures that only the solute is detected by the sensor.
- Measurement of one of the several bulk physical properties by means of careful reference compensation and temperature control (refractive index detectors)
- Finding solute properties for which the mobile phase does not interfere.

Based on the above, detectors can be grouped into two categories:

i. Bulk property detectors which changes in the overall physical property of the mobile phase.
ii. Solute property detectors which are sensitive to physical properties of the solute which are not exhibited by the mobile phase to any extent e.g. UV-absorption and radioactive detectors.

Bulk property detectors are generally less sensitive compared to solute property detectors. The criteria for choice of detectors are:

- the noise level of the detector
- the sensitivity
- detectors linearity
- ease of operation
- solute properties

Noise**:** This is defined as the variation in output signal of the detector which cannot be attributed to the solute passing through the cell. It may be a result of temperature fluctuation, line voltage changes or electronic consideration in the instrument. Noise may fall into three categories:

- Short term noise; due to variation on the recorder tracing that tends to widen the width of the trace and appears as a 'fuzz' on the baseline.
- Long term noise; this may appear as peaks or valleys on the baseline.
- Drift; this noise variation may due to a steady movement up or down scale (GNDP, 1999).

Sensitivity**:** The best detector for analysis of a particular solute is the detector that is capable of detecting the smallest solute concentration. This produces a peak that is double the value of the noise.

Linearity**:** For a detector to function effectively, it is desirable that the signal output is linearly proportional to solute concentration. All recommended detectors for liquid chromatography approach this goal although none is perfectly linear over its entire range. In practice, it is recommended that a detector is used within its linear range. Data handling is primarily based on linearity. The chromatogram may be considered as a plot of continuously varying signal from the detector against time. Computers for signal processing and evaluation of data have simplified the difficulties in data handling.

Ease of Operation**:** It is important the detector be easy to handle and can be operated at ambient temperature.

Solute Properties**:** The choice of detectors may also be based on the nature of the solute. E.g. if the solute is highly absorbing in the UV region the best detector would be the UV detector. It must be emphasized that no detector is the universally accepted one. However, based on the above-mentioned and the types of detectors available, the UV detector is the most widely used. It is relatively insensitive to temperature and flow changes and has a high sensitivity to many drug substances. It also allows monitoring and continuous registration of the UV absorbance at a selected wavelength or over a span of wavelengths (diode array detection).

Appearance of the analyte in the detector flow-cell causes the change of the absorbance. If the analyte absorbs greater at than the background (mobile phase), a positive signal is obtained (Kazakevich and Lorutto, 2007).

Data Acquisition and Control System**:** Computer-based system that control all parameters of HPLC instrument (eluent composition (mixing of different solvents): temperature, injection sequence. etc.) and acquires data from the detector and monitors system performance (continuous monitoring of the mobile-phase composition, temperature, backpressure, etc.) (Rasmussen and Ahujah, 2007).

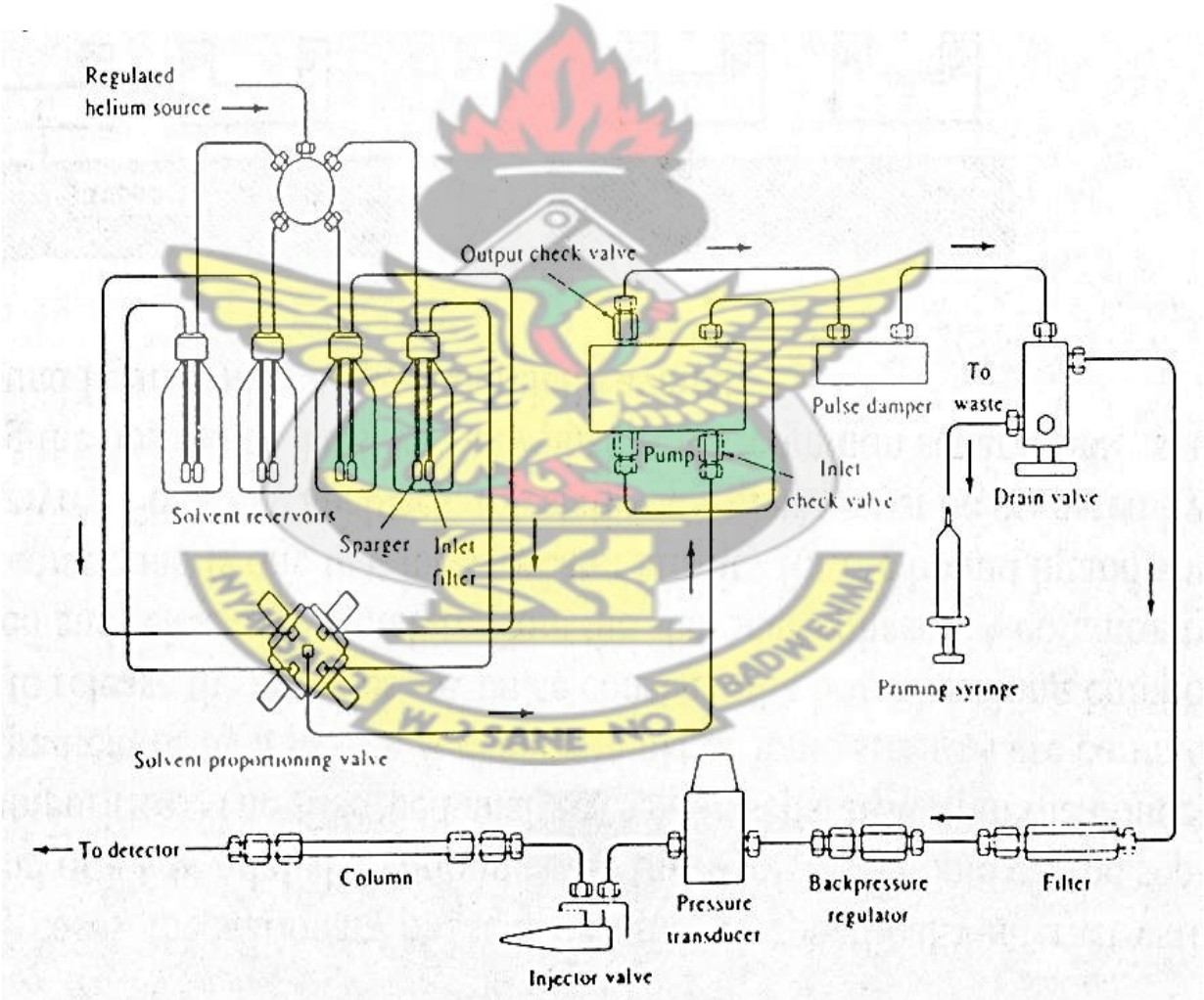


Figure 5HPLC Chromatograph (*Perkin-Elmer Corp)*

2.5.1.2 Types of HPLC

There are four main types of HPLC techniques; Normal Phase, Reversed Phase, Ion Exchange, and Size Exclusion Chromatography. The principal characteristic defining the identity of each technique is the dominant types of molecular interactions employed.

2.5.1.2.1 Normal-Phase Chromatography (NP-HPLC)

Normal-phase HPLC explores the differences in the strength of the polar interactions of the analytes in the mixture with the stationary phase (Coradini and Phillips, 2011). The greater the analyte-stationary phase interaction, the longer the retention of the analyte in the stationary phase (Kromidas, 2006). Comparatively to any liquid chromatographic technique, NP-HPLC separation is a competitive process. Analyte molecules compete with the mobile-phase molecules for the adsorption sites on the surface of the stationary phase (Coradini and Phillips, 2011).

The stronger the mobile-phase interactions with stationary phase, the lower the difference between the stationary-phase interactions and the analyte interactions, and thus the lower the analyte retention (Kromidas, 2006). Mobile phase in NP-HPLC are based on non-polar solvents (such as hexane, heptanes, etc.) with the small addition of polar modifier (i.e. methanol, ethanol) (Kromidas, 2006). These polar modifiers (methanol, ethanol, or isopropanol) which are also referred to as polar additives are also added to the mobile phase in relatively small amounts (Rasmussen and Ahujah, 2007). Since polar forces are the dominant type of interaction employed and these forces are relatively strong, even only 1 v/v% variation of the polar modifier in the mobile phase usually results in a significant shift in the analyte retention. Variation of the polar modifier concentration in the mobile phase allows for the control of the analyte retention in the column (Kazakevich and Lorutto, 2007).

Traditionally, the packing materials used in NP-HPLC are usually porous oxides such as silica (SiO_2) or alumina (Al_2O_3).

The surface of this stationary phase is covered with the dense population of OH groups which makes these surfaces highly polar. Analyte retention on these surfaces is very sensitive to the variations of the mobile-phase composition (Kromidas, 2006). Chemically modified stationary phases can be used in normal-phase HPLC (Rasmussen and Ahujah, 2007). Silica modified with glycidoxypropyltrimethoxysilanes (common name; diol-phase) is a typical packing material with decreased surface polarity (Coradini and Phillips, 2011). Surface density of OH groups on diol phase is on the level of 3-4 µmol/m^2, while on bare silica silanols surface density is on the level of 8 µmol/m^2 (Martin, *et al.*, 2009). The use of diol-type stationary phase and low-polarity eluent modifiers (esters (ethyl acetate) instead of alcohol] allow for increase in separation ruggedness and reproducibility, compared to bare silica (Kazakevich and Lorutto, 2007). Selection of using normal-phase HPLC as the chromatographic method of choice is usually related to the sample solubility in specific mobile phase (Kromidas, 2006). Since NP uses mainly non-polar solvents, it is the method of choice for highly hydrophobic compounds (which may show stronger interaction in reversed-phase HPLC), which are insoluble in polar or aqueous solvents (Kazakevich and Lorutto, 2007).

2.5.1.2.2 Reversed-Phase HPLC (RP-HPLC)

Contrast to normal-phase HPLC, reversed-phase chromatography employs mainly dispersive forces (hydrophobic or Van der Waals interactions) (Kazakevich and Lorutto, 2007). The polarities of mobile and stationary phase reversed such that the surface of the stationary phase in RP-HPLC is hydrophobic and mobile phase is **polar**, where mainly water-based solutions are employed (Kazakevich and Lorutto,2007).. Reversed-phase HPLC is by far the most popular mode of chromatography (Rasmussen and Ahujah, 2007). Almost 90% of all

analyses of low-molecular-weight samples are carried out using RP-HPLC (Martin *et al.*, 2009) One of the main drivers for its enormous popularity is the ability to discriminate very closely related compounds and the ease of variations of retention and selectivity (Kazakevich and Lorutto, 2007). The origin of these advantages could be explained from an energetic point of view. Dispersive forces employed in this separation mode are the weakest intermolecular forces, thereby making the overall background interaction energy in the chromatographic system very low compared to other separation techniques (Rasmussen and Ahujah, 2007). This low background energy allows for distinguishing very small differences in molecular interactions of closely related analytes (Kazakevich and Lorutto, 2007). Adsorbents employed in this mode of chromatography are porous rigid materials with hydrophobic surfaces (Rasmussen and Ahujah, 2007). In all modes of HPLC with positive analytes surfaces interactions, the higher the adsorbent surfaces area, the longer the analyte retention and in most cases the better the separations (Kazakevich and Lorutto, 2007). The majority of packing materials used in RP-HPLC are chemically modified porous silica (Kromidas, 2006).

