KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

THE USE OF SURROGATE REFERENCE STANDARDS IN QUANTITATIVE RP-HPLC FOR THE ANALYSIS OF MULTICOMPNENT FORMULATIONS: A CASE OF ARTEMETHER AND LUMEFANTRINE

BY

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DECLARATION

I declare that this thesis is my own work and that it does not contain materials published by any other investigator except where due references and acknowledgement have been given. I further declare that this work has not submitted to any institution or body for the award of any degree or certificate.

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DEDICATION

I dedicate this project to the Almighty God and my family.

ABSTRACT

A simple, rapid isocratic RP-HPLC method was developed for the quantitative analysis of Artemether and Lumefantrine using Diclofenac and Ibuprofen as surrogate reference standards. The assay of Artemether and Lumefantrine was done by the use of a surrogate constant calculated for each surrogate in relation to the particular analyte of interest. The analysis was performed on a Kromasil C-18 (4.6 x 250 mm) 5µm column using a mobile phase composition of methanol and acetate buffer (pH 2.8) in a ratio of 85:15 (v/v). An isocratic mode of elution was employed using a flow rate of 1 ml/min and a UV wavelength of detection at 230 nm. The mean retention times in minutes obtained for Diclofenac, Ibuprofen, Artemether and Lumefantrine were 4.60 ± 0.031, 5.08 ± 0.017,7.48 ± 0.056 and 8.63 ± 0.028 minutes respectively. The surrogate constant obtained for Diclofenac and Ibuprofen when used as surrogate for Artemether were 0.007637 ± 0.00045 and 0.02477 ± 0.00074 respectively. The surrogate constant obtained for Diclofenac and Ibuprofen when used as surrogate for Lumefantrine were 2.919989 ± 0.1847 and 10.513 ± 0.3051 respectively. The effect of concentration on the surrogate constant was investigated and concentration ratio limits were specified for each surrogate. Percentage contents obtained for the four commercial brands of tablets AL 1, AL 2, AL 3 and AL 4 using Diclofenac as surrogate for Artemether were 96.76 ± 0.7132, 97.92 ± 0.7186, 98.16 ± 0.6411 and 96.26 ± 0.9900 respectively while percentage contents of 105.47 ± 0.6044, 103.45 ± 0.8272, 102.62 ± 0.6251 and 104.77 ± 0.3760 respectively were obtained for Lumefantrine using the same surrogate. Percentage contents obtained for the four commercial brands AL 1, AL 2, AL 3 and AL 4 using Ibuprofen as surrogate for Artemether were 98.81 ± 0.9661, 97.83 ± 0.6382, 98.64 ± 1.2596 and 97.03 ± 0.5989 respectively while

percentage contents of 104.12 ± 0.8054, 102.42 ± 0.9090, 101.99 ± 0.4496 and 103.79 ± 0.5443 respectively were obtained for Lumefantrine using the same surrogate. The method was validated in accordance to the ICH guidelines and was shown to have acceptable levels of accuracy, precision, robustness, linearity over the given concentration range and sensitivity for Artemether and Lumefantrine using Diclofenac and Ibuprofen as surrogate reference standards. The results obtained for the assay of Artemether/Lumefantrine tablets using the developed method showed no statistical difference when compared to results obtained for the same brands assayed with the standard method in the International Pharmacopeia.

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LIST OF ABBREVIATIONS

ACT	Artemisinin Combination Therapy
BP	British Pharmacopeia
GC-MS	Gas Chromatography – Mass Spectrometry
ITN	Insecticide-treated Mosquito Net
IP	International Pharmacopeia
ICH	International Conference on Harmonization
HPLC	High Performance Liquid Chromatography
SRS	Surrogate Reference Standards
RDT	Rapid Diagnostic Test
LC –MS	Liquid Chromatography- Mass Spectrometry
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RSD	Relative Standard Deviation
UV	Ultraviolet

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Drug counterfeiting is an age old problem which is constantly crippling the pharmaceutical sector and the advancement of technology has made drug counterfeiting more complicated than ever. One of the factors that propel counterfeiting is drugs in high demand. Thus drugs that are fast moving and have a ready market are always the target. It is estimated that 10% of global pharmaceutical sales are counterfeit and it is worth about US$ 21 billion (WHO, 1999).

The World Health Organization estimates that about 3.2 billion people are at risk of getting malaria infections with young children, pregnant women and travelers being the most vulnerable group. Sub-Saharan Africa alone carries about 88% of the global disease burden(WHO, 2016). Anti-malarial drugs no exception to the counterfeiting menace because it has been estimated antimalarial drugs constitute about 7% of counterfeit drugs sold globally (WHO, 1999) .

However the Drug Quality and Information Program in 2009 discovered counterfeit Coartem, an ACT, on the Ghanaian market and this is a clear indication that this menace is not far from us (Nyarko and Nettey, 2013).

Oral monotherapy for treatment of malaria was faced out due to the development artemisinin drug resistance in the malaria parasites. This led to the introduction of Artemisinin Combination therapy (ACT) by W.H.O. in the management of malaria. This appears to be the last resort for acute malaria management even though there is ongoing research into the development of

malaria vaccines. There is therefore the need to ensure that these anti-malarial drugs manufactured and imported into the country are of the right quality.

Poor quality or counterfeit anti-malarial drugs poses a lot of risk to the clients such as treatment failure, adverse side effects, prolonged disease conditions, development of drug resistance and eventually death as a result of complications. The health care system will also be greatly affected since clients will distrust the system and there will be waste of financial resources when medicines used for clients exhibit high treatment failure rates. There is therefore the need as a country to stop this counterfeiting menace or reduce it to the barest minimum. Thus onus task lies on regulatory authorities to monitor the quality of anti-malarial drugs manufactured in the country as well as those imported into the country.

Drug quality control entails the totality of all efforts made to ensure that medicines conform to established specifications of identity, potency, purity, uniformity, stability and safety. This is done by using analytical procedures that discriminate as well as allow the detection and measurement of only the active ingredient in a formulation. Most regulatory authorities and manufacturing companies however use standards and analytical methods prescribed in the pharmacopeias to ascertain the quality of all drugs.

Analytical methods for pharmaceuticals has evolved of the years from simple titrimetry, UV-vis spectroscopy for quantitation and Infrared spectroscopy for qualitative analysis to more sophisticated techniques like mass spectrometry (GC-MS, LC-MS), chromatography, atomic absorption, fluorescence and flame emission. Analytical methods found in the official books (pharmacopeias)

heavily rely on chromatographic techniques. The chromatographic technique mostly used is High Performance Liquid Chromatography (HPLC) because this technique provides rapid and accurate quantitative and qualitative analysis.

HPLC techniques for the analysis of drugs found in the pharmacopeias even though very efficient require the use of tedious extraction procedures, complex solvent systems and very expensive reagents. Some also require the use of complex gradient elution method. Quantitative analysis using HPLC requires the use of Chemical reference standards which are sometimes very expensive and difficult to access.

These factors coupled with inadequate financial resources hampers the ability of the regulatory authorities in the country to effectively monitor the quality antimalarial drugs manufactured in the country as well as those imported into the country. The gap created in checking counterfeit antimalarial drugs can be bridged by developing cheaper alternative methods which can be used in routine analysis.

Asare Nkansah *et al*, 2011 came up with a proposal that compounds with similar physicochemical properties can be used as surrogate reference standards in quantitative HPLC analysis(Asare-Nkansah et al., 2011). This study seeks to develop alternative method for quantitative analysis of anti-malarial drugs using surrogate reference standards.

1.2 PROBLEM STATEMENT

Surrogate reference standards (SRS) in the past have been used for the assay of single analyte for single component pharmaceutical dosage forms. There is

however the need to investigate the feasibility of using surrogate reference standards for simultaneous assay of multi-component pharmaceutical dosage forms since most formulations nowadays come in multi-component to improve patient compliance and synergic therapeutic effect in treating any disease condition.

1.3 RESEARCH OBJECTIVES

1.3.1 General Objective

Investigate the feasibility of using Ibuprofen and Diclofenac as SRS in RPHPLC analysis of Artemether and Lumefantrine as well as investigate concentration effect of both surrogates and analytes on the surrogate constant.

1.3.2 Specific Objectives

- Develop and design a RP- HPLC method for quantitative assay of Artemether and Lumefantrine using Ibuprofen and Diclofenac as surrogate reference standards
- Validate the developed method according to the ICH guidelines
- Determine surrogate constants (K) that can be effectively used for the analysis
- Investigate the concentration ratios for which the surrogate constant can be used
- Determine the percentage content of various brands of Artemether/ Lumefantrine tablets using the developed method
- Compare the developed method statistically to standard methods in the International Pharmacoepia (IP)

1.4 HYPOTHESIS OF STUDY

The study as proposed by Asare-Nkansah *et al* for the estimation of percentage content of analyte using a surrogate reference standard indicates that;

In HPLC;

The peak area (A) in a chromatogram is directly proportional to the concentration of the analyte (C).

This implies

A α C ……. Eqn 1

For similar compounds the constant remains the same hence,

$$\frac{A(analyte)}{C(analyte)} = \frac{A(standard)}{C(standard)} \text{……. Eqn 2}$$

However, for different compounds

$$\frac{A(analyte)}{C(analyte)} \neq \frac{A(standard)}{C(standard)} \text{…….Eqn 3}$$

Thus introducing a constant of proportionality **K**, known as the surrogate constant;

$$\frac{A(analyte)}{C(analyte)} = \boldsymbol{K}\frac{A(standard)}{C(standard)} \text{…… Eqn 4}$$

$$\boldsymbol{K} = \frac{A(analyte)xC(standard)}{C(analyte)xA(standard)} \text{……Eqn 5}$$

The concentration of the analyte can easily be calculated when the surrogate constant is determined using the developed method. Hence equation 5 above when rearranged becomes;

$$C(analyte) = \frac{C(standard)xA(analyte)}{KxA(standard)} \quad \text{……..Eqn 6}$$

1.5 JUSTIFICATION

Pharmaceutical formulations in the past were mostly single component formulations. The advancement of technology, drug resistance, improvement of patient compliance and therapeutic efficacy has made it imperative to manufacture multi-component pharmaceutical formulations. Monitoring the quality of Artemether/Lumefantrine drugs on the market by regulatory authorities using standard methods in the pharmacopeia requires the use of Chemical reference standards for both Artemether and Lumefantrine. The cost of these reference standards and difficulty importing them into the country seriously hampers monitoring and checking of counterfeit Artemether/ Lumefantrine on the Ghanaian market. These analytical methods invariably become expensive and very tedious to use for routine analysis.

Table 1.1 Price quotes for Chemical Reference Standards from USP daily Reference Standards Catalog February, 26 2016

Catalogue No.	Reference Standard	Current Lot	Quantity/Unit (mg)	Unit Price ($)
1042780	Artemether	H0M313	100	222.0
1370746	Lumefantrine	G0L394	100	222.0

Alternative analytical methods which are relatively cheaper and less time consuming ought to be developed to enhance monitoring.

Surrogate reference standards (SRS) in the past has been used to assay single component formulations. Thus the possibility of using surrogate reference

standards in the assay of multicomponent formulations ought to be investigated with Artemether/ Lumefantrine as a case study.

CHAPTER TWO

LITERATURE REVIEW

2.1 MALARIA

Malaria is a disease caused by Plasmodium parasites which is transmitted from person to person through the bites of infected anopheles female mosquitoes. There are five (5) species of the Plasmodium parasite which causes malaria in humans. Of the five parasite species P. falciparum and P. vivax are responsible for most malaria infections however P. falciparum is the most virulent (Health, 2016).

The initial signs and symptoms of malaria are non-specified and usually presents as a minor illness which resembles a viral infection. The initial symptoms are fatigue, headache, abdominal discomfort, muscle and joint aches. These are followed by chills, fever, headache, anorexia and vomiting. However in children malaria presents as lethargy, cough and poor feeding (WHO, 2015).

It is estimated by WHO that about 3.2 billion of the world's population are at risk of being infected with malaria. Thus malaria affects all classes of people from young to old but the most vulnerable group are pregnant women, children under five years, travelers from malaria-free areas and people living with HIV/AIDS. From the year 2000 to the year 2015, people at risk of getting malaria fell by 37% while people at risk of death from malaria reduced by 60%(WHO, 2016).

Malaria is prevalent in most developing countries and countries in Sub-Saharan

Africa are no exception. Sub-Saharan Africa alone caries about 80% of the global malaria disease burden and malaria related deaths are about 90% (WHO, 2016).

2.2 TRANSMISSION

Malaria infection is transmitted from person to person through its vector which is the female Anopheles mosquito. The most important malaria vectors are active from dusk till dawn. The rate of malaria transmission is dependent on the host, the parasite, the vector and the environment.

When an infected female Anopheles mosquito feeds on a person, it injects the sporozoites form of the parasite into the human bloodstream. The sporozoites move along with the blood and finally reside in the liver cells. The sporozoites grow within five to sixteen days. They multiply rapidly to produce several haploid forms (merozoites) for every liver cell. These merozoites move from the liver cells into the blood where they undergo asexual reproduction in the red blood cells. They develop and mature into schizonts in the red blood cells. The red blood cells rapture releasing the newly formed merozoites which attack other red blood cells. This cycle of attack and rapture of the red blood cells repeats every 3 days. The merozoites in some of the red blood cells develop into male and female gametocytes. These sexual forms of the parasite are then released into the blood. When the mosquito sucks the infected blood into its body, the red blood cells break up to release the gametocytes. The gametocytes which are released into the mosquito develop into gametes. The male and female gamete fuse to form zygotes. They then mature into oocyts in the mosquito midgut wall and are then released into the body of the mosquito after 8-15days.

The asexual form of the parasite then migrate to the salivary glands and the cycle then starts all over when the Anopheles mosquito bites humans (Health, 2016).

Anopheles mosquito breeds in habitats with fresh water, shallow collection of water in tins, car tyres, muddy areas or marshy areas and puddles. They lay eggs in water which eventually hatch into larvae. Larvae then develop to adult mosquitoes. Human blood is used to nurture eggs.

Malaria transmission increases in the rainy seasons where climatic conditions in Africa, such as rainfall patterns, humidity and temperature, favor the survival of the mosquito. These conditions allow for the parasite to develop completely inside the mosquito and mosquitoes have a longer life span. During this season, people with low or no immunity are affected by malaria.

Immunity in humans is another factor that influences the transmission of malaria. Partial immunity is acquired after years of exposure to the parasite. This usually happens amongst people staying in areas which are noted for high transmission. This kind of immunity does not provide complete protection against malaria but reduces the risk of malaria developing into severe malaria.

2.3 PREVENTION

2.3.1 Vector Control

One of the main ways to reduce malaria transmission is to control the vector. If the vector (Anopheles mosquito) is properly controlled in a particular area, then a level of protection will be conferred on that area. There are two forms of vector control. They are indoor residual spraying and insecticide-treated mosquito nets.

Insecticide-treated mosquito nets (ITNs)

People in areas where malaria can be easily acquired are given ITNs for free under public health programmes intended to reduce the risk of people of all classes from getting malaria. They are educated on the proper use of ITNs and are supposed to sleep under these treated nets every night as well as maintain them properly.

Indoor spraying with residual insecticides

Malaria transmission can be effectively reduced by spraying with very powerful insecticides to kill mosquitoes. The type of insecticide used will indicate how effective indoor residual spraying will be thus indoor residual spraying can be effective for 3-6 months. This is mostly done during seasons where malarial transmission is highest to give some level of protection to people at risk.

2.3.2 Prophylaxis with Anti-malarial drugs

Chemoprophylaxis with anti-malarial medicines can be used in malaria prevention for non-immune travelers. Anti-malarials used for prophylaxis include Atovaquone/Proguanil (Malarone), Doxycycline, Mefloquine and Primaquine. Pregnant women residing in vicinities that have moderate to high malaria transmission rates are at a very high risk of being infected with malaria. They are to be given intermittent preventive treatment. The drug of choice for this form of treatment is Sulphadoxine-Pyrimethamine as recommended by WHO. Intermittent preventive treatment scheduled at each antenatal visit after the first trimester. Three (3) doses of Sulfadoxine-Pyrimathamine is also recommended for infants residing in areas with high malaria transmission rates (WHO, 2016).

2.4 TREATMENT

Malaria is a highly treatable and an easily preventable disease. Any form of malaria treatment should;

- Rapidly and completely eliminate the plasmodium parasite from the bloodstream of a person diagnosed with malaria so as to prevent the condition from moving from acute malaria to severe malaria.
- To avoid malaria infection from becoming chronic which invariably leads to some complications such as anaemia.

Patients suspected to have malaria should be properly diagnosed by confirmation of the presence of Plasmodium parasite either by microscopy or rapid diagnostic test (RDT) (WHO, 2015). Treatment can then be started immediately after diagnosis and should be started within 24 hours of the onset of fever to avoid any complications as a result of malaria.

The treatment of malaria with oral artemisinin-based monotherapy is responsible for development of resistance to artemisinin derivatives. The WHO has tasked regulatory authorities to ensure that the production and marketing of oral monotherapy is halted. They are also to promote the availability of artemisinin-based combination therapies of the highest quality on the market. An effective antimalarial therapy reduces morbidity and mortality as well as reduces the risk of antimalarial resistance.

The mainstay of all malaria treatment nowadays is artemisinin based therapies and steps must be taken to ensure that their efficacy is preserved as no new classes of antimalarial drugs have been discovered or produced in recent times.

Artemisinin based combination therapies (ACT) are now required for the management of uncomplicated malaria in adults and in children except pregnant women in their first trimester.

These include;

- Artemether + Lumefantrine
- Artesunate + Amodiaquine
- Artesunate + Mefloquine
- Dihyroartemisinin + Piperaquine
- Artesunate + Sulfadoxine-Pyrimethamine (SP)

For complicated or severe malaria, treatment involves intravenous or intramuscular artesunate for at least 24 hours. This form of treatment is given until the patient can take oral medications. ACT is given for 3 days after parenteral Artesunate therapy once the patient can take oral medication (WHO, 2015).

2.4.1 Classification of Antimalarial Drugs.

Antimalarial drugs can be classified under two (2) main categories;

1. Type of antimalarial activity
    a. **Tissue schizonticides** -

        For casual prophylaxis and prevention of relapse: Primaquine and Pyrimethamine

    b. **Blood schizonticides** - Chloroquine, quinine, mefloquine, halofantrine, pyrimethamine, sulfadoxine, sulfones and tetracyclines.

    c. **Gametocytocides** – Chloroquine, quinine, Primaquine.

    d. **Sporontocides** – Primaquine and Chloroguanide

2. Chemical structure of the Compound

a. **Aryl amino alcohols -** Quinine, quinidine, mefloquine, halofantrine.

b. **4-aminoquinolines –**Chloroquine and amodiaquine

c. **Folate synthesis inhibitors –** Pyrimethamine, sulphonamides, chloroproguanil, proguanil and Sulphones

d. **8-aminoquinolines –** Primaquine

e. **Antimicrobials -** doxycycline, clindamycin, tetracycline, azithromycin, fluoroquinolones

f. **Peroxides -** Artemether, Arteether, and Artesunate.

g. **Naphthoquinones –** Atovaquone

h. **Iron chelating agents:** Desferrioxamin (Srinivas, 2016).

2.5 HPLC METHOD DEVELOPMENT

HPLC is a chromatographic separation technique in which a sample mixture or solvent (mobile phase) is pumped under very high pressure through a column with tightly packed fine particles (stationary phase) in other to achieve high resolution separations (Harris, 2010). It basically consists of reservoirs which houses the mobile phase, a pump which pumps the mobile phase through the stationary phase at high pressures, a column which is the stationary phase and this is where separation of components or compounds occurs, a detector which senses mixture components that elute from the column and an integrator which acquires data from the detector and displays a chromatogram based on the data received (Kenkel, 2010). The advancement of technology has led to development of advanced and sophisticated HPLC chromatographs with higher efficiency.

HPLC is one of the most widely used analytical techniques in the pharmaceutical sector because it has a lot of advantages over other separation techniques. It is mostly used for qualitative as well as quantitative purposes.

HPLC method development plays a very critical role in the pharmaceutical industry in relation to discovery, development and manufacture of pharmaceutical products. However, there are some steps to consider during method development. These include;

2.5.1 Information on the Sample

Information about the nature of the sample is vital in selecting the initial separation conditions. It minimizes time used in method development and also waste of reagents.

There are two approaches to obtaining information about the nature of the sample to be analysed either by the theoretical or the empirical approach. In the theoretical approach, information about the sample is obtained from literature whiles in the empirical approach information used in development is based on previous knowledge or past experience about working with the sample. The type of information acquired include;

- Chemical composition
- Physical characteristics – solid, liquid or semi-solid
- Molecular mass, chemical structure, pKa value
- Whether the sample contain impurities, degradation products or metabolites
- Whether is an acid, base or salt
- Solubility properties – solubility in water or organic solvents

2.5.2 Define separation goals

The goals of the method to be developed should be properly defined in order to skew the development process in a particular direction since there are different methods for different analytes. Defining these goals must be done having in mind the equipment and the type of reagents available. The overall cost of the method should also be taken into consideration whether the institution can afford it or not.