USP SALMOUS edition method employs gradient elution using acetonitrile and an ion-pairing reagent. Cesar *et al.* also described a method for the simultaneous determination of Artemether and Lumefantrine in fixed dose combination formulations (Cesar *et al.*, 2008). All the above methods require the use of acetonitrile as the major component of the mobile phase.

2.5.1.2.3 Ion-Exchange Chromatography (IEX)

Ion-exchange chromatography, as indicated by its name, is based on the different affinities of the analyte ions for the oppositely charge ionic centers in the resin or adsorbed counterions in the hydrophobic stationary phase (Coradini and Phillips, 2011).

Four major types of ion-exchange centers are usually employed;

I. SO_3^- : Strong cation-exchanger

II. CO_3^- : weak cation-exchanger

III. Quaternary amines : strong anion-exchanger

IV. Tertiary amine : Weak anion-exchanger

Analyte retention and selectivity in ion-exchange chromatography are strongly dependent on the pH and ionic strength of the mobile phase (Kazakevich and Lorutto, 2007).

2.5.1.2.4 Size-Exclusion Chromatography (SEC)

Size-Exclusion Chromatography (SEC) is the method for dynamic separation of molecules according to their size; as the name indicates. The separation is based on the exclusion of the molecules from the porous space of packing materials due to their steric hindrance (Coradini and Phillips, 2011). Hydrodynamic radius of the molecules is the main factor determining its retention. This is the only chromatographic separation method where any positive interaction of the analyte with the stationary phase should be avoided (Coradini and Phillips, 2011). In SEC, the higher the molecular weight of the molecules, the greater its hydrodynamic radius, which results in faster elution (Coradini and Phillips, 2011).

2.5.1.3 Basic Chromatographic Descriptors

There are four major descriptors which are commonly used to report characteristics of the chromatographic column, systems, and particular separations.

I. Efficiency *(N)*

II. Retention Factor *(K)*

III. Selectivity *(α)*

IV. Resolution *(R)*

2.5.1.3.1 Efficiency

It is the measure of the degree of peak dispersion in a particular column: as such, it is essentially the characteristics of the column. This parameter determines the reliability of the column in separating the components of a mixture. It describes the rate at which the solute molecules spread out as they travel through the column (Ritchel, 1986). Efficiency is expressed in the number of theoretical plates (N) calculated as (Kazakevich and Lorutto, 2007).

$$N = 16\left(\frac{t_R}{w}\right)^2$$

Where t_R is the analyte retention time and w is the peak width at the baseline.

Column efficiency is mainly dependent on the kinetic factors of the chromatographic systems such as molecular diffusion, mass-flow dynamics, properties of the column packing bed, flow rate, etc. (Kazakevich and Lorutto ,2007). The smaller the particles, the more uniform their packing in the column, the higher the efficiency (Kromidas, 2006). The faster the flow rate, the less time analyte molecules have for diffusive band-broadening.

2.5.1.3.2 Retention Factor

The retention factor is a measure of the retention of a particular compound on a particular chromatographic system at given conditions. It is defined as:

$$K = \frac{V_R - V_0}{V_0} = \frac{tR - t_0}{t_0}$$

Where V_R is the analyte retention volume. V_0 is the volume of the liquid phase in the chromatographic system or the void volume. t_R is the analyte retention time, t_0 is sometimes defined as the retention time of non-retained analyte (Barbara *et al.*, 1982). The retention

factor is convenient because it is independent on the column dimensions and mobile phase flow rate. Note that all other chromatographic conditions significantly affect retention factor (Ahuja and Dong .2005).

2.5.1.3.3 Selectivity

It is the ability of a chromatographic system to discriminate between two different analytes. It is defined as the ratio of corresponding retention factors (Rasmussen and Ahujah, 2007).

$$\alpha \equiv \frac{k2}{k1}$$

Where K_1 and K_2 are the retention factors of the two analytes.

The selectivity is primarily dependent on the nature of the analytes and their interaction with the stationary phase (Rasmussen and Ahujah, 2007). If a dramatic change of the selectivity is needed for a particular separation, the best solution is the replacement of the type of the stationary phase (Coradini and Phillips, 2011).

2.5.1.3.4 Resolution:

It is a measure of the separation of two compounds which include peak dispersion and selectivity. Resolution is defined as:

$$R = \frac{2(t2 - t1)}{(w2 + w1}$$

Where t_1 and t_2 are the respective retention times and w_1 and w_2 the distance between the respective peak maxima.

Now, the distance between the peak maxima reflects the selectivity of the system. The greater the distance, the higher the selectivity (Kazakevich and Lorutto, 2007).

Improvement of the resolution of a poorly resolved analyte then could be pursued in two different ways: either by increasing the efficiency or by improving the selectivity

(Kazakevich and Lorutto, 2007). A resolution value of 1.5 is usually regarded as sufficient for the baseline separation of closely eluted peaks: and if the typical average efficiency of modern HPLC column is equal to 10,000 theoretical plates, then the selectivity necessary for this separation to get a resolution of 1.5 can be calculated. It will also be useful to compare what would be required in terms of efficiency and selectivity to improve the resolution from 1 to 1.5 (Rasmussen and Ahujah, 2007).

2.5.1.4 HPLC DETECTORS

2.5.1.4.1 UV-Visible Detectors

The most widely used detectors in modern HPLC are photometers based on ultraviolet (UV) and visible light absorption. These detectors have high sensitivity for many solutes but the samples must be capable of absorbing in the UV (or visible) region (Ahuja and Dong, 2005).

UV-visible spectrophotometric detectors can respond throughout a wide wavelength range (e.g. 190-600 nm), which enables the detection of a broad spectrum of compound types (Coradini and Phillips, 2011).

Reversed-phase mobile phases of acetonitrile plus water or phosphate buffer can be used routinely for detection at 200 nm, whereas methanol-containing mobile phases cannot be used below 210 nm, depending on the concentration of the methanol (Kromidas, 2006). A proper selection of the mobile phase makes it possible to operate UV detectors in a near-universal detection mode in the 200-215 nm region, where most organic compound exhibit some UV absorbance (Coradini and Phillips, 2011). UV detectors come in three common configurations. Fixed-wavelength detector rely the chosen wavelength. The differential refractive index (RI) detector responds to a difference in the refractive index of the column effluent as it passes through the detector flow cell (Foulstone, 2001). The RI detector is a bulk-property detector that responds to all solutes. If the refractive index of the solute is

sufficiently different from that of the mobile phase (Kazakevich and Lorutto,2007). Refractive index detector characteristics include but are not limited to the following:

Excellent versatility: all solutes can be detected, moderately sensitivity but generally not useful for trace analyses, not useful for gradient elution, efficient heat-exchanger required, sensitive to temperature changes (Kazakevich and Lorutto, 2007). It is reliable, fairly easy to operate, non-destructive (Rasmussen and Ahujah, 2007).

2.5.1.5 Review of various HPLC works on Artemether and Lumefantrine

César and coworkers were the first authors to propose a HPLC method allowing a separation of the two analytes (within 5 min.) using a cyano stationary phase, an acetonitrile-0.05% trifluoroacetic acid in water (60:40, v/v) mobile phase and a 210 nm detection wavelength. The method proved to be linear, precise, accurate, specific and robust. Four batches of Artemether-lumefantrine tablets were assayed by the validated method. The Artemether content in the tablets varied from 98.1% to 103.35% while lumefantrine were 97.92-100.48 %(Cesar*et al.*, 2008).

Vikas P *et al* developed a simple and precise HPLC method for the determination of Artemether and Lumefantrine in pure drug and pharmaceutical dosage form using Cyano stationary phase with mobile phase comprising of Phosphate Buffer (p H 2.6) and Acetonitrile in the ratio of 40:60 (v/v) with a flow rate of 1mL/min, detection was done using PDA at 215nm and 234 nm. The retention times were 3.3 and 4.9 mins for AM and LU respectively, with recovery being 98.9% and 99.8% for AM and LU respectively (Vikas P *et al.*, 2011).

Sridhal ***et*** *al* developed a reverse phase HPLC method for the determination of Artemether and Lumefantrine in pharmaceutical dosage form using a mobile phase comprising of phosphate Buffer (p H 3.0) and Acetonitrile in the ratio of 40:60 (v/v) with a flow rate of 1.5 ml/min and detection wavelength of 303nm (Sridhal *et al.*, 2010).

Prasanna *et al* developed an HPLC method for the determination of Lumefantrine in solid dosage form using a mobile phase comprising Methanol and Acetonitrile in the ratio of 50:50 (v/v) with a flow rate of 2.0 ml/min, detection wavelength at 235nm. The method was linear over a concentration range of 50-150µg/ml for lumefantrine, and a recovery of 99.76%. The method was successfully employed for the analysis of lumefantrine- containing pharmaceutical formulations and can be employed for bioequivalence study for the same formulation. (Prasanna *et al.*, 2010)

Kakde *et al* developed anHPLC method for the determination of Artemether in combination with Lumefantrine in solid dosage form using Hypersil ODS column with mobile phase of methanol and 0.05% triflouro acetic acid with triethylamine buffer (p H 2.8) adjusted with orthphosphoric acid in the ratio of 80:20(v/v), detection was done with PDA at 210nm.The retention times were 6.15 and 11.31 for AM and LU respectively. Linearity was over a concentration range of 20-120 and 120-720 µg/ml for AM and LU respectively, with recovery being 99.5-101.16% and 99.78-101.21 for AR and LU respectively. The statistical analysis proved that the method was suitable for analysis of AM and LU as a bulk drug and in pharmaceutical formulation without any interference from the exepients (Kakde *et .l*, 2011).

Sunil *et al* developed an HPLC method for the determination of Artemether and Lumefantrine in pharmaceutical dosage form using a mobile phase comprising Phosphate buffer and Acetonitrile with a flow rate of 1.5 ml/min, detection wavelength of 235nm with UV detector. Percent recovery was 98.87 and 99.78% for AM and LU respectively. The method was validated by evaluation of different parameters. (Sunil *et al.*, 2010).

A simple, precise and rapid HPLC method for the determination of Artemether and Lumefantrine in pharmaceutical dosage form using a mobile phase comprising 0.01M tetra butyl ammonium hydrogen sulphate and Acetonitrile in the ratio of 20:80 (v/v) with a flow

rate of 1.0 ml/min, detection wavelength at 222nm with UV/VIS detector, with retention times being 4.19 and 5.22 for AM and LU respectively (Pankaj *et al.*, 2012).

Khalil *et al*. developed the measurement of Lumefantrine& its metabolite in plasma by HPLC with UV detection. Chromatographic separation was carried out on a Synergi polar-RP column (250 x 300mm, particle size 4μm). The mobile phase consisted of acetonitrile-0.1 M ammonium acetate buffer (85:15v/v). Absorbance of the compounds was monitored at 335nm using a reference wavelength of 360nm (Khalil *et al.*, 2011).

2.5.2 Spectroscopy

The light absorption spectra (ultraviolet and visible spectra)are characteristic for the individual compounds, and are readily determined by use of a spectrophotometer. The absorption spectrum of a compound in solution gives an indication of its purity by comparison of the shape of the spectrum with that of a known compound, and a measure of its concentration. This is particularly valuable for confirming purity and determining concentration of solutions for use as standards on HPLC.