The analyst should have in mind some set of questions to help develop a method. Some of these questions are;

- Is the method for qualitative or quantitative purposes? Will it be used to characterize an unknown sample or to isolate a pure compound?
- Is it important to resolve all component of the sample to be analyzed?
- Which sample matrix will be analyzed? Organic solvent, plasma or urine.
- How many samples are to be analyzed?

2.5.3 Sample Pretreatment and Detector selection

Pretreatment of the sample is very important in HPLC. Due to the fragile and sophisticated nature of some components (pump and column) of the equipment it is necessary to remove any impurities from the sample. The sample should be properly prepared to aid in easy separation and detection. The nature of the sample determines the pretreatment method to be selected.

Samples to be used for analysis present in a myriad of different forms such as;

- Solutions which can be injected readily unto the column.

- Very dilute samples which need to be concentrated.
- For some samples, they are to be diluted, a buffer is to be added and other require an internal standard.
- Some are solids in nature and require further extraction.
- Others require removal of insoluble impurities by filtration (Snyder et al., 2012).

The detector selected should be sensitive enough to all sample components of interest. The UV detector is normally the first choice because it is very convenient and applicable to most samples to be analyzed. However other detectors can be used based on the properties of the sample to be analyzed. Some types of detectors are fluorescence, mass spectrometer, refractive index and electrochemical detectors.

2.5.4 Optimizing separation conditions

Optimization of separation conditions is based on the information obtained about the sample and the goals of the method to be developed. This is done to ensure good separation with complete resolution. A short run time should be targeted and should not be as short as 1 minute because good separation cannot be achieved at that run time. There are certain parameters to be looked at while optimizing a method. These are usually acquired through training and experience. They are;

Stationary phase – The type of stationary phase whether normal phase or reverse phase (C-18, cyano or phenyl) should be chosen but most samples are best separated on the reverse phase due to their intermediate polarity.

Column length and pore size also have an effect on separation. The higher the number of theoretical plates of a column the more efficient it is. If a short run time is needed, a shorter column is used or vice versa. The pore size has an influence on the rate at which the mobile phase moves along the column. The stationary phase is changed only after exhausting all mobile phase compositions.

Mobile phase – Good resolution is highly dependent on the mobile phase composition. The mobile phase must be continually modified to achieve good separation. Some variables to look at include; changing organic solvent, mixing two organic solvents, mixing an organic solvent with water, modifying the pH using different types of buffers, adding an ion pair reagent or a complexing agent.

Flow rate and temperature – Depending on the column length, a high flow rate gives a shorter run time and sometimes poor resolution between peaks while a low flow rate gives a longer run time and good resolution between peaks. Thus appropriate flow rate should be chosen.

A degree rise in temperature will reduce retention by 1 to 2 %(Snyder et al., 2012). This makes temperature an important parameter to consider after all other parameters mentioned above failed to improve resolution or separation.

Checking for problems

Most HPLC methods developed are to be used for routine analysis and a myriad of problems might arise as it is being used. It is important to anticipate these problems and find solutions if any before the method is finally released for use.

Problems that may arise include; split peaks, new interferences, retention drift, poor quantitative precision and short column life.

2.5.5 Quantitation and method validation

The developed method is validated before release. Validation is done based on accuracy, precision and linearity. Many HPLC method are used for quantitative analysis and require the use of chemical reference standards.

In quantitative analysis, the area of the peak is directly proportional to the amount of the component being separated which passes through the detector. This is in turn proportional to the concentration of that component in the mixture being analyzed. This implies that the larger the peak areas the higher the concentration of the component separated or vice versa.

2.6 REVIEW OF HPLC METHODS USED FOR ASSAY OF ARTEMETHER/ LUMEFANTRINE

A number of researchers have made an attempt to develop HPLC methods for the assay of Artemether/Lumefantrine in formulations such as tablets and suspensions. These are a review of the various methods developed;

Suleman *et al,* 2013 developed an isocratic elution method on a Halo RP-Amide column with a mobile phase composition Acetonitrile and 1 M phosphate buffer pH 3.0 (52:48 v/v). The injection volume was 3µl at a flow rate of 1.0 ml/min. Artemether was analyzed at 210nm and Lumefantrine at 335nm (Suleman et al., 2013). An HPLC- UV method for simultaneous determination of Artemether Lumefantrine at 254nm was developed by Arun R. *et al,* 2011 using a C-18 column and an isocratic mobile phase composition of Acetonitrile; 0.01 M potassium dihydrogen orthophosphate buffer at pH 4 (70:30)(Arun and Smith, 2011). Sridhar *et al* 2010 developed a method using a mobile phase composition

of Acetonitrile; 0.1%v/v orthophosphoric acid at pH 3 (60:40) on a C-18 column and a UV detection wavelength of 303 nm(Sridhar et al., 2010). Da Costa Cesar *et al, 2008* also developed an optimized method on a Zorbax SB-Ciano column with a mobile phase composition of Acetonitrile; 0.05%Triflouroacetic acid (60:40) at pH 2.35 using a flow rate of 1ml/min and UV detection of 210 nm(da Costa César et al., 2008). An isocratic mobile phase composition of Acetonitrile; phosphate buffer pH 3 (60:40) at a flow rate of 1.5ml/min using a dual UV detection of 210nm and 303nm was optimized on a C-18 column by Sunil *et al* , 2010(Sunil et al., 2010). Kalyankar *et al,* 2011 developed a method on a reverse phase column C-18 using 0.05% Triflouroacetic acid with triethlyamine buffer at pH 2.8; methanol (20:80) at a flow rate of 1.5 ml/min and UV detection at 210 nm (Kalyankar and Kakde, 2011). Shah *et al,* 2013 developed a RP-HPLC method on a C-18 column using a mobile phase combination of 0.05M potassium hydrogen phosphate ; Acetonitrile (70:30) adjusted to pH 3.0 with phosphoric acid at a flowrate of 1.5 ml/min and UV detection at 210 nm (Shah et al., 2013).

However, Daniel Afosah*, 2010*, used different methods in the estimation of Artemether Lumefantrine on a C-18 column. An isocratic mobile phase composition of methanol; 0.1% Triflouroacetic acid (90:10) at 335 nm for Lumefantrine and methanol; 0.04% Triflouroacetic acid (90:10) at 235 nm for Artemether was used all at a flow rate of 2.5 ml/min(Afosah, 2010).

2.7 ANALYTICAL METHOD VALIDATION

Many HPLC methods are developed for routine analysis in the industry thus they should be validated. Analytical method validation is a very important regulatory requirement in the pharmaceutical industry before the drug is

approved. Validation is done to determine how suitable the analytical method will be for its intended purpose.

Analytical method must be validated or revalidated when;

- The method is newly developed
- Conditions in the method have been changed or modified.
- The method is tested in a different laboratory with different equipment and different analysts
- Equivalence between a standard method and a new method ought to be demonstrated (Kalra, 2011).

Various analytical methods are developed for different procedures but there are four types of analytical methods that can be validated (ICH, 2005)

These are;

- Identification tests;
- Quantitative tests for impurities;
- Limit tests for the control of impurities;
- Methods that are designed to determine quantitatively the major component(s) in a drug substance

Validation parameters that should be considered according to ICH 2005 are as follows

Linearity, Limit of Detection, Limit of Quantification, Precision, Repeatability, Intermediate precision, Accuracy, Range and Specificity

2.7.1 Linearity

Linearity is a validation parameter that expresses the ability of an analytical method to produce responses that are directly proportional to analyte concentration within a given concentration range. The linearity of all active substances, additives or preservatives be established for any given analytical method. Linearity can be determined with a minimum of 5 concentrations. These concentrations can be prepared by diluting a standard stock solution and analyzing the solutions using the proposed method.

It can be determined by assessing visually a plot of responses against the analyte concentration. The plot is then subjected to regression analysis which is a statistical method. The correlation coefficient, y-intercept, slope of regression line and residual sum of squares are calculated in regression analysis. (ICH, 2005)

2.7.2 Limit of detection (LOD)

This is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The ICH describes three other methods used in limit of detection. These are visual evaluation, signal to noise ratio and standard deviation of the response and the slope (ICH, 1997).

In HPLC, there is baseline noise and the detection limit can be calculated based on the signal-to-noise ratio. This is expressed as concentration. Determination of signal-to-noise ratio is done by comparing already calculated signals of solutions with low concentrations to blank samples. From this comparison, the lowest concentration at which the analyte can be easily detected is established. A signal-to-noise ratio of between 3:1 and 2:1 is accepted (McPolin, 2009).

Other scientist define LOD in chromatography as the lowest concentration of the analyte that can be detected above baseline noise which is normally three times the noise level (Shabir, 2004).

2.7.3 Limit of quantitation (LOQ)

This is the level above which responses are easily determined with an acceptable level of accuracy and precision. The ICH describes three methods that can be used to determine the LOQ. These are visual evaluation, signal-to-noise ratio as well as standard deviation of the slope and the response(ICH, 2005). LOQ is usually expressed as concentration and this parameter is mostly used for determining of impurities or degradation products.

In HPLC, there is baseline noise thus the LOQ can be calculated based on the signal-to-noise ratio. The signal-to-noise ratio is determined by comparing measured signals of solutions with low concentrations to blank samples. This comparison is to aid in establishing the minimum concentration at which the analyte can be quantified with ease. Signal-to-noise ratio should be 10:1(Bliesner, 2006).

2.7.4 Precision

Precision is the agreement between a set of replicate measurements without assumption of knowledge of the true value. The precision of a proposed method can be represented as standard deviation and sometimes as coefficient of variation of the replicate measurement. Precision is performed under two (2) different levels. They are;

Repeatability (Intra-day Precision)

This is determined as the precision of an analytical method under the same operating conditions for a short period of time. This kind of variation is carried out by one analyst on an instrument. It is determined from a minimum of nine (9) concentrations over a specified concentration range or six (6) determinations of 100% of the target concentration (ICH, 1997)

Intermediate precision

This validation procedure seeks to assess the effect random events will have on the precision of an analytical method. A second analyst can repeat the analysis by making use of within-laboratory variations. These include carrying out or trying the developed method on different days using different equipment (Bliesner, 2006).

2.7.5 Accuracy

Accuracy is the nearness of a result or the mean of a set of measurements to the true value. It also gives an indication of any bias or systemic error in the developed method. According to ICH accuracy should be established across the range specified for the analytical method(ICH, 2005). Accuracy can be measured by determining the recovery of the active pharmaceutical ingredient (API), or the recovery of active drug component in a drug matrix. Other procedures used in accuracy determination involves spiking of the drug product matrix with equal amounts of the API (Bliesner, 2006). The API can also be spiked with known amount of impurities or a blank sample matrix spiked with known amount of the API. This type of accuracy measurement is a function of the efficiency of sample preparation (Kalra, 2011).