The concentration of a solution may be calculated from its absorbance (at the wavelength of maximum absorbance) by using either its molar extinction coefficient (∈) or its absorption coefficient ($E^{1\%}$).

Beer-Lambert Law states that, $A = \in b\, c$

Where A is absorbance, ∈is the molar absorptivity with units of L mol^{-1} cm^{-1}, b is the path length of the sample - that is, the path length of the cuvette in which the sample is contained. Expressed in centimeters and c is the concentration of the compound in solution, expressed in mol/L. The ∈ is the calculated absorbance of a 1 M solution at the designated wavelength in a 1-cm light-path spectrophotometer cuvette. The concentration in μM of a solution can be calculated by dividing the absorbance by ∈ (10^{-6}).

The $E^{1\%}$ is the calculated absorbance of a 1 % solution at the designated wavelength in a 1-cm light-path spectrophotometer cuvette. The concentration in μg/ml of a solution can be calculated by dividing the absorbance at the designated wavelength by $E^{1\%}10^{-4.}$

Due to its lack of such chromophore groups, Artemisinin and its derivatives, unlike lumefantrine absorb weakly in the low wavelength region and this makes their quantification difficult. The available UV Spectrophotometric methods for the analysis of Artemether make use of its HCl decomposition product. This acid decomposition product of Artemether has been described as an α β unsaturated decalone and absorbs at a wavelength of 254nm (Thomas*et al*., 1992).

2.5.3Thin Layer chromatography

Thin layer chromatography (TLC) is a chromatographic technique used to separate mixtures. TLC can be both analytical and preparative. TLC is performed on a sheet of glass, plastic, or aluminum foil, coated with a thin layer of adsorbent material. The substances frequently used as coating materials are silica gel, alumina and cellulose. To give stable layers they often contain binders such as calcium sulphate or starch (Beckett *et. al*., 1988).

2.5.4 Non Aqueous titrations

Non-aqueous titration is the titration of substances dissolved in non-aqueous solvents. It is the most common titrimetric procedure used in pharmacopoeial assays and serves a double purpose:

1. It is suitable for the titration of very weak acids and very weak bases, and
2. It provides a solvent in which organic compounds are soluble.

The most commonly used procedure is the titration of organic bases with perchloric acid in anhydrous acetic acid. The end point of most titrations is detected by the use of visual indicator but the method can be inaccurate in very dilute or colored solutions. However under

the same conditions, a potentiometric method for the detection of the equivalence point can yield accurate results without difficulty.

2.5.5 Other Methods

Nondestructive portable spectroscopic technologies are available for Field testing of poor quality medicines. Infrared spectroscopy and Raman spectroscopy are currently being evaluated for rapid detection of poor quality medicines. With these techniques, drug samples may be scanned through the plastic of the blister pack while still in its original packaging and no toxic chemicals or flammable solvents are necessary.

Raman spectroscopy is based on the Raman Effect, the scattering of light interacting with the different vibrational modes of the drug and excipient molecules contained in the tablet. One potential drawback of using Raman spectroscopy is that only the sample surface is probed, so that if the active pharmaceutical ingredient is not distributed homogeneously throughout the entire tablet, the resulting content information may be inaccurate.

The spectra obtained using Raman spectroscopy cannot be deconvoluted into specific signals from different chemicals as it presents information regarding the functional groups in a molecule. In order to identify genuine samples, a fingerprinting method is used where a Raman spectrum is compared against a spectral database. It is also important to ensure that interference from an excipient does not cause the sample to be wrongly characterized as a fake. Because of this, it is crucial to have a database of every genuine formulation from every manufacturer, thus decreasing the risk of incorrectly identifying a genuine drug as a poor quality medicine. A common disadvantage seen when Raman has been tested to analyze pharmaceutical preparations is that many drugs contain highly fluorescent excipients, thus negatively affecting the quality of the spectrum.

Nevertheless, Raman spectroscopy has been successfully tested in the field for detection of counterfeits.

Infrared spectroscopy utilizes the fact that different drug molecules absorb differently when excited with infrared radiation. Unlike Raman spectroscopy, the infrared radiation has a larger penetration depth, with the potential advantage that the larger area examined can detect an active ingredient that is not perfectly homogeneous throughout the entire tablet. Infrared spectroscopy, like Raman, uses the fingerprinting method in order to match the sample spectrum to a compound in the database. Near-infrared spectroscopy uses the near-infrared region of the electromagnetic spectrum (from approximately 800–2500 nm) and entails exciting the molecules in a sample and recording the unique fingerprint obtained. The method has been used to analyze components and may be used to demonstrate that they are not in the correct proportion, thus suggesting that the medicine is a counterfeit. X-ray fluorescence (XRF) is a nondestructive technique that utilizes X-rays to determine which chemical elements are contained in a sample.

When X-ray fluorescence is used for analysis, X-rays bombard the sample and characteristic emissions result from different elements. This technique requires no sample preparation and is most commonly used for elemental analysis and to detect metals present. Although the active pharmaceutical ingredient is not measured directly, the elemental composition of counterfeit drugs tends to be quite different from that of the genuine. XRF, like Raman and infrared methods, requires a genuine tablet to verify whether a medicine can be classified as genuine or poor quality. X-ray diffraction (XRD) is based on the elastic scattering of X-rays by the crystalline structures organized and aligned in crystals.

Powder diffraction is often used in identifying unknown samples. This technique compares the spectrum obtained from a specific sample to a database of spectra from every expected possibility. This method is destructive, as the tablet must be crushed into a powder. This

method can also be used to measure the relative abundance of the major components in the sample, and usually provides information regarding excipients that is not easily obtained by mass spectrometry and other common analytical methods.

2.6 Validation of HPLC Methods

For the accuracy and reliability of HPLC methods developed, they must be developed in accordance with ICH guidelines. Many factors are taken into account when validating a method. The factors include:

1. Accuracy
2. Precision
3. Robustness
4. Linearity
5. Limit of Detection and Limit of Quantitation

Accuracy is defined as the closeness of a test results obtained by the method of interest to the true value. For drug substances and drug products, the accuracy can be inferred in some instances once the precision, linearity, and specificity have been established. Accuracy is measured using a minimum of five determinations per concentration.

The precision of an analytical method describes the closeness of individual measurements of an analyte when the procedure is applied repeatedly to multiple aliquots of a sample. Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, inter-batch precision or repeatability, which measures precision with time, and may involve different analysts, equipment, reagents, and laboratories.

Linearity: A linear relationship should be evaluated across the range of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard

stock solution) and/or separate weighing of synthetic mixtures of the drug product components, using the proposed procedure.

Robustness can be described as the ability to reproduce the analytical method in different laboratories or under different circumstances without the occurrence of unexpected differences in the obtained results. Robustness tests were originally introduced to avoid problems in inter laboratory studies and to identify the potentially responsible factors. The robustness test was considered a part of method validation related to the precision (reproducibility) determination of the method.

Limit of Detection (LOD): the detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The limit of detection can be found based on

1. Visual Evaluation
2. Signal-to-Noise
3. The Standard Deviation of the Response and the Slope

Limit of Quantitation (LOQ): the quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products. The limit of quantitation may be determined by

1. Deviation of the Response and the Slope Visual Evaluation
2. Signal-to-Noise
3. The Standard.

CHAPTER THREE

METHODOLOGY

3.1 Instruments and Materials

350 pH meter JENWAY

UV-1800 Shimadzu Spectrophotometer

Shimadzubalance(ModelAY-120).

Humaqua 5 Water bath

JASCO LC- NetII ADS HPLC

2089-Chromatograph pump

UV 2070 PLUS Detector

Ultracarb 3µ ODS(20), 200×3.20mm Column

3.2 Reagents and Samples

Acetonitrile

Ethanol

Hydrochloric acid (36%w/w) (Elitech Clinical Systems, France)

Methanol (HPLC Grade)

Phosphate buffer tablets

Sodium dihydrogen Phosphte buffer tablet

Sulphric acid

TFA

Vinillin

Table 1: Pure samples used

SAMPLE	SOURCE	BATCH NO.	MAN. DATE	EXP. DATE
Artemether	PHARMANOVA	AT-R-12023	Dec 2012	May 2016
Lumefantrine	PHARMANOVA	2010LU3RM	Mar 2012	Feb 2017

Table 2: Brands of tablets used (20mgAM/120mg LM)

BRAND	ID	BATCH NO.	MAN. DATE	EXP. DATE	COUNTRY OF ORIGIN
MALAR 2DS	A	1911N	NOV 2013	NOV2016	GHANA
DANMETHER	B	1309263	SEP2013	SEP2015	GHANA
MALAFANTRINE	C	S3213	NOV 2013	OCT 2016	INDIA
COATERM	D	X1650	MAY 2013	APR 2015	CHINA
LONART	E	LD511	MAR 2013	FEB 2015	INDIA
ARTEMOS PLUS	F	LOT120401	APR 2012	APR 2015	CHINA

3.3 Study Area

The Kintampo North municipality has a population of 140 000 with a relatively poor socio-economic status. It lies in the transitional zone between the south and north of Ghana. As such, the population is composed of migrants settlements and transiting passengers plying the south-north corridors. It is located between latitudes 8º45'N and 7º45'N and Longitudes 1º20'W and 2°1'E and shares boundaries with five districts in the Country:, namely; Central

Gonja District to the North; Bole District to the West; East Gonja District to the North-East (all in the Northern Region); Kintampo South District to the South; and Pru District to the South- East (both in the Brong Ahafo Region). The Municipality has a surface area of about 5,108km², thus occupying a land area of about 12.9% of the total land area of Brong Ahafo (39,557km²).This work was carried out at the Kintampo Health Research Centre Bioanalytic laboratory.

3.4 Collection and Purchasing of Artemether Lumefantrine Antimalarial Tablets

All drugs were purchased in tablet form.

3.4.1 Sampling of Antimalarials

Ten packets each of six brands of Antimalarial drug containing 20mg AM and 120 mg LU were purchased from three pharmaceutical shops and four chemical shops. Opportunistic sampling was used so as to get all drugs available in each shop.

3.5 Identification and Assay of Pure Samples

To compare artemether and lumefantrine to standard samples, reference standards were obtained from Pharmanova. These standards ensured the authenticity of these drugs for both qualitative and quantitative works.

Artemether was identified by color tests stated in the IP. Lumefantrine was identified as stated in the IP (Draft). The methanolic solution of the lumefantrine gave a specific absorbance of 324 which falls within the reference range of 314–348 as stated in the IP Draft.

3.6 Preparation of Reagents

3.6.1 Preparation of Sodium Dihydrogen Phosphate Buffer

1.26g of the sodium dihydrogen phosphate buffer tablet was dissolved in 1L distilled water. The pH was checked by means of 350 JENWAY pH meter. The desired pH was attained by adjusting with phosphoric acid.

3.6.2 Preparation of the Mobile Phase

The mobile phase is composed of methanol and sodium dihydrogen Phosphate buffer (72:28).To prepare 500mL of the mobile phase, 360mL of Methanol and 140mL of the buffer were measured and mixed in a suitable measuring cylinder. The mobile phase was sonicated to expel gases and the solution was filtered using the sintered glass filter.

3.6.3Preparation of Standard Solutions of Artemether and Lumefantrine

4mg lumefantrine and 24mg artemether were accurately weighed and transferred into a 25mL volumetric flask, sonicated and diluted to volume with the mobile phase to give a solution of 160µg/mL of artemether and 960µg/mL of lumefantrine. The solution was then filtered using a sintered glass filter.

3.6.4 Preparation of mixed standard solutions of Artemether and Lumefantrine

1 mL of stock solution was put into a 10 mL volumetric flask and made volume up to the mark with diluent to obtain a final concentration of 16µg/mL and 96µg/mL for artemether and lumefantrine, respectively.

3.6.5 Preparation and Analysis of Tablet Formulations

Twenty Tablets of artemether and lumefantrine were weighed and finely powdered. A quantity equivalent to 4mg of Artemether and 24mg of Lumefantrine was transferred into 25 mL volumetric flask and appropriate amount of diluent was added. The contents were sonicated to dissolve completely and the volume was made up to the mark with diluent and filtered through sintered glass filter. 1 mL of stock solution was transferred to a 10 Ml volumetric flask and made volume up to the mark with diluents to get final concentration of 16µg/mL and 96µg/mL for artemether and lumefantrine, respectively.

3.6.6 Preparation of working standard solution

1 mL of stock solution was put into a 10 mL volumetric flask and made volume up to the mark with diluent to obtain final concentration of 16µg/mL and 96µg/mL for artemether and lumefantrine, respectively.

20mL of sample and standard solutions were injected into HPLC in triplicate and the consequent chromatograms were recorded.

3.7 Method Design Consideration

The literature was carefully searched for information on the chemical properties of the active components (artemether and lumefantrine) and all the chemical and reagents mentioned under reagent and samples above.

3.7.1 Establishment of Chromatographic Conditions

3.7.1.1 Selection of Stationary Phase

The chromatographic mode used in the method development is Reversed-Phase and as a result an **Ultracarb OctadecylSilane (ODS)20, 3µm 100A, 200 x 3.2mm,** was used.

3.7.1.2 Determination of Mobile Phase Conditions

In establishing optimal mobile phase conditions to separate the API's (artemether and lumefantrine), different buffers were employed and the one that gave optimal resolution of both compounds was selected. Methanol was used in combination with varying amounts of triflouroacetic acid, acetonitrie, and phosphate buffer. The pH of the resulting solutions was as well varied. The combination that gave an optimal resolution of both compounds was therefore selected.

3.7.1.3 Determination of flow rate of mobile phase

Different flow rates of the mobile phase were investigated. The flow rates included from 0.2 mL/min, 0.5 mL/min, 0.8 mL/min, 1.0 mL/min, 1.2 mL/min, 2.5mL/min and 2.9 mL/min.

However, the flow rate that yielded the best separation and retention times of the active pharmaceutical compounds was selected.

3.7.1.4 Determination of wavelength of detection

Detection wavelength was selected by scanning standard drug over a wide range of wavelength from 200nm to 400nm. A fixed concentration of analyte (10µg/mL) was analyzed at different wavelength. As per the response of analyte, the λ max value was found to be 209nm and 335nm for Artemether and Lumefantrine, respectively. Using this data 222nm was selected as a detection wavelength at which the components showed well resolved peaks.

3.7.1.5 Studies on Sensitivity of the method

The Sensitivity of the method was investigated by varying recorder range of the detector (AUFS i.e. Absorbance Units Full Scale) to determine which value of AUFS would give the best response. The range was varied from 0.005 to 20 AUFS. (NB: This is always done on the computer connected to HPLC and a good starting point is about 0.1 AUFS).

3.8Assay of Artemether and Lumefantrine

3.8.1 Chromatographic condition

Column: Ultracarb 3µ ODS, (20) 200*3.2mm)

Mobilephase: Methanol: 0.1% Sodium Dihydrogen Phosphate buffer (72:28)

Flow rate: 2.7ml/min

Wavelength of detection: 222nm

Injection volume: 20µL

Run time: 10min

Upon setting the above condition on the HPLC, 20µL of samples and the resulting chromatograms recorded and used for the quantitation of the APIs.

3.9 Method validation

The method was validated for linearity, accuracy, Precision (intra-day and inter-day) and robustness, in accordance with the ICH guidelines (ICH Harmonized Tripartite Guidelines, 2005).

3.9.1 Linearity

3.9.1.1 Plotting of Lumefantrine Calibration Curve

Aliquot portions of standard stock solution 0.2, 0.4, 0.6, 0.8 and 1.0 mL were taken in separate 10 mL volumetric flasks. The volume was adjusted to the mark with diluent to obtain concentrations of 3.2, 6.4, 9.6, 12.8, 16.0µg/mL and 19.2, 38.4, 57.6, 76.8, 96.0, 115.2 µg/mL for Artemether and Lumefantrine, respectively. Calibration curve was plotted over a concentration range of 3.2-16µg/mL for artemether and 19.2-115.2µg/mL for lumefantrine. Calibration curve was constructed by plotting peak area v/s concentration, the graph must be linear and the regression equation was calculated.

3.9.2 Precision

One set of three different concentrations of mixed standard solutions of artemether and lumefantrine were prepared. All the solutions were analyzed in triplicates, in order to record any intraday variations in the results. For inter-day variations study, three different concentrations of the mixed standard solutions in linearity range were analyzed on three consecutive days. The peak areas were recorded and the Relative Standard deviation (RSD) was calculated for both series of analyses**.**

3.9.3 Robustness

In the robustness study, the influence of small, deliberate variations of the analytical parameters on retention time of the drugs was examined. The following factors were selected:

1. Flow rate of the mobile phase (2.7±0.02ml/min)

2. Wavelength at which the drugs were recorded (222±1nm).

3.9.4 Accuracy

The accuracy of the method was determined by calculating the recovery of the analyte of interest by the standard addition method: Known amounts of working standard of artemether (1.6µg) and lumefantrine (9.6µg) were added to solutions of various concentrations like: 3.2µg, 6.4µg and 9.6µg of artemether and 19.2µg, 38.4µg and 57.6µg of lumefantrine. Each sample was prepared in triplicate and injected.

3.9.5 Sensitivity

Limit of detection (LOD) and quantification (LOQ) were estimated from the signal-to-noise ratio. The LOD and LOQ were calculated by the use of the equations:

LOD = 3 **σ/s.**

LOQ = 10 σ/s

Where **σ** is the standard deviation of intercept of calibration plot and **s**is the average of the slope of the corresponding calibration plot.

3.9.6 Ruggedness

Sample solutions of artemether (16µg/mL) and lumefantrine (96µg/mL) were prepared and analyzed using slightly different operational and environmental conditions.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Identification Tests for Reference standards

4.1.1.1 Color Test

Table 3Results for Color Test for Pure Powders Artemether and Lumefantrine

API'S	Test	Result	Inference
AM	ethalonic solution of sample + vanillin	Pink color observed	AM present
LU	Methalonic solution of sample	specific absorbance at 324nm	LU present

4.1.2 HPLC Method Development

4.1.2.1 Optimization of chromatographic mode

Artemether and lumefantrine, both the API's are non-polar in nature, hence either reversed phase or ion-pair or non-aqueous chromatography could be used. Reversed phase HPLC (RP-HPLC) was selected for the initial separations because of its simplicity and suitability.

4.1.2.2 Optimization of detection wavelength

Detection wavelength was selected by scanning reference standards over a wide range of wavelength from 200nm to 400nm. A fixed concentration of AM-LU (10µg/ml) was analyzed at different wavelengths. From the responses, the λ max value was found to be

209nm and 335nm for artemether and lumefantrine, respectively. Using this data 222nm was selected as a detection wavelength at which the components showed well resolved peaks.

Table 4Selection of Optimum wavelength of detection

Peaks	**Wavelength of absorption (nm)**							
	200	209	222	259	264	270	335	340
AM	X	√	√	x	√	√	X	X
LU	X	X	√	√	x	x	√	X

KEY:

√→ Peak is detected at that wavelength

x→ Peak is not detected at that wavelength

4.1.2.3 Optimization of chromatographic condition

The standard solution of Artemether and Lumefantrine was prepared and run through the system and different combinations of mobile phase and column were tried for isocratic mode to get well resolved, symmetric peaks. The mobile phase was filtered through 0.45 µ membrane filter. As indicated earlier, there are a few HPLC methods of analysis for fixed-dose artemether lumefantrine combination formulations. These methods also require working at very low wavelengths of detection and hence employ acetonitrile as the main solvent in the mobile phase. Also, some of the methods employ gradient elution for analysis. The method therefore employed methanol/phosphate buffer (72:28), which is economical but have well resolved peaks.

4.1.2.3.1 Optimal HPLC conditions established for AM-LU analysis

- Column: Ultracarb 3µ ODS(20), 200×3.20mm
- Mobile phase: Methanol: Phosphate buffer pH2.8 (72:28)
- Flow rate: 2.7ml/min
- Detector: UV detector
- Wavelength of detection: 222nm
-

Table 5:Retention times of Artemether and Lumefantrine using various mobile phases and flow rates.

Mobile Phase Composition	**Flow Rate (ml/min)**	**Artemether R_t(min)**	**Lumefantrine R_t(min)**
Water/Methanol(50:50)	1.0	2.7	19.8
Methanol/Phosphate buffer pH 2.8 (72:28)	**2.7**	**4.2**	**5.2**
Methanol/Phosphate buffer pH 2.8 (72:28)	1.5	10.2	11.5
Methanol/Phosphate buffer pH 5.0 (72:28)	2.7	7.0	6.5
Methanol/Phosphate buffer pH 2.8 (72:28)	2.0	9.8	10.2
Methanol/Phosphate buffer pH 2.8 (60:40)	1.5	7.4	8.5
Methanol/0.05%TFA (90/10)	2.7	5.7	8.1
Methanol/0.05% TFA (90/10)	2.0	6.4	8.6
Methanol/0.04% TFA (90/10	2.0	16.2	12.2
Methanol/0.05%TFA (95/5)	2.5	3.7	5.6
Methanol/Acetonitrile (60:40)	2.5	7.1	4.1
Methanol/Acetonitrile/ 0.05%TFA (60/30/10)	2.5	6.9	2.6

4.1.2.4 Chromatograms of Method Development

Figure 6:Trial using mobile phase of methanol: buffer (95:5) using Artemether alone