2.7.6 Range

A range for any analytical method is the interval between the highest and lowest concentration of analyte in sample for which it has been demonstrated that the analytical method has an acceptable level of sensitivity, accuracy, precision, and linearity. The minimum specified range for analytical method when used for the assay of drug substance or finished product is 80-120% of the test solution (ICH, 1997).

2.7.7 Robustness

Robustness measures the analytical method's ability to remain unchanged by small and deliberate changes in the analytical method conditions. This provides an indication of how reliable the method is during normal usage.

In initial stages of method development, the effect of major factors on the reliability or accuracy of the method should be investigated. The type of variations which are investigated include; monitoring how stable analytical solutions will be over a specified period, extraction time, varying equipment and varying analysts.

The variations investigated in High Performance Liquid Chromatography (HPLC) are; effect of pH of the mobile phase, deliberately modifying mobile phase composition, varying columns, temperature and flow rate(ICH, 1997). Other examples are Buffer concentration (ionic strength), injection volume, equilibration time and column age (Shabir, 2004)

Robustness is usually investigated in the initial stages of method development. Variations which cause significant changes in the developed method should be

closely monitored or clearly specified as a precautionary statement in the method procedure (Kalra, 2011, Schmauser, 2010).

2.7.8 Specificity

It is the ability of the analytical method to detect solely the analyte of interest in the presence of other components. These components include impurities, excipients and degradation products.

An analytical method is said to be specific when it measures only the desired analyte without any form of interference from other components of the formulation present in the sample matrix. Specificity is determined during validation by analyzing the blank, sample matrix and known quantities of impurities together to find out whether there will be any interferences (Bliesner, 2006).

There are other parameters that may be investigated in method validation which are not highlighted in the ICH guidelines. They are

2.7.9 Stability of Sample Solution

The stability of analyte or sample solutions should be closely monitored under normal bench conditions and normal storage conditions over a period of time. If there are any degradation products present, then special storage conditions such as refrigeration and protection from light can be specified in other to maintain the integrity of the solution during analysis.

2.8 SYSTEM SUITABILITY DETERMINATION

In instances where a method is modified, method is used on a different equipment in a different lab or for a newly developed method, system suitability must be determined. This is done by evaluating the components of the analytical

system to find out whether the performance of the system meets the standards required by the method. Each analytical method has its own system suitability parameters. For HPLC assays, the system suitability parameters include; tailing factor, resolution, precision of standard peak areas, comparison to a confirmation standard, retention time, theoretical plates and capacity factor (Bliesner, 2006).

2.9 A REVIEW OF CHEMICAL COMPOUNDS USED

2.9.1 Artemether

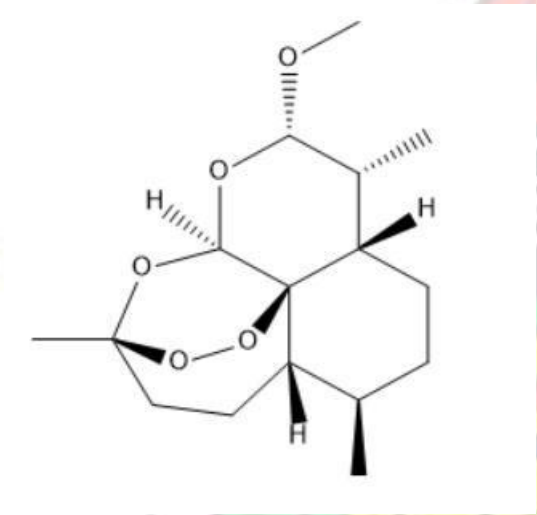


Figure 2.1 Structure of Artemether

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

Chemical Formula $C_{16}H_{26}O_5$

Relative Molecular Mass 298.37

Artemether is a sesquiterpene lactone which belongs to the artemisinin- based class of antimalarials. It is a methyl ester of dihydroartermisinin which comes as white crystals or a white crystalline powder. It is very soluble in dichloromethane, acetone, ethyl acetate and dehydrated ethanol but practically insoluble in water. It also has a melting point range of 86 – 90^{o}C. (IP, 2015)

Artemether comes in two formulations; parenteral form and as tablets which is administered in combination with Lumefantrine. It is used in the management of acute uncomplicated malaria and severe complicated malaria (Basco et al., 1998). Like other artemisinin-based compounds, artemether exhibits very rapid activity against blood schizonts of both *Plasmodium falciparum* and *Plasmodium vivax* . It is well absorbed after oral administration. Mean plasma concentration of Artemether is reached in about two hours. Concomitant intake of a high fat meal increases the bioavailability of artemether by 2 folds (Buck, 2010).

It undergoes extensive first pass metabolism and is converted to dihydroartemisinin which contributes to its antimalarial activity. The elimination half-life of artemether is about two hours.

2.9.2 Lumefantrine

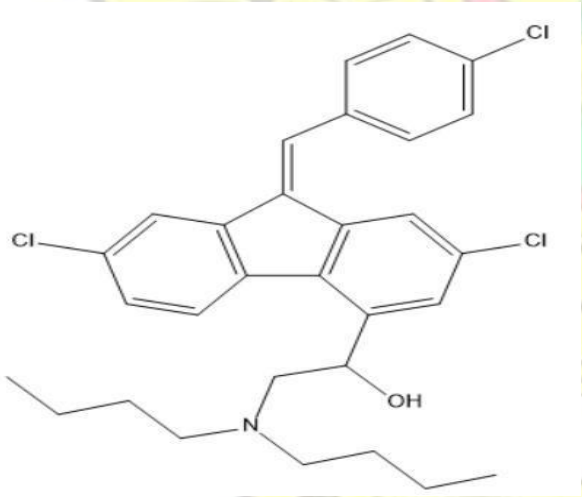


Figure 2.2 Structure of Lumefantrine

(1RS)-2-(dibutylamino)-1-(9Z)-2,7-dichloro-9-[(4chlorophenyl)methylidene]-9H-fluoren-4-yl) ethanol

Chemical formula $C_{30}H_{32}Cl_3NO$

Relative molecular mass 528.94

Lumefantrine, also known as benflumetol, is an antimalarial agent. It is a racemic mixture of a synthetic dibutyl aminoethanol fluorine derivative and belongs to the aminoalcohol class of compounds. It structurally resembles quinine, halofantrine and mefloquine (Buck, 2010). It is a yellow crystalline powder slightly soluble in methanol, soluble in dichloromethane but practically insoluble in water. It has a melting point range of 128 – 132 ºC.(IP, 2015)

Lumefantrine is manufactured as tablets in combination with Artemether and used in the management of acute uncomplicated malaria. It acts by preventing the breakdown of haemoglobin to the non-toxic hemozoin in the parasite. Thus accumulation of heme and free radicals eventually leads to parasitic death (Buck, 2010). It is slowly absorbed after oral administration. The peak plasma concentration of Lumefantrine is achieved in about 6 – 8 hours. Concurrent administration with a high- fat meal and milk increases bioavailability of Lumefantrine by 16-fold.

Lumefantrine has an elimination half-life of 2- 4 days. It is metabolized to the active desbutyl-lumefantrine by CYP3A4 enzymes. Desbutyl-lumefantrine is responsible for the antimalarial effect of Lumefantrine (Hoglund et al., 2015)

2.9.3 Ibuprofen

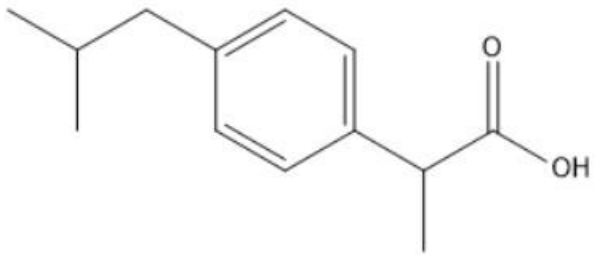


Figure 2.3 Structure of Ibuprofen

(2*RS*)-2-[4-(2-Methylpropyl)phenyl]propanoic acid.

Chemical Formula $C_{13}H_{18}O_2$

Relative Molecular Mass 206.28

Ibuprofen is a propionic derivative that was discovered in the year 1969 as an alternative to indomethacin and aspirin. It is a non-steroidal anti-inflammatory drug (NSAID) that comes as white crystalline powder or colourless crystals. It is freely soluble in methylene chloride, methanol and acetone but practically insoluble in water. It also dissolves in alkali hydroxide and carbonate solutions. It has a melting point range of 75 – 78ºC.

Ibuprofen is manufactured in different formulations; cream, gel, oral capsule, suspension and tablet. Prostagladins play a vital role in the mediation of fever, pain and inflammation. Ibuprofen prevents the production of prostaglandins by selective inhibition of cyclo-oxygenase – 1 (COX-1) and cyclo – oxygenase -2 (COX 2) involved in the synthesis of these mediators. Ibuprofen has profound antipyretic and analgesic activity but weaker anti-inflammatory effect compared to other NSAIDs. It has several indications such as management of pain in menstrual disorders, headache, fever, dental pain, rheumatoid arthritis, cystic fibrosis, prophylaxis of Alzheimer's disease, migraine and osteoarthritis (Bushra and Aslam, 2010).

It is well absorbed after oral administration. The mean plasma concentration is achieved within one to two hours after administration. It is also highly bound to plasma protein and metabolized extensively by the liver. The serum half-life of Ibuprofen is 1.8 to 2 hours (Bushra and Aslam, 2010).

2.9.4 Diclofenac Potassium

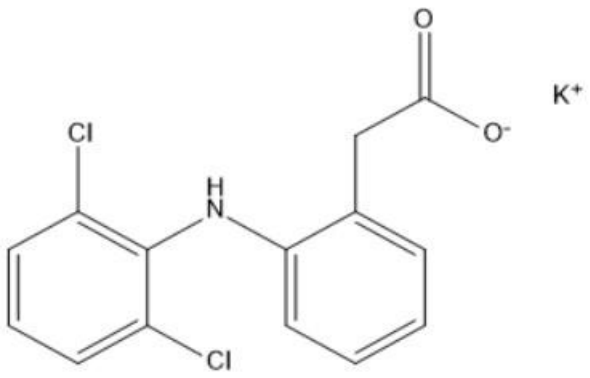


Figure 2.4 Structure of Diclofenac Potassium

Potassium [2-[(2,6-dichlorophenyl)amino]phenyl]acetate.

Chemical Formula $C_{14}H_{10}C_{l2}KNO_2$

Relative Molecular Mass 334.34

Diclofenac is classified as a non-steriodal anti-inflammatory drug (NSAID) and was first synthesized in 1973 as Voltaren by Rudolf Pfister and Alfred Sallmann. It appears as white or slightly yellowish crystalline powder and is slightly hygroscopic in nature. It is readily soluble in ethanol and methanol but slightly soluble in water and acetone. It has a melting point of about 280^0C with decomposition.