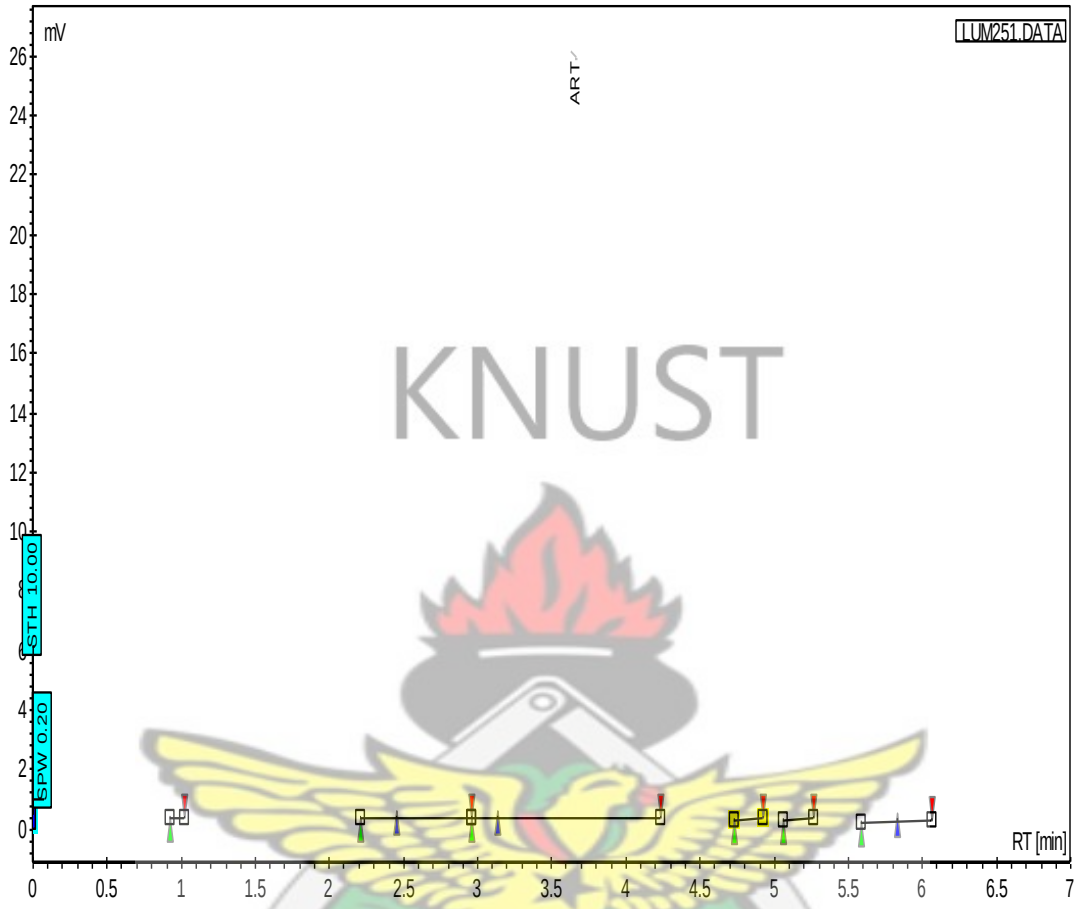


Figure 7: Mobile phase of Methanol: water (50:50)

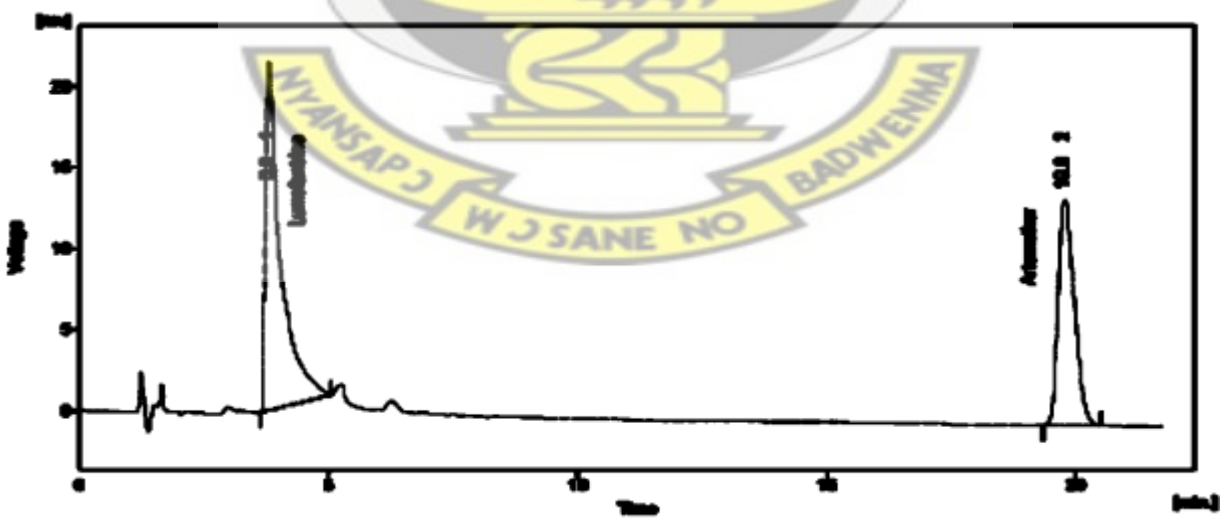


Figure 8:Methanol/Phosphate buffer pH 5.0 (72:28)

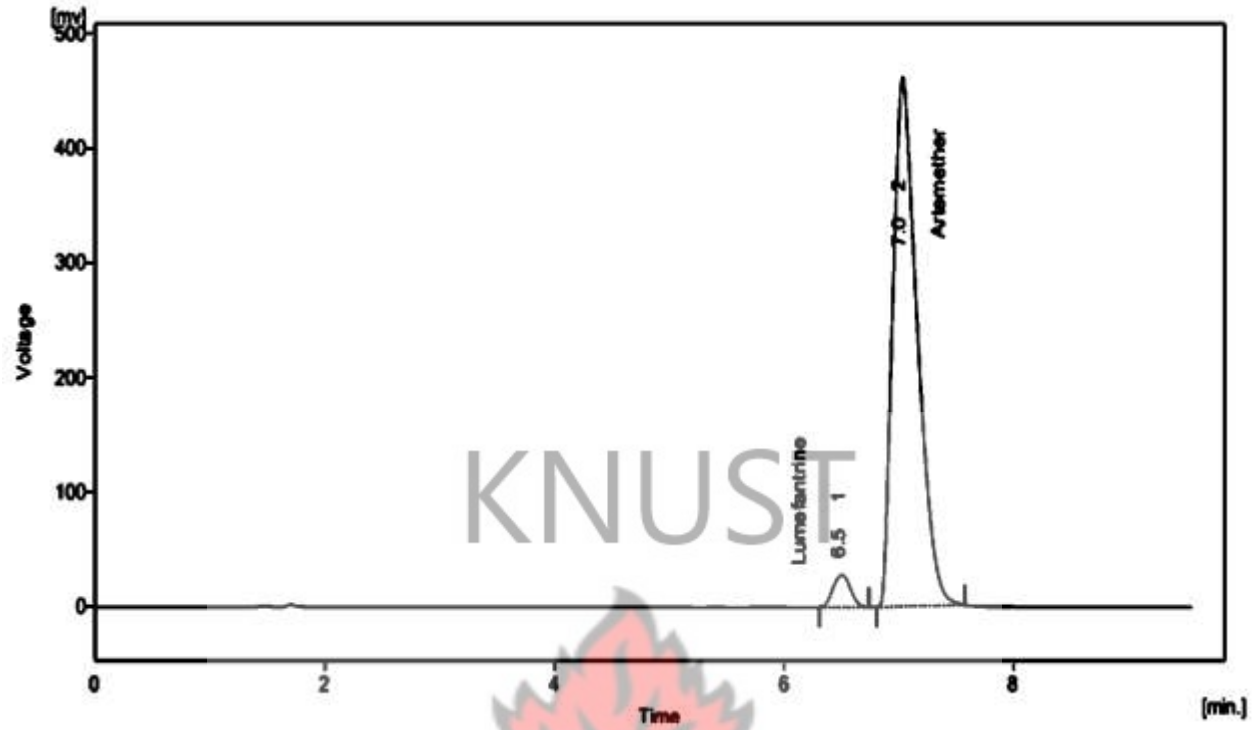
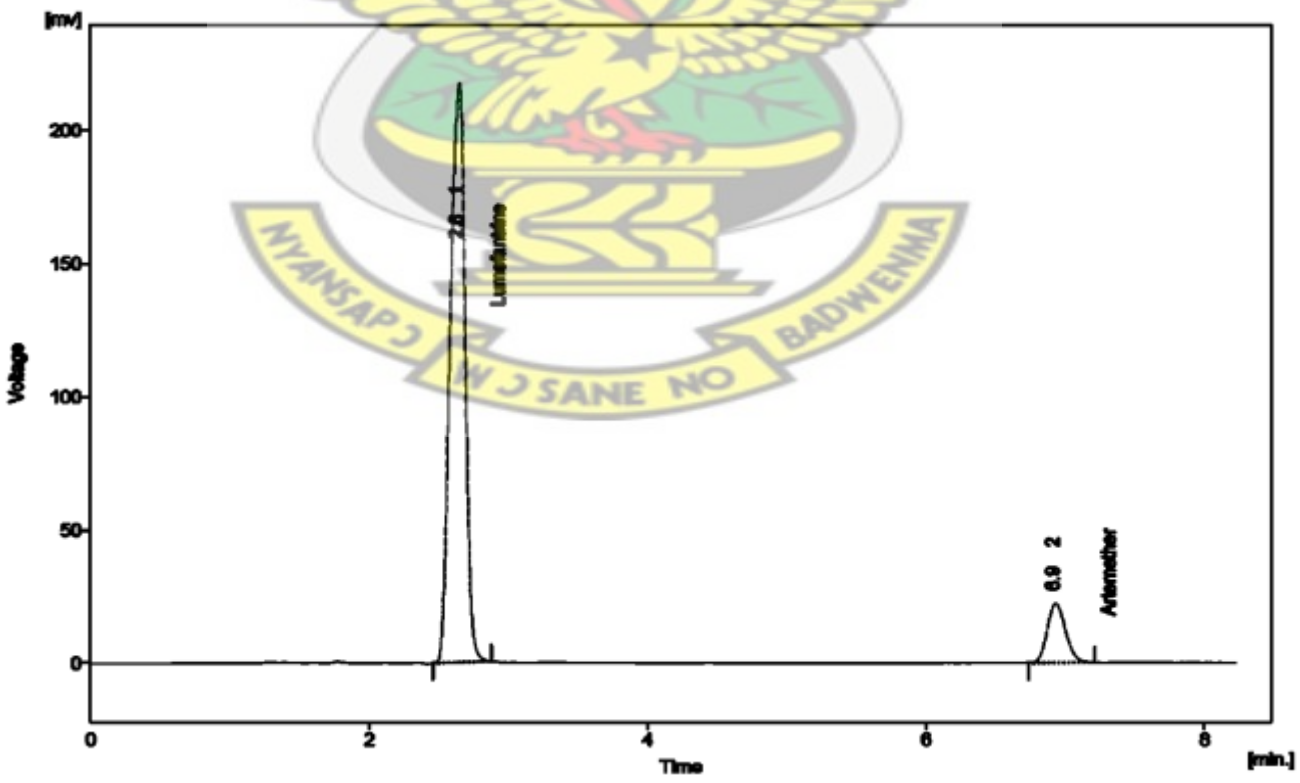


Fig 9: Methanol:ACN:TFA(60:30:10)



4.1.3 HPLC Method Validation

4.1.3.1 Precision

In the prepared solutions for analysis, 100% label claim (lc) represents 20 mg/ml Artemether and 120 mg/ml Lumefantrine solution.

4.1.3.1.1 Intra-day precision

Mean contents and RSD of Artemether and Lumefantrine in the intra-day precision analysis (n=6) were 98.2.6% with RSD = 0.55% and 100.3% with RSD = 1.18%, respectively as shown in table 6.

Table 6: Intra-day Precision

	LU	AM
Determination	**% Recovered**	**% Recovered**
1	100.43	99.33
2	100.19	98.15
3	101.37	98.05
4	101.65	97.85
5	100.84	96.30
6	101.12	99.50
Mean	100.93	98.20
RSD	0.55	1.18

The mean $\bar{x}$ is given by $\bar{x} = \sum \frac{x}{n}$, where x is concentration and n is number of samples.