As an NSAID, diclofenac has antipyretic, analgesic and anti-inflammatory activities. It is a potent cyclo-oxygenase – 2 (COX-2) inhibitor compared to other NSAIDs. Diclofenac is manufactured in different formulations such as tablet, cream, suppository and injection. It is well absorbed after oral administration and has a short half-life. It also undergoes extensive first-pass metabolism and is metabolized by CYP2C enzyme to 4- hydroxydiclofenac which is the active metabolite.

Diclofenac has several therapeutic uses such as long-term treatment of gout, arthritis, rheumatoid arthritis, ankylosing spondylitis and osteoarthritis. It is also used to treat mild to moderate pain, postoperative pain and menstrual pain (Goodman L. , 2006).

CHAPTER THREE

METHODOLOGY

3.1 MATERIALS

EQUIPMENTS

Spectra Series P 100 isocratic pump, Perkin Elemer 785A programmable UV/Visible absorbance detector, eDAQ Power Chrom 280 integrator, Sartorius SE623P analytical weighing balance, Kromasil C-18 column 4.6 x 250 mm, FS 28H Fischer Scientific Sonicator, Stuart melting point apparatus, Hanna HI 2211 pH meter, Vacuum pump

REAGENTS

Materials and reagents were provided by the Department of Pharmaceutical Chemistry, Faculty of Pharmacy and they include; HPLC grade methanol (BDH), Glacial Acetic acid (BDH), Anhydrous sodium acetate, Ethanol (BDH), HPLC grade Acetonitrile (Fisher Scientific), Hydrochloric acid (E. Merck), Perchloric acid (Qualikems Chem Limited)

Samples used for analyses were donations from Ernest Chemist Limited, Ghana.

3.2 METHODS

3.2.1 Identification of Compounds Used

Identification of Artemether pure powder

i. Melting point was determined ii. 30 mg of Artemether was weighed into a test tube and 1ml of dehydrated ethanol added to it. 0.1g of potassium iodide was added to the solution and mixture heated on a water bath.

iii. 30 mg of Artemether was weighed into a beaker and 6 ml of dehydrated methanol was used to dissolve the powder. About three drops of the mixture was placed on a white porcelain tile and 1 drop of vanillin/sulphuric acid was added.

Identification of Lumefantrine pure powder

i. Melting point was determined ii. IR spectrum of Lumefantrine was obtained and compared to standard.

Identification of Diclofenac pure powder

i. Melting point was determined ii. 10 mg of Diclofenac pure powder was weighed and dissolved in 10 ml of ethanol. 1 ml of the resulting solution was pipetted and 0.2 ml of freshly prepared 0.6 %w/v potassium ferricyanide and 0.9%w/v ferric chloride solutions. The mixture was protected from light and allowed to stand for 5 minutes. 3 ml of 10 g/L of Hydrochloric acid solution was also added to the mixture. The mixture was then protected from light and allowed to stand for 15 minutes.

Identification of Ibuprofen pure powder

i. Melting point was determined ii. IR spectrum was obtained and compared to standard.

3.2.2 Assay of Pure Compounds Used

Assay of Diclofenac pure powder

250 mg of Diclofenac pure powder was weighed and dissolved in 30ml of glacial acetic acid. The resulting solution was titrated with 0.1 M perchloric acid and the end point was determined using potentiometry.(BP, 2013)

1 mL of 0.1 M perchloric acid is equivalent to 33.42 mg of $C_{14}H_{10}Cl_2KNO_2$.

Assay of Ibuprofen pure powder

450 mg of Ibuprofen pure powder was weighed and dissolved in 50 ml of methanol. The solution was titrated with 0.1 M sodium hydroxide using phenolphthalein as an indicator. A blank titration was carried out.(BP, 2013)

1 mL of 0.1 M sodium hydroxide is equivalent to 20.63 mg of $C_{13}H_{18}O_2$

Assay of Artemether pure powder

50 mg of Artemether pure powder was accurately weighed and dissolved in 20 ml of dehydrated ethanol. The solution was transferred into a 100 ml volumetric flask and made up to volume using dehydrated ethanol. 2ml of this solution was pipetted into a 100 ml volumetric flask and made to volume using 0.1 M hydrochloric in ethanol. The flask was stoppered and heated at 55 ^{0}C in a water bath for 5 hours. The solution was allowed to cool and absorbance measured at a wavelength of 254 nm. The percentage purity was calculated using A (1%, 1cm) value of 385.(IP, 2015)

Assay of Lumefantrine pure powder

450 mg of Lumefantrine pure powder was accurately weighed and dissolved in 50 ml of glacial acetic acid. The mixture was stirred continuously for 15 mins

to ensure complete dissolution of the powder. The solution was titrated with 0.1M perchloric acid and the end point was determined using potentiometry.(IP, 2015)

1 mL of 0.1 M perchloric acid is equivalent to 52.89 mg of $C_{30}H_{32}C_{l3}NO$.

3.2.3 HPLC Method Development

Chromatographic Conditions

An optimized condition was developed for the analysis of both surrogates and analytes on a reverse phase Kromasil C-18 (4.6 x 250 mm) 5µm. A mobile phase composition of methanol: acetate buffer (pH 2.8) in a ratio 85:15 v/v was used. The mobile phase was pumped through the column at a flow rate of 1.0 ml/min and the injection volume for sample solution used was 20µl. The wavelength of detection used for the analysis of both surrogates and analytes was 230 nm. Mode of elution used was isocratic. Sample preparation was done using the mobile phase (methanol: acetate buffer pH 2.8) (85:15 v/v) and methanol as a dissolution solvent. Extraction of Artemether/Lumefantrine from tablets was done with methanol.

Preparation of Acetate Buffer (pH 2.8)

4g of anhydrous sodium acetate was weighed and dissolved in 840 ml of filtered distilled water and transferred into a 1L volumetric flask.155 ml of glacial acetic acid was measured using a measuring cylinder and transferred into the volumetric flask and made up to the 1L mark with the filtered distilled water.

3.2.4 HPLC Method Validation

LINEARITY

Preparation of Standard solutions for Diclofenac, Lumefantrine and

Artemether for calibration curve

10 mg of Diclofenac pure powder, 4 mg of Lumefantrine pure powder and 400 mg of Artemether pure powder were accurately weighed, dissolved with 40 ml of methanol and transferred into a 100 ml volumetric flask (0.1mg/ml Diclofenac, 0.04mg/ml Lumefantrine and 4mg/ml of Artemether). The stock solution was sonicated for 10 mins and allowed to cool. The solution was then made up to the 100 ml mark with the mobile phase and mixed thoroughly. Serial dilutions were prepared from the stock solution by pipetting 5ml, 2.5 ml, 2 ml, 1 ml and 0.5 ml into separate 10 ml volumetric flasks to obtain various concentrations of Diclofenac, Lumefantrine and Artemether. The solutions were made up to the 10 ml mark using the mobile phase. The stock solution together with the other solutions prepared serially were filtered and analyzed using the chromatographic conditions developed. Multiple runs were made for each solution and the average peak areas calculated. Calibration curves were drawn for Diclofenac, Lumefantrine and Artemether and subsequently subjected to regression analysis.

Preparation of Standard solutions of Ibuprofen, Lumefantrine and Artemether for calibration curve

20 mg of Ibuprofen pure powder, 4 mg of Lumefantrine pure powder and 400 mg of Artemether pure powder were accurately weighed, dissolved with 40 ml of methanol and transferred into a 100 ml volumetric flask (0.2 mg/ml Ibuprofen, 0.04mg/ml Lumefantrine and 4mg/ml of Artemether). The stock solution was sonicated for 10 mins and allowed to cool. The solution was then made up to the 100 ml mark with the mobile phase and mixed thoroughly. Serial dilutions were prepared from the stock solution by pipetting 5ml, 2.5 ml, 2 ml,

1 ml and 0.5 ml into separate 10 ml volumetric flasks to obtain various concentrations of Ibuprofen, Lumefantrine and Artemether. The solutions were made up to the 10 ml mark using the mobile phase. The stock solution together with the other solutions prepared serially were filtered and analyzed using the chromatographic conditions developed. Multiple runs were made for each solution and the average peak areas calculated. Calibration curves were drawn for Ibuprofen, Lumefantrine and Artemether and subsequently subjected to regression analysis.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limit of Detection and Quantitation were obtained from the linearity using equation 7 and 8 below as stated by the ICH guidelines

$$LOD = \frac{3.3\sigma}{S} \quad \text{..... Eqn 7}$$

$$LOQ = \frac{10\sigma}{S} \quad \text{.....Eqn 8}$$

Where σ is the standard deviation of the response and S is the Slope of the calibration curve

ACCURACY

Accuracy of the developed method was determined at three concentration levels (120%, 100% and 80%) of the working concentration.

Concentration level I (120%)

a. 3 ml of the stock solution of Diclofenac, Lumefantrine and Artemether was pipetted into a 10 ml volumetric flask. 5 ml of the mobile phase was added and the solution made up to volume with tap water and mixed.

The concentrations obtained were 0.03mg/ml Diclofenac, 1.2mg/ml Artemether and 0.012mg/ml Lumefantrine. Triplicate injections were made and peak areas recorded. The percentage recoveries were calculated

b. 3 ml of the stock solution of Ibuprofen, Lumefantrine and Artemether was pipetted into a 10 ml volumetric flask. 5 ml of the mobile phase was added and the solution made up to volume with tap water and mixed. The concentrations obtained were 0.06 mg/ml Ibuprofen, 1.2mg/ml Artemether and 0.012mg/ml Lumefantrine. Triplicate injections were made and peak areas recorded. The percentage recoveries were calculated

Concentration level II (100 %)

a. 2.5ml of the stock solution of Diclofenac, Lumefantrine and Artemether was pipetted into a 10 ml volumetric flask. 5 ml of the mobile phase was added and the solution made up to volume with tap water and mixed. The concentrations obtained were 0.025 mg/ml Diclofenac, 1.0mg/ml Artemether and 0.010mg/ml Lumefantrine. Triplicate injections were made and peak areas recorded. The percentage recoveries were calculated.

b. 2.5 ml of the stock solution of Ibuprofen, Lumefantrine and Artemether was pipetted into a 10 ml volumetric flask. 5 ml of the mobile phase was added and the solution made up to volume with tap water and mixed. The concentrations obtained were 0.05 mg/ml Ibuprofen, 1.0mg/ml Artemether and 0.010mg/ml Lumefantrine. Triplicate injections were made and peak areas recorded. The percentage recoveries were

calculated.