For LU,

$$\bar{x} = \frac{100.43+100.19+101.37+101.65+100.84+101.12}{6} = 100.93$$

This implies that the mean is thus 100.93%.

Standard deviation S, is also given by S = $\sqrt{\frac{\sum(x-\bar{x})^2}{n-1}}$

Relative Standard deviation (RSD) is given by %RSD = S/$\bar{x}$,

%RSD= 0.561/100.93= 0.55

4.1.3.1.2 Inter-day precision

Mean contents and RSD values of Artemether and Lumefantrine in the inter-day precision analysis (n=3×6) were 98.1% with RSD = 1.01% and 101.4% with RSD = 0.99%), respectively.

Table 7: Inter-day Precision: HPLC ASSAY of LM and AM

	LU	AM
Determination	**% Content**	**% Content**
1	100.43	99.33
2	100.19	98.15
3	101.37	98.05
4	101.65	97.85
5	100.84	96.30
6	101.12	99.50
7	102.33	98.83
8	101.65	97.64
9	102.72	98.87
10	101.65	97.19
11	100.84	97.47
12	103.54	99.74
13	100.45	99.09
14	99.49	97.39
15	101.37	99.01
16	102.34	97.19
17	100.98	96.78
18	102.48	97.46
Average	101.41	98.10
RSD	0.99	1.01

For both compounds, the intra-day and inter-day precision % RSD values were lower than 2.0%, revealing good precision of the method.

4.1.3.2 Accuracy (recovery test)

Known amounts of working standard of Artemether (1.6µg) and lumefantrine (9.6µg) were added to solutions of various concentrations as: 3.2µg, 6.4µg,9.6ug, 12.8ug and 16.0µg of artemether and 19.2µg, 38.4µg, 57.6, 76.8ug and 96.0µg of lumefantrine. Each sample was prepared in triplicate and injected. The chromatograms were recorded and from the peak area of the drug, % recovery was calculated. Artemether mean recovery (n=6) was 98.2% (RSD = 1.18%) and lumefantrine mean recovery was 100.93% (RSD = 0.55%), indicating the accuracy of the method.

Table 8 Accuracy

				AM			LU
Amount added to (ug/ml)		**Amount recovered (ug/ml) AM**	**% Recovered**	**SD Mean=98.88**	**Amount Recovered LU (ug/ml)**	**% Recovered**	**SD Mean= 98.4**
AM	**LU**						
3.20	19.2	3.16	98.80	0.02	18.88	98.30	0.05
6.4	38.4	6.39	99.86	0.24	37.44	97.50	0.22
9.6	57.6	9.49	98.92	0.39	57.14	99.20	0.23
12.8	76.8	12.69	99.20	0.01	75.42	98.20	0.05
16.0	96.0	15.62	97.60	0.3	94.80	98.80	0.10

RSD LU =0.13 AM = 0.19

4.1.3.3 Robustness

Two factors, with deliberate small deviations from the method settings, were considered: percentage V/V of methanol in mobile phase (72 and 80%), flow (from 2.7 and 2.6 ml/min).

Table 9 Robustness (Flow rate)

	LU	AM
Flow rate (ml/min)	**%Recovery**	**%Recovery**
2.7	97.60	96.80
3.0	95.56	95.56

Table 10: Robustness - MP Composition

Flow rate (ml/min)		**%Recovery LU**	**%Recovery AM**
Methanol: (72:28)	**Buffer**	97.20	98.10
Methanol: (80:20)	**Buffer**	96.45	96.76

4.1.3.4 Linearity

4.1.3.4.1 Calibration of the developed method

Figure 10: Calibration Curve for LU

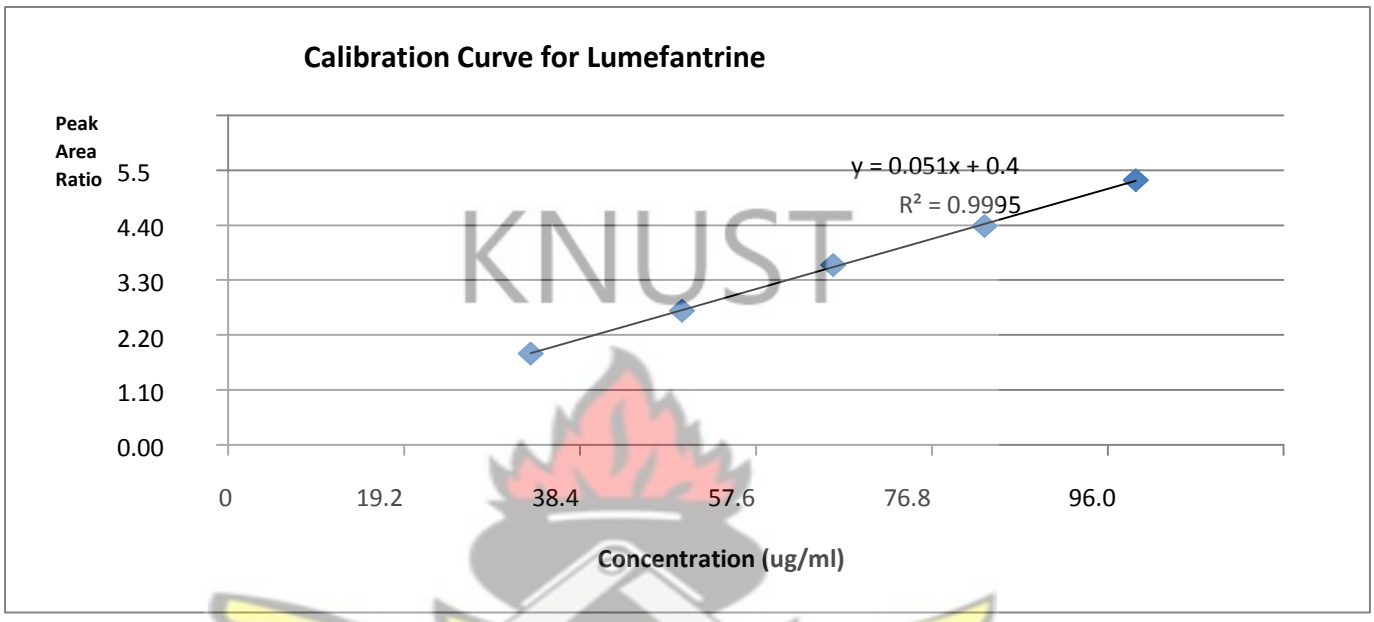


Table 11: Parameters of Lumefantrine Calibration Curve

Concentration(mg/ml)	**Peak Area Ratio**
19.2	1.1
38.4	2.2
57.6	3.3
76.8	4.4
96	5.5

Slope = 0.051±0.00045

Intercept = 0.4±0.0053

R^2 =0.9995

RSD = 0.96

4.1.3.4.2.1 Determination of the equation of the curve for LU

- Sample Calculation y = mx + c, Where
- y = Peak Area Ratio
- m = Slope of Calibration Curve
- x = Concentration
- c = y - intercept

From graph, equation of curve: **y = 0.051x + 0.4**

For y = 3.3

3.3= 0.051x + 0.4

(3.3 - 0.4)/0.051= x

x = 56.8ug/ml

% Content = (56.8/57.6) × 100

= 98.4% purity.

LOD =3SD/Slope

=3x0.96/0.051

=338ug/ml

LOQ = 10x0.96/0.051

=**1129ug/ml**

Figure 11:Calibration Curve for AM

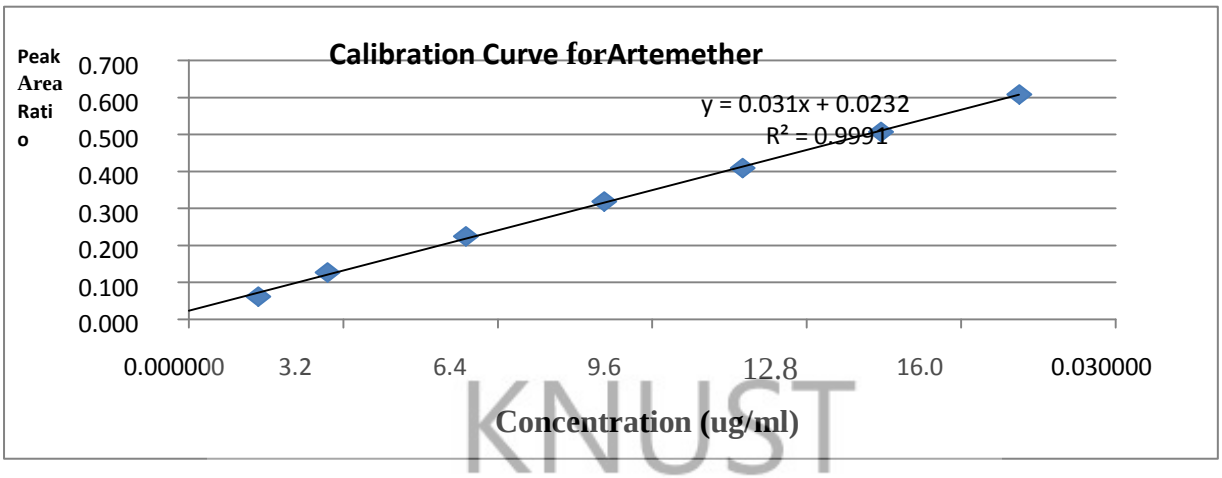


Table 12:Parameters Artemether of Calibration Curve

Concentration(ug/ml)	**Peak Area Ratio**
3.2	0.10
6.4	0.20
9.6	0.30
12.8	0.40
16.0	0.50
Slope =0.031	
Intercept =0.0232	
R^2 =0.9991	
RSD =0.96	

4.1.3.4.2.2 Determination of the equation of the curve for AM

Using the equation y=mx+ c,

For y = 0.3,

0.3 =0.032x + 0.023

0.3-0.023 =0.032x

0.277= 0.032x

x= 0.94

=94% purity

LOD =3SD/Slope

=3x0.96/0.032

=90ug/ml

LOQ = 10SD/Slope

= 10x0.96/0.032

=300ug/ml

Table 13:LOD and LOQ for AM and LU

Parameter	**AM**	**LU**
LOD (ug/ml)	90	338
LOQ (ug/ml)	300	1129

Figure 12:Accuracy chromatogram

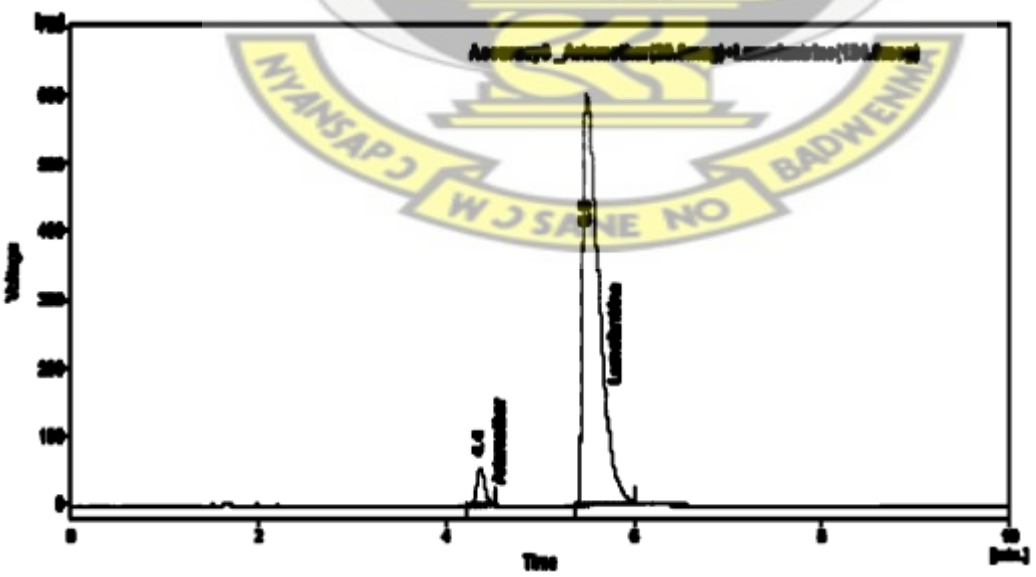
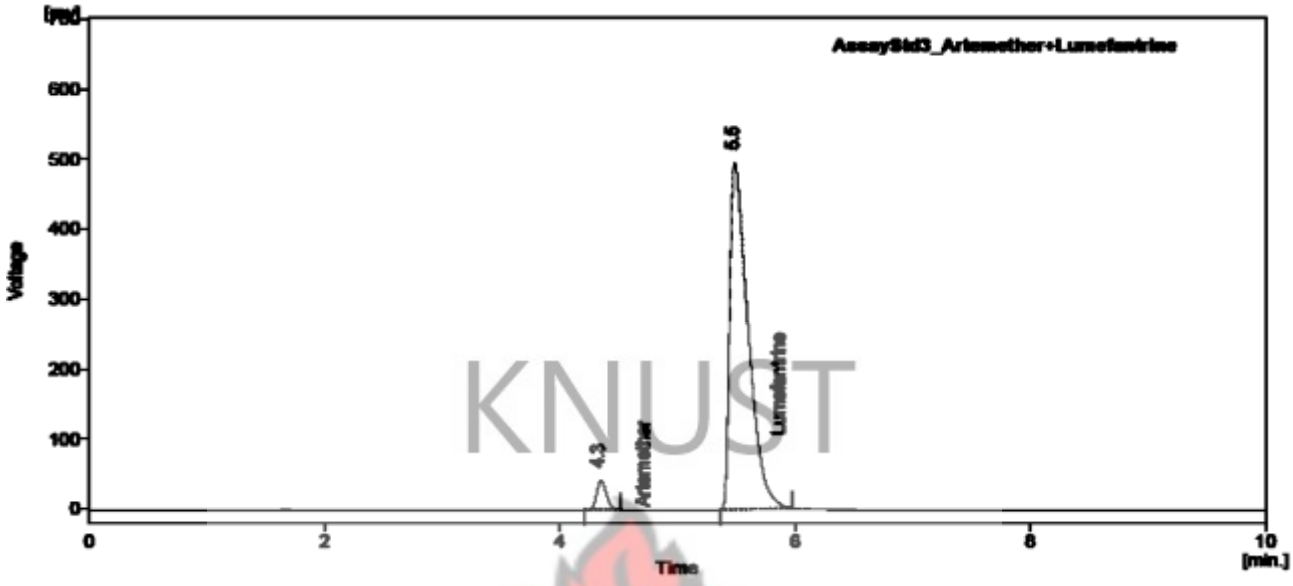


Figure 13: Linearity Chromatogram



4.1.2 Artemether Lumefantrine tablets analysis

4.1.2.1 Physical test on AM-LU tablets

Table 14 Assay of AM in Tablets Using Developed HPLC Method

Brand	Label Claim(mg)		Amount(mg)		% Content		SD	
	AM	LU	AM	LU	AM	LU	AM	LU
A	20	120	18.7	109.6	**93.50**	**91.3**	0.58	0.89
B	20	120	19.8	119.0	**99.20**	**97.2**	0.36	0.10
C	20	120	19.3	116.4	**96.62**	**97.0**	0.07	0.07
D	20	120	19.2	118.2	**95.82**	**98.5**	0.20	0.32
E	20	120	21.1	119.9	**105.0**	**99.9**	1.33	0.55
F	20	120	18.4	115.0	**92.05**	**95.8**	0.83	0.13

4.1.4.1 Chromatograms of sample analysis

Figure 14: Chromatogram of Sample A

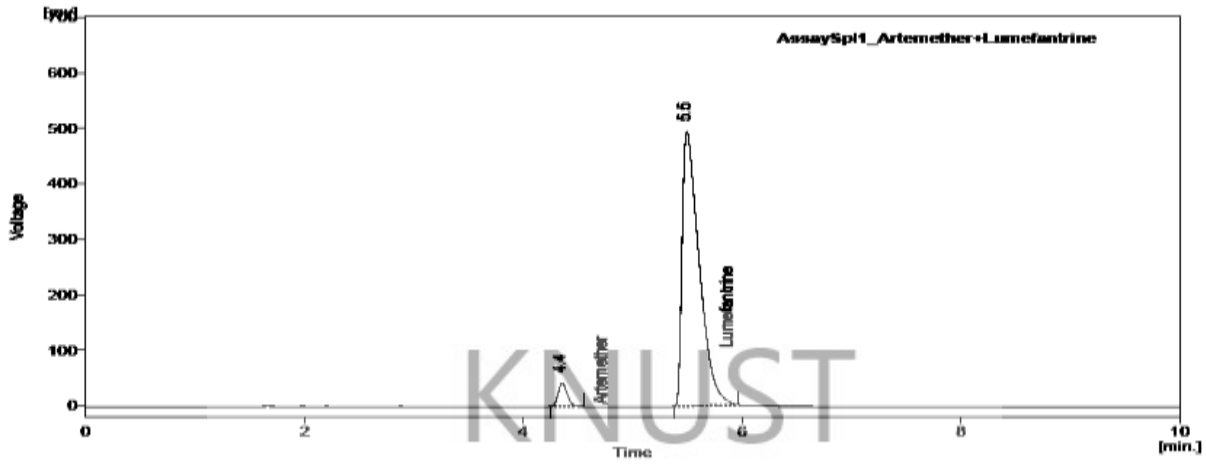


Figure 15: Chromatogram of sample B

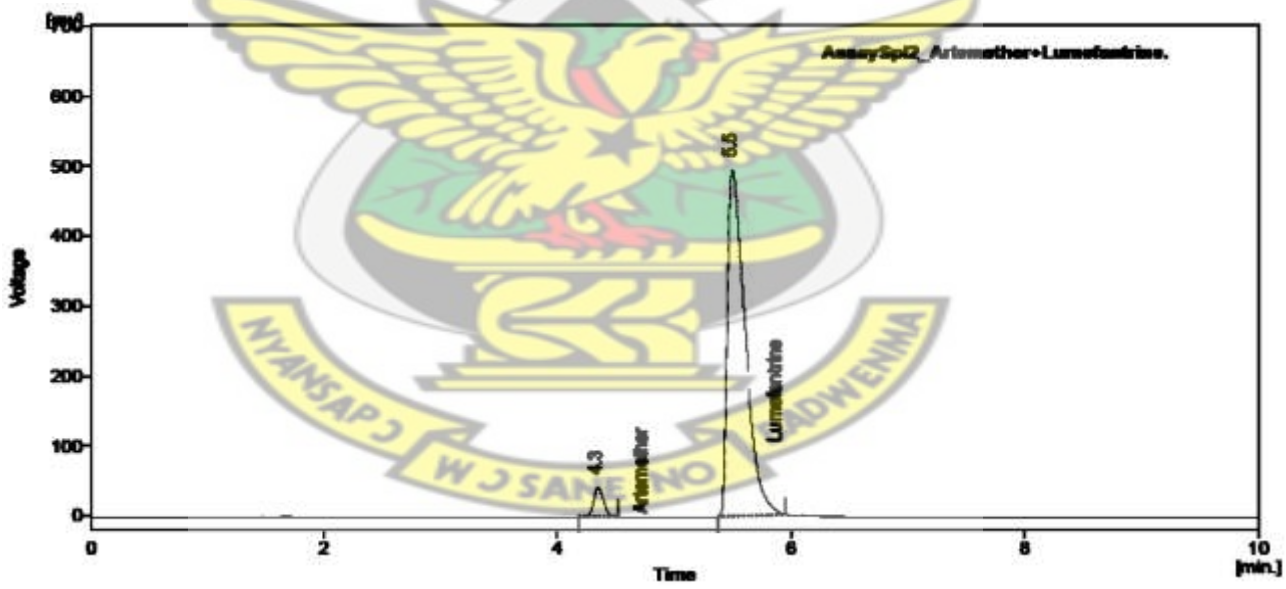


Figure 16: Chromatogram of sample C

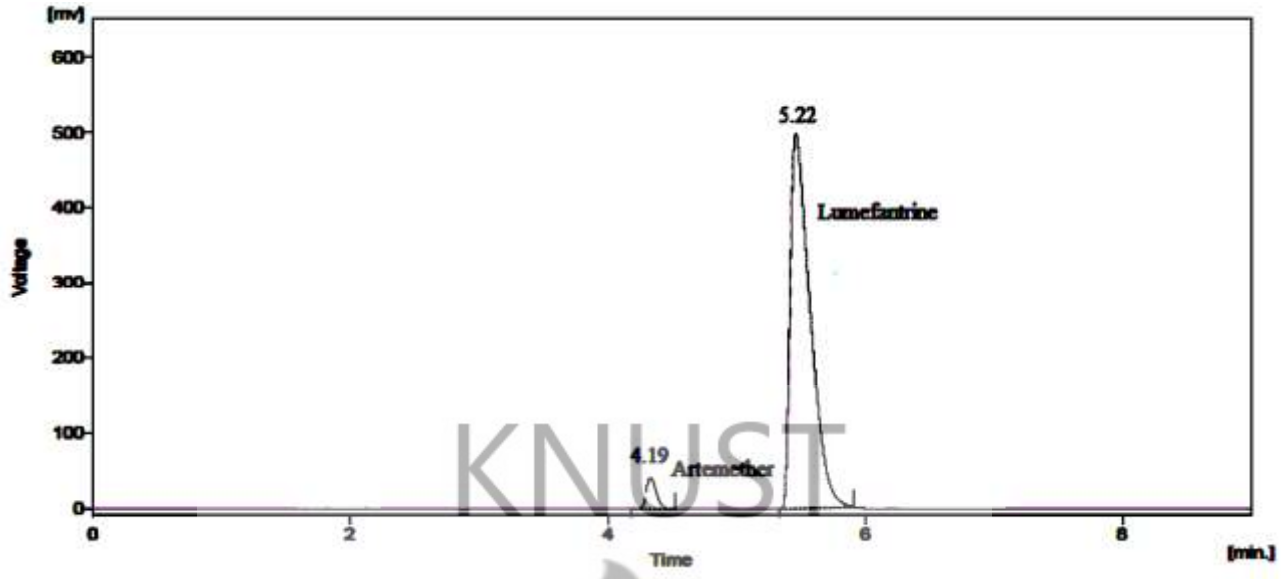
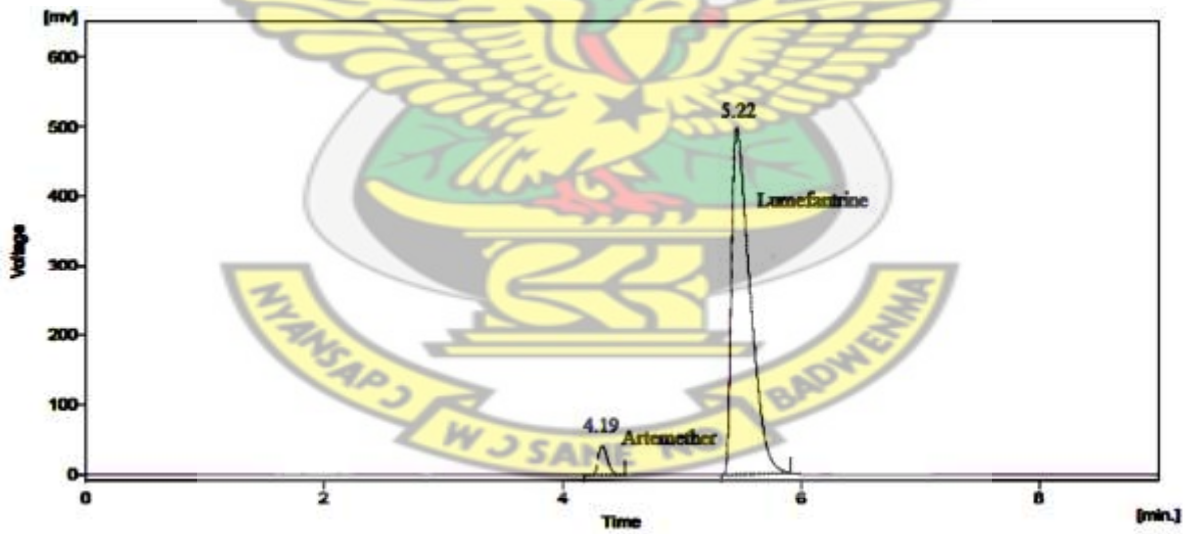