Concentration level III (80%)

a. 2.0 ml of the stock solution of Diclofenac, Lumefantrine and Artemether was pipetted into a 10 ml volumetric flask. 5 ml of the mobile phase was added and the solution made up to volume with tap water and mixed. The concentrations obtained were 0.020 mg/ml Diclofenac, 0.8mg/ml Artemether and 0.008 mg/ml Lumefantrine. Triplicate injections were made and peak areas recorded. The percentage recoveries were calculated.

b. 2.0 ml of the stock solution of Ibuprofen, Lumefantrine and Artemether was pipetted into a 10 ml volumetric flask. 5 ml of the mobile phase was added and the solution made up to volume with tap water and mixed. The concentrations obtained were 0.04 mg/ml Ibuprofen, 0.8mg/ml Artemether and 0.008mg/ml Lumefantrine. Triplicate injections were made and peak areas recorded. The percentage recoveries were calculated.

PRECISION

Intra-Day Precision (Repeatability)

a. 2.5 ml and 0.5 ml of the stock solution of Diclofenac, Lumefantrine and Artemether (0.1 mg/ml Diclofenac, 4mg/ml Artemether and 0.04mg/ml Lumefantrine) was pipetted into separate 10 ml volumetric flasks. The solutions were made up to volume using the mobile phase and mixed. The stock solutions including the prepared solutions were assayed using the developed method. The three solutions were injected three times

over a 24-hour period. The peak areas were determined and the RSD calculated.

b. 2.5 ml and 0.5 ml of the stock solution of Ibuprofen, Lumefantrine and Artemether (0.2 mg/ml Ibuprofen, 4mg/ml Artemether and 0.04mg/ml Lumefantrine) was pipetted into separate 10 ml volumetric flasks. The solutions were made up to volume using the mobile phase and mixed. The stock solutions including the prepared solutions were assayed using the developed method. The three solutions were injected three times over a 24-hour period. The peak areas were determined and the RSD calculated.

Inter-Day Precision (Reproducibility)

a. Standard solution of the working concentration was prepared by weighing 2.5 mg of Diclofenac, 100 mg Artemether and 1 mg Lumefantrine. 40 ml of methanol was used to dissolve the pure powders and transferred into a 100 ml volumetric flask. The solution was sonicated for 10 mins and allowed to cool. The solution was made up to volume using the mobile phase.

b. Standard solution of the working concentration was prepared by weighing 5 mg of Ibuprofen, 100 mg Artemether and 1 mg Lumefantrine. 40 ml of methanol was used to dissolve the pure powders and transferred into a 100 ml volumetric flask. The solution was sonicated for 10 mins and allowed to cool. The solution was made up to volume using the mobile phase.

The working standard solutions were injected six consecutive times on three different days. The RSDs were calculated.

ROBUSTNESS

The standard solutions of working concentrations of both surrogates and analytes were assayed using Kromasil C-18 column (original condition). The standard solutions were then assayed using the developed method but done on a phenomenex C-18 column by a different analyst (varied condition).

STABILITY

The stability of the surrogate and analytes in solution were studied over a period of 6 hours. Triplicate injections were made each hour and the peak area recorded.

3.2.5 Determination of Surrogate Constant (K) Using the Surrogate Reference Standards

Diclofenac and Ibuprofen were as surrogate reference standards for Artemether and Lumefantrine.

Stock solution of Diclofenac, Lumefantrine and Artemether was prepared by weighing 10 mg, 4 mg and 400 mg respectively and dissolved in 40 ml methanol and transferred into a 100 ml volumetric flask. The solution was sonicated for 10 mins and allowed to cool. The solution was made up to volume using the mobile phase and mixed thoroughly. The stock solution was diluted serially five times to obtain different concentrations of the surrogate and analytes. The solutions were assayed using the developed method. Peak areas obtained from the various chromatograms and the corresponding concentrations were used to calculate the surrogate constant (K).

The same procedure above was repeated for stock solution of Ibuprofen, Artemether and Lumefantrine. The amount of powder taken for a 100 ml solution was 20 mg Ibuprofen, 4mg Lumefantrine and 400 mg Artemether.

3.2.6 Uniformity of Weight of Tablets

Twenty tablets each of four brands of Artemether/Lumefantrine were used in the study. The tablets were weighed individually and the weights recorded. The twenty tablets were then weighed together and the average weight per tablet calculated. The deviation and percentage deviation of each of the tablets were calculated.

3.2.7 Assay of Commercial Brands of Artemether Lumefantrine Using the Surrogate Reference Standards

A stock solution of Diclofenac was prepared by weighing 5 mg of the powder and dissolving in 20 ml of methanol. The solution was transferred into a 50 ml volumetric flask and made up to volume using methanol to obtain a concentration of 0.1mg/ml.

Stock solution of Ibuprofen was prepared by weighing 10 mg of the powder and dissolving in 20 ml of methanol. The solution was transferred into a 50 ml volumetric flask and made up to volume using methanol to obtain a concentration of 0.2mg/ml.

Assay of Artemether using surrogate reference standards

Twenty tablets of each brand of Artemether/Lumefantrine were weighed and powdered. A weight of powdered tablets equivalent to 25 mg was taken and dissolved in 15 ml of the mobile phase. The solution was sonicated for 10 mins and allowed to cool. Filtration was done using Whatman No. 1 filter paper and

the filtrate transferred into a 25 ml volumetric flask. 6.25ml of Diclofenac stock solution was pipetted into the 25 ml volumetric flask and the mixture made up to volume with the mobile phase. The solution was assayed using the developed method. The peak areas for the surrogate and Artemether were obtained from the chromatograph. The percentage content of Artemether in the tablets was calculated using the surrogate constant (K).

The same procedure was applied when Ibuprofen is used as a surrogate for Artemether.

Assay of Lumefantrine using surrogate reference standards

A weight of the powdered Tablets equivalent to 5 mg of Lumefantrine was taken and dissolved in 20 ml of methanol. The solution was sonicated for 10 mins and allowed to cool. Filtration was done using Whatman No. 1 filter paper and the filtrate transferred into a 50 ml volumetric flask. The solution was made up to volume with methanol. 2.5 ml of the solution was pipetted into and 25 ml volumetric flask and 6.25 ml of Diclofenac stock solution was also pipetted and added. The resulting solution was made up to the 25 ml mark using the mobile phase. The solution was assayed using the developed method. The peak areas for the surrogate and Lumefantrine were obtained from the chromatograph. The percentage content of Lumefantrine in the tablets was calculated using the surrogate constant (K). The same procedure was applied when Ibuprofen is used as a surrogate for Lumefantrine.

3.2.8 Assay of Brands of Artemether/Lumefantrine Tablets Using the Standard Method (International Pharmacopeia)

The standard method as stated in the International Pharmacopeia for the assay of Artemether/Lumefantrine was slightly modified.

Chromatographic conditions for Artemether

- **Pump:** Spectra Series P100 Isocratic pump
- **Detector**: Perkin Elmer 785A Programmable UV/VIS absorbance detector
- **Elution mode**: Isocratic
- **Stationary Phase**: Waters Nova-Pak C-18 (3.9 mm x 150 mm) 5µm
- **Mobile Phase for Artemether:** Mobile Phase A: Mobile Phase B (60:40 v/v)
- **Flow rate**: 1.5 ml/min.
- **Injection volume**: 20µl
- **Wavelength of absorption for Artemether:** 210 mn
- **Analytical software**: eDAQ PowerChrom 280

Chromatographic conditions for Lumefantrine

- **Pump:** Spectra Series P100 Isocratic pump
- **Detector**: Perkin Elmer 785A Programmable UV/VIS absorbance detector
- **Elution mode**: Isocratic
- **Stationary Phase**: Waters Nova-Pak C-18 (3.9 mm x 150 mm) 5µm
- **Mobile Phase for Lumefantrine:** Ion Pair reagent: Acetonitrile (30:70 v/v)
- **Flow rate**: 1.5 ml/min.
- **Injection volume**: 20µl
- **Wavelength of absorption for Lumefantrine:** 320 nm
- **Analytical software**: eDAQ PowerChrom 280

Preparation of Ion Pair reagent

5.65 g of Sodium hexane sulfonate and 2.75g of Sodium dihydrogen phosphate were accurately weighed and dissolved in 900 ml of distilled water. The pH of the solution was adjusted to 2.3 using phosphoric acid. The solution was transferred into a 1L volumetric flask and made up to volume with distilled water. The resulting solution was filtered using a 0.45µm filter before use.(Goodman L. , 2006)

Preparation of Mobile phase A

700 ml of Ion Pair reagent and 300 ml of Acetonitrile were measured and mixed thoroughly. The solution was sonicated to expel gases and filtered.

Preparation of Mobile Phase B

300 ml of the Ion Pair reagent and 700 ml of Acetonitrile were measured and mixed thoroughly. The solution was sonicated to expel gases and filtered.

Preparation of Diluent

100 ml of Ion Pair reagent, 30 ml of water and 100 ml of 1-propanol were measured and thoroughly mixed together. The solution was made up to 500 ml using Acetonitrile. The solution was sonicated and filtered.

ASSAY OF ARTEMETHER USING THE MODIFIED METHOD

Assay of Artemether pure powder

20 mg of Artemether pure powder was weighed and dissolved in 85 ml of the diluent. The solution was transferred into a 100 ml volumetric flask. The solution was sonicated for 20 mins, allowed to cool and made up to volume with the diluent. The solution was assayed using the chromatographic conditions developed for Artemether.

Assay of Artemether in formulation

Twenty tablets of each brand of Artemether/Lumefantrine was weighed and powdered. An amount of the powdered tablets equivalent to 20mg Artemether was taken and dissolved in 85 ml of the diluent. The solution was transferred into a 100 ml volumetric flask and sonicated for 20 minutes. It was then allowed to cool and made up to volume with the diluent. The solution was assayed using the chromatographic conditions developed for Artemether.

ASSAY OF LUMEFANTRINE USING THE MODIFIED METHOD

Assay of Lumefantrine pure powder

120 mg of Lumefantrine pure powder was weighed and dissolved in 85 ml of the diluent. The solution was transferred into a 100 ml volumetric flask. The solution was sonicated for 20 mins, allowed to cool and made up to volume with the diluent. The solution was assayed using the chromatographic conditions developed for Lumefantrine.

Assay of Lumefantrine in formulation

An amount of the powdered tablets equivalent to 120 mg of Lumefantrine was weighed and dissolved in 85 ml of the diluent. The solution was transferred into a 100 ml volumetric flask. The solution was sonicated for 20 mins, allowed to cool and made up to volume with the diluent. The solution was assayed using the chromatographic conditions developed for Lumefantrine

The peak areas of Artemether and Lumefantrine in the pure powders and formulation were obtained from the various chromatograms. The percentage content of Artemether and Lumefantrine in the tablets were calculated.

CHAPTER FOUR

RESULTS AND CALCULATIONS

4.1 IDENTIFICATION OF PURE SAMPLES USED

Melting point Determination

Table 4.1 Melting points of surrogates and analytes

Sample	(^{0}C)	Value in Literature (^{0}C)
Diclofenac	280 – 281 with decomposition	About 280 with decomposition
Ibuprofen	75 -77	About 76
Lumefantrine	128 – 130	128 – 132
Artemether	87 – 90	86 – 90

Diclofenac

- A blue coloured precipitate was formed.

Artemether

- A yellow coloured solution was produced.
- A pink colour was produced upon addition of vanillin/ sulphuric acid.

4.2 ASSAY OF PURE COMPOUNDS USED

Table 4.2 Percentage purity of Analytes used

Sample	Permissible Range (IP 2015)	Assayed Value (% w/w)	Inference
Artemether	98.0 – 102.0	99.50	Passed
Lumefantrine	98.5 – 101.0	99.20	Passed

Table 4.3 Percentage purity of Surrogates used

Sample	Permissible Range (BP 2013)	Assayed Value (% w/w)	Inference
Diclofenac	99.0 – 101.0	99.40	Passed
Ibuprofen	98.5 – 101.0	98.90	Passed

4.3 HPLC METHOD DEVELOPMENT

Table 4.4 Mean retention times of Surrogates and Analytes used

Sample	Mean Retention Times* (mins)
Ibuprofen	5.08 ± 0.017
Diclofenac	4.60 ± 0.031
Lumefantrine	7.48 ± 0.056
Artemether	8.63 ± 0.028

******indicates mean of three determinations*

SAMPLE CHROMATOGRAMS

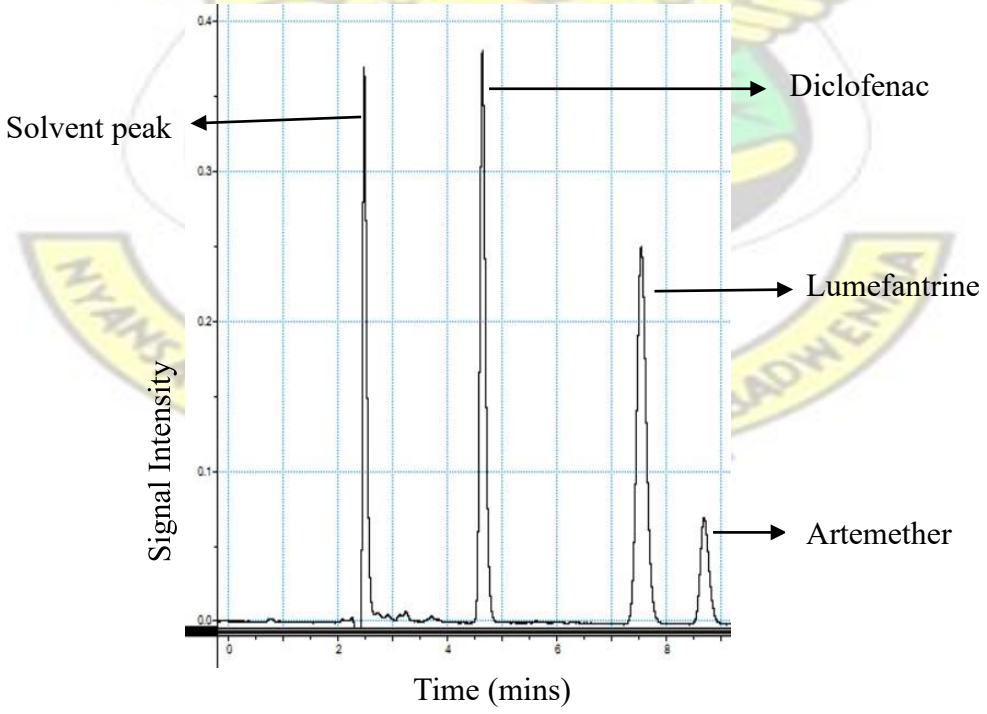


Figure 4.1 Chromatogram of pure Diclofenac, Artemether and Lumefantrine

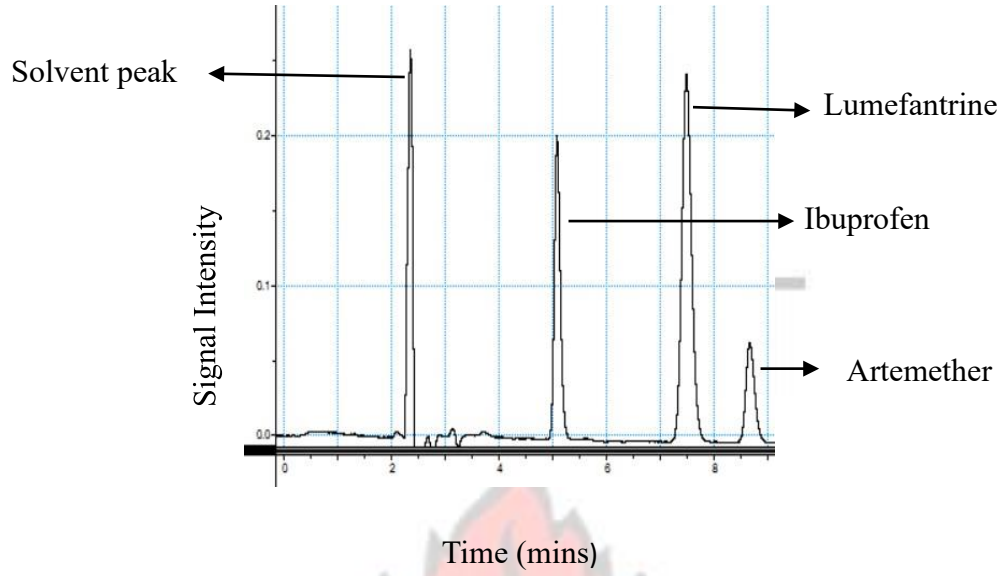


Figure 4.2 Chromatogram of pure Ibuprofen, Artemether and Lumefantrine

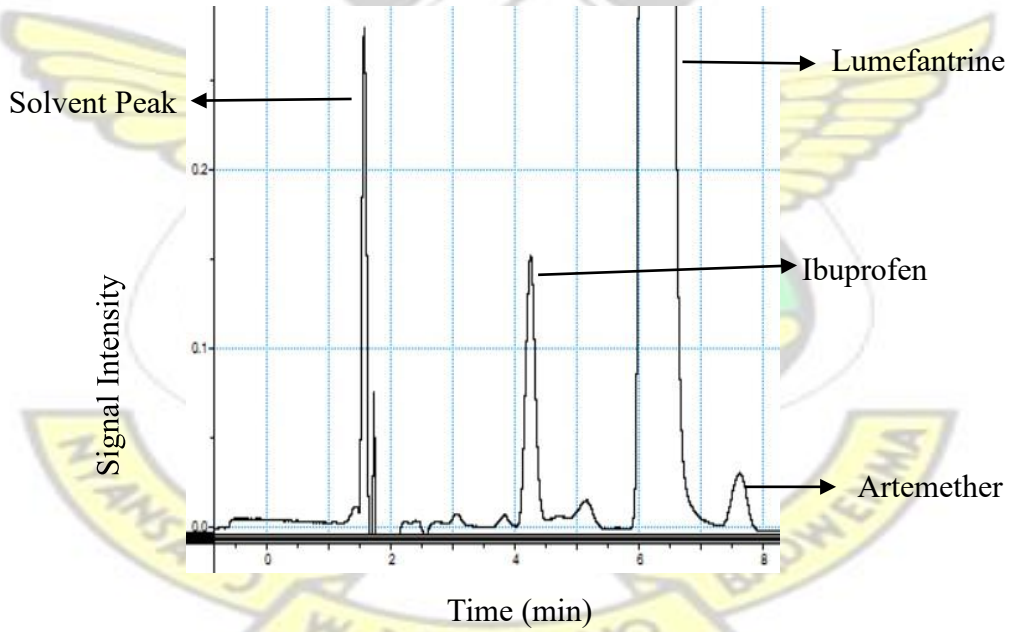


Figure 4.3 Chromatogram of Ibuprofen, Lumefantrine and Artemether in tablets

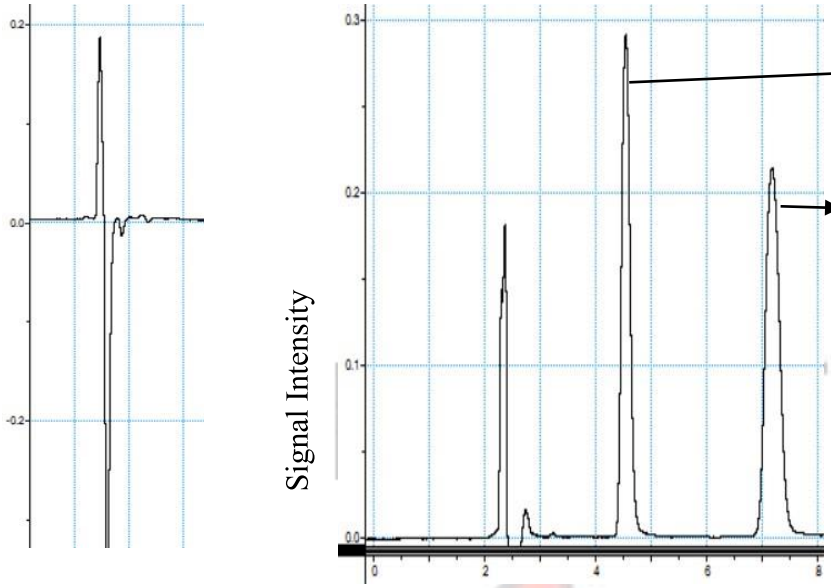


Fig. 4.4 Solvent Front

Figure 4.5 Chromatogram of Diclofenac and Lumefantrine in formulation

4.4 HPLC METHOD VALIDATION

4.4.1 Linearity

Sample Calibration Curves

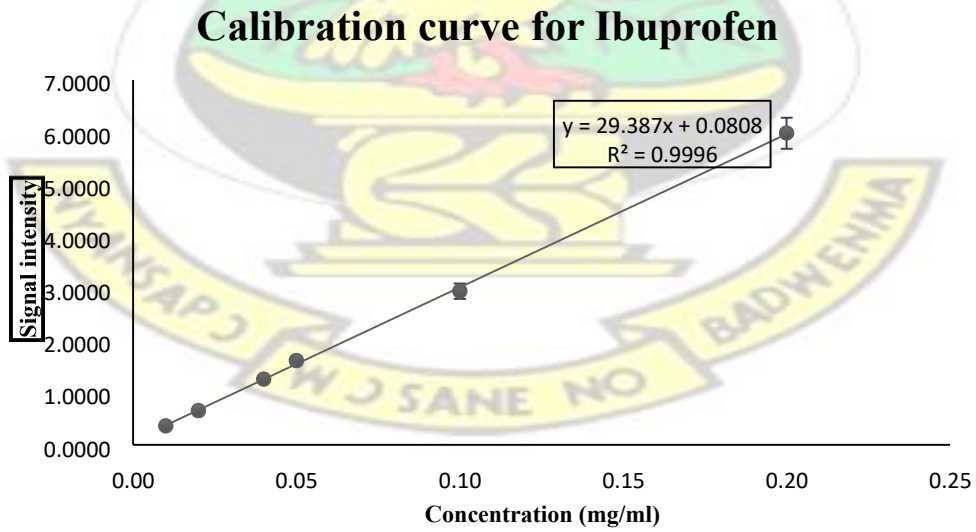


Figure 4.6 Calibration curve for Ibuprofen when used as a surrogate for Artemether and Lumefantrine