Figure 17: Chromatogram of sample E



4.2 Discussion

4.2.1 Discussion

4.2.1.1 Identification of Reference Standard

The relevance of the use of reference standards in pharmaceutical analysis as a mean of ensuring the validity of an analytical data cannot be overestimated. They serve as the basis of comparison with active pharmaceutical ingredients in formulated drug products for establishing the quality of these products. The purity and authenticity of these reference standards are of prime importance and it is imperative that thereare pharmaceutical analytical method development such as that conducted in this study. It was to this relevant effect that all reference standards used under this study were qualitatively assessed. In orderto compare Artemether and Lumefantrine to standard samples, reference samples were obtained from Pharmanova. The use of these standards ensured the authenticity of these drugs for both qualitative and quantitative works.

4.2.1.1.1 Artemether

Artemether was identified by color tests stated in the IP and also by its melting point. A yellow color was produced when potassium iodide was added to the ethanolic solution of the sample and heated. Adding a drop of vanillin/sulphuric acid TS1 to the ethanolic solution of the sample also produced a pink color.

4.2.1.1.2 Lumefantrine

Lumefantrine was identified as stated in the IP (Draft). The methanolic solution of the Lumefantrine gave a specific absorbance of 324nm (by means of a spectrophotometer) which falls within the reference range of 314–348 as stated in the IP Draft.

4.2.2 HPLC Method Development

4.2.2.1 Optimization of chromatographic mode

Artemether and Lumefantrine, both the API's are non-polar in nature, hence either reversed phase or ion-pair or non-aqueous chromatography can be used. Reversed phase HPLC (RP-HPLC) was selected for the initial separations because of its simplicity and suitability. Several mobile phase compositions were investigated (Table 3) after which the methanol/buffer (72:28) was chosen due it's reduced cost and a smaller retention time. This method is cheaper as compared to methods developed by Cesar *et al*, 2008; Vikas *et al*, 2011; Sridhal *et al* 2010; Khalil et al, 2011; Pankaj et al, 2012 and Prasanna et al, 2010. In these methods, Acetonitrile was used as the major component of the mobile phase. The relatively high cost of Acetonitrile makes these methods expensive.

The PDA detection used by (Kakde *et al*, 2011) makes it expensive as compared to this developed method.

In designing the conditions for this method inorganic buffers were considered and after thorough investigation the Sodium Phosphate buffer which gave optimal results was chosen. The caution however was that, Phosphate buffers forms crystals that block the plumbing system of the chromatograph generating undesirable back pressure and causing the wear and tear of moving parts of HPLC pumps and eventually may break down the expensive HPLC chromatograph [Foulstone, 2001]. Therefore thorough long wash out periods are usually a basic requirement after using mobile phase systems which contains phosphate buffer and inorganic buffers in general [Standard Treatment Guideline, 2005].

The buffer was used in combination with methanol which is also cheaper as compared to other HPLC organic modifiers such as acetonitrile. From the results, a mobile phase system that consists of 72:28Methanol: Phosphate (pH 2.8) gave the optimal separation of artemether from lumefantrine with retention times of 4.20 ± 0.15 and 5.20 ± 0.15 minutes respectively.

For any HPLC method, the cost of reagents is a major challenge especially for developing countries like Ghana. This method was designed using 72% of methanol (a cheaper solvent compared to acetonitrile in moderate amount) and 28% of phosphate buffer which is also cheaper. In choosing a stationary phase, the polarity of the two compounds was considered. Both compounds have polar groups and a reversed phase stationary phase was thus suitable. An Octadecylsilane (ODS), C18 (2) Ultracarb 3µ was used as mobile phase. The results showed that artemether was eluted faster than lumefantrine. This could be explained from the mechanism of separation that the main underlying factor for separation of artemether from lumefantrine is the control of pH of the mobile phase. The compound that is fully ionized easily has a greater affinity for the mobile phase but lower interaction with stationary phase and is thus eluted faster. Also, artemether was readily soluble in the phosphate buffer solution than lumefantrine and with a mobile phase system composing of abuffer, it is be able to elute faster the compounds that are more soluble in the buffer (BP, 2007).

For ionizable compounds, since changes in solvent content of mobile phase affect the retention behavior (Posner *et al*., 2004). Optimization of the separation of these mixtures is often carried out at a fixed pH and most compounds get ionized at a pH within 1-2 units of their pKa values. Lumefantrine can be ionized if at least one or two of the ionizable groups are ionized. However, at a mobile phase pH of 2.8 none of the groups in Artemether (-CH3), becomes ionized and thus the compound is not partially ionized and this decreases its affinity for the mobile phase.

Other conditions of the method that were established include the sensitivity of the recorder also designated as the Absorbance Units Full Scale (AUFS) and pump pressure which is very critical in the design of an HPLC method. A pump pressure of 1950-2750 psi was established and the recorder sensitivity was 0.05 AUFS.

4.2.2.2 Optimization of detection wavelength

Detection wavelength was selected by scanning standard drug over a wide range of wavelength from 200nm to 400nm. A fixed concentration of analyte (10µg/ml) was analyzed at different wavelength. As per the response of analyte, the λ max value was found to be 209nm and 335nm for Artemether and Lumefantrine, respectively. Using this data 222nm was selected as a detection wavelength at which the components showed well resolved peaks.

4.2.3 Validation

The linearity, precision (reproducibility and repeatability), accuracy, and specificity data obtained after using an analytical method. As a result, this method was validated as per the ICH guidelines on analytical method validation.

The method demonstrates linearity over a concentration range of 10-100ug/ml for lumefantrine and 3-20ug/ml for artemether. From the calibration curve the R^2 value over this range was found to be 0.9991 and 0.9995 for Artemether and Lumefantrine respectively. This indicates a linear relationship between the concentrations of the two analytes.

When some conditions of the mobile phase such as pH and column used for the method were varied, there was no statistically significant difference between results of the varied and old conditions of mobile and stationary phases using the student t-test. The flow rate and wavelength of detectionwere varied, with no significant difference in the peak areas. This indicates that the method is robust under varying condition of both flow rate and wavelength of detection (USP SALMOUS edition 2008).

The developed method was validated using the ICH guidelines. The method's accuracy, inter day and intraday precision, robustness have recoveries falling in the range of (90-110)% of the USP SALMOUS edition.(Tables 7 to 11).

Precision is reflected by percentage RSD values less than 2. These low values suggest high sensitivity of the developed method (USP SALMOUS edition 2008).

With Robustness, it was observed that, there was no marked changes in the chromatograms characteristics which demonstrated that the method I srobust.

With the developed method, the LOD and LOQ for LU were 338 and 1129ug/ml (0.333 and 1.129mg/L) respectively. AM had 90 and 300ug/ml (0.090 and 0.033mg/L as LOD and LOQ respectively.

The percent recoveries for Accuracy was within range of (97.6 -99.86)% for AM and 97.5-99.2 for LU which indicates that the method was accurate.

The percent purities of the standards were determined from the calibration curve. LU had a higher purity of 98.4%, whereas AM had purity of 94%.

The recoveries obtained for Accuracy, intra and inter day precision, robustness all fell within the USP SALMOUS edition range of (90-110)%. The method is therefore reliable and accurate (IP, 2007).

4.2.4 Assay of Tablets

According to the USP SALMOUS standard, Artemether-Lumefantrine tablets should contain not less than 90.0 percent and not more than 110.0% of the labeled amounts of Artemether and of Lumefantrine.(USP SALMOUS edition 2008).

Six commercial brands of (two local and four foreign) AM-LU tablets were analyzed for active substances using the developed method. Triplicate determinations were carried out. The respective contents of AM and LU were 93.5/91.3, 99.2/97.2, 96.62/97.0, 95.82/98.5, 105.2/99.9, and 92.05/95.8 percentage of the declared contents for Malar2DS, DANMETHER, Malafantrine, Coartem, Lonart and Artemos plus. The formulations complied with the (90-110%) of the label claimof the IP

From the HPLC method of, the mean contents of artemether in the brands of tablets assayed ranged from 93.5 to 105.0%w/w of the labeled amount with standard deviations from 0.07 to 1.33. The mean contents of lumefantrine in the tablets from the HPLC method ranged from 91.3 to 99.9%w/w with standard deviations from 0.07 to 0.89

Both local and foreign brands of the tablets passed with respect to both AM and LU contents, with China's Lonart having the highest percentage content for both AM and LU.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Considering the increasing use of ACT to treat malaria in endemic areas, the availability of simple and rapid analytical method is essential to evaluate the quality of formulations being used currently. From the present study it can be concluded that the optimized and validated RP-HPLC method is simple, sensitive, precise, accurate and reproducible. The developed method has been validated as per the ICH guidelines and it meets all the acceptance criteria given in ICH guidelines.

Hence the method can be used in routine analysis for the simultaneous determination of Artemether and Lumefantrine in bulk as well as in pharmaceutical preparations.

5.2 Recommendation

Further studies could be performed to quantify the amount of Artemether and Lumefantrine in in-vivo analysis to see how much of these actives is actually excreted and also perform studies to quantify the amount of degradation products in drug tablet so as to advise health professionals on the temperature condition to store the tablets

In addition to the above, other artemether lumefantrine tablets (over the counter drugs) on the local markets containing these API's should be analyzed using this method ascertain its quality.

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