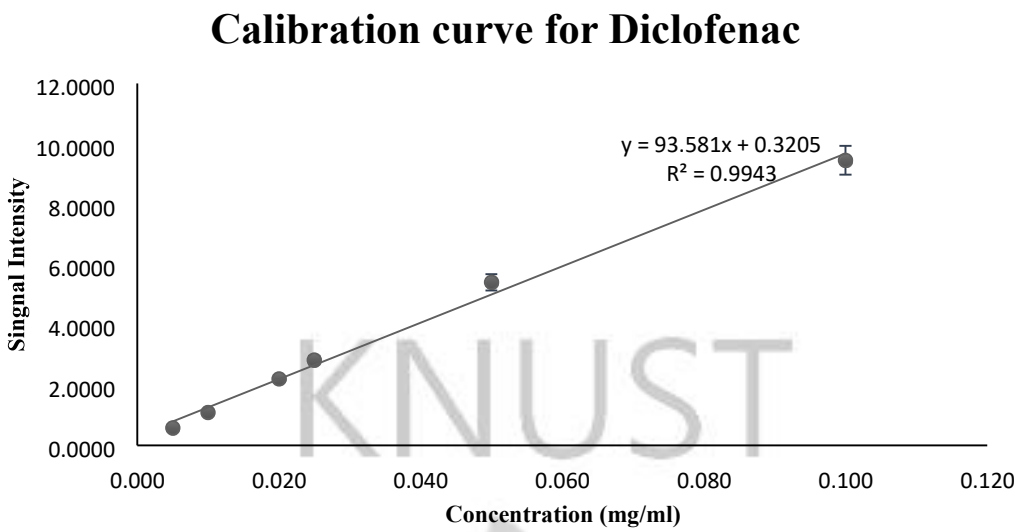


Figure 4.7 Calibration curve for Diclofenac when used as surrogate for Artemether and Lumefantrine

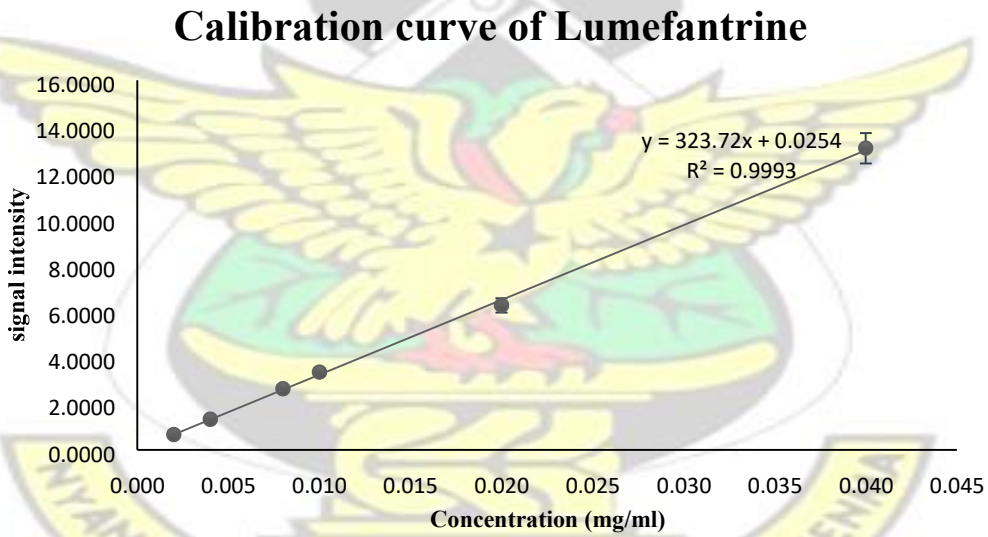


Figure 4.8 Calibration curve for Lumefantrine when Ibuprofen is used as a surrogate

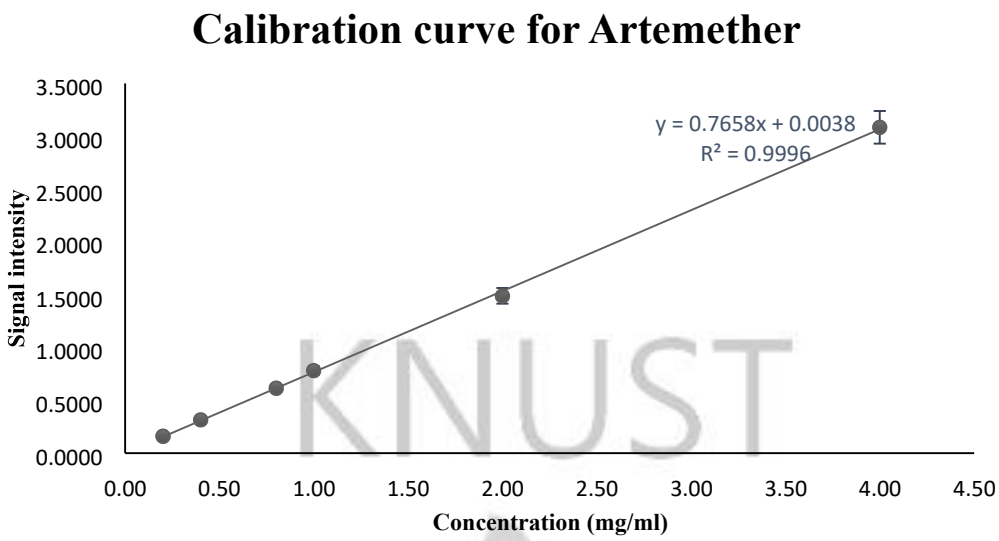


Figure 4.9 Calibration curve for Artemether when Ibuprofen is used as a surrogate

Table 4.5 Calibration equations and correlation coefficient of samples used

Sample	Calibration equation	Correlation Coefficient
Ibuprofen used as a surrogate for Artemether and Lumefantrine		
Artemether when Ibuprofen is used as a surrogate	Y = 0.7658x + 0.0038	0.9996
Lumefantrine when Ibuprofen is used as a surrogate	Y = 323.72x + 0.0254	0.9993
Diclofenac used as a surrogate for Artemether and Lumefantrine	Y = 93.581x + 0.3205	0.9943
Artemether when Diclofenac is used as a surrogate	Y = 0.7916x + 0.022	0.9997
Lumefantrine when Diclofenac is used as a surrogate	Y = 306.49x + 0.0708	0.9998

Table 4.6 Limit of Detection (LOD) and Limit of Quantitation (LOQ) of sample used

Sample	LOD (mg/ml)	LOQ (mg/ml)
Ibuprofen used as a surrogate for Artemether and Lumefantrine	3.2521×10^{-3}	9.8547×10^{-3}
Artemether when Ibuprofen is used as a surrogate	0.0665	0.2014
Lumefantrine when Ibuprofen is used as a surrogate	8.4794×10^{-4}	2.5695×10^{-3}
Diclofenac used as a surrogate for Artemether and Lumefantrine	5.9581×10^{-3}	0.01805
Artemether when Diclofenac is used as a surrogate	0.0579	0.1753
Lumefantrine when Diclofenac is used as a surrogate	4.4177×10^{-4}	1.3387×10^{-3}

4.4.2 Accuracy

Table 4.7 Mean Percentage recoveries of Surrogates and Analytes at different concentration levels

Sample	Concentration (mg/ml)	Amount Recovered (mg/ml)	Mean %Recovery*	RSD (%)
	0.030	0.02995	99.84	**0.90**
Diclofenac	0.025	0.02578	103.13	**1.26**
	0.020	0.02016	100.78	**0.81**
	0.06	0.06020	100.34	**0.57**
Ibuprofen	0.05	0.05125	104.30	**1.36**
	0.04	0.04047	101.17	**0.84**
Artemether	1.20	1.19640	99.70	**1.09**
	1.00	1.01360	101.36	**1.28**
	0.80	0.80096	100.12	**0.93**
	0.012 0.010	0.11897	99.14	**0.40**
Lumefantrine		0.01031	103.11	**1.42**
	0.008	0.00814	101.73	**0.39**

**indicates mean of three (3) determinations*

4.4.3 Precision

Intra-day Precision (Repeatability)

Table 4.8 Mean Peak Areas of Surrogates and Analytes at different concentrations

Sample	Concentration (mg/ml)	Mean Peak Area*	RSD (%)
Ibuprofen	0.20	6.02	**1.16**
	0.05	1.61	**1.02**
	0.01	0.37	**1.50**
Diclofenac	0.10	9.48	**0.45**
	0.025	2.78	**1.06**
	0.005	0.58	**0.89**
Lumefantrine	0.04	13.02	**1.13**
	0.01	3.32	**0.54**
	0.002	0.67	**0.81**
Artemether	4.00	3.12	**0.83**
	1.00	0.80	**1.46**
	0.02	0.16	**1.49**

**indicates mean of three (3) determinations*

Inter-day Precision (Reproducibility)

Table 4.9 Mean Peak areas of Surrogates and Analytes determined on different days

Sample	Days	Mean Peak Areas*	RSD (%)
Ibuprofen	1	1.62	**0.94**
	2	1.60	**1.23**
	3	1.61	**1.29**
Diclofenac	1	2.78	**0.78**
	2	2.78	**1.06**
	3	2.79	**0.62**
Lumefantrine	1	3.39	**0.58**
	2	3.38	**0.54**

	3	3.38	**0.44**
Artemether	1	0.79	**1.56**
	2	0.80	**1.46**
	3	0.79	**1.05**

**indicates mean of six (6) determinations*

4.4.4 Robustness

Table 4.10 Mean recoveries of Surrogates and Analytes when conditions were varied

Sample	**Concentration (mg/ml)**	**Original Condition**	**Varied Condition**
		Mean Recovery (%)*	**Mean Recovery (%)***
Ibuprofen	0.050	103.13	102.87
Diclofenac	0.025	104.30	103.92
Artemether	1.000	101.36	100.91
Lumefantrine	0.010	103.11	102.75
RSD (%)		**1.18**	**1.22**

**indicates mean of three (3) determinations*

4.4.5 Stability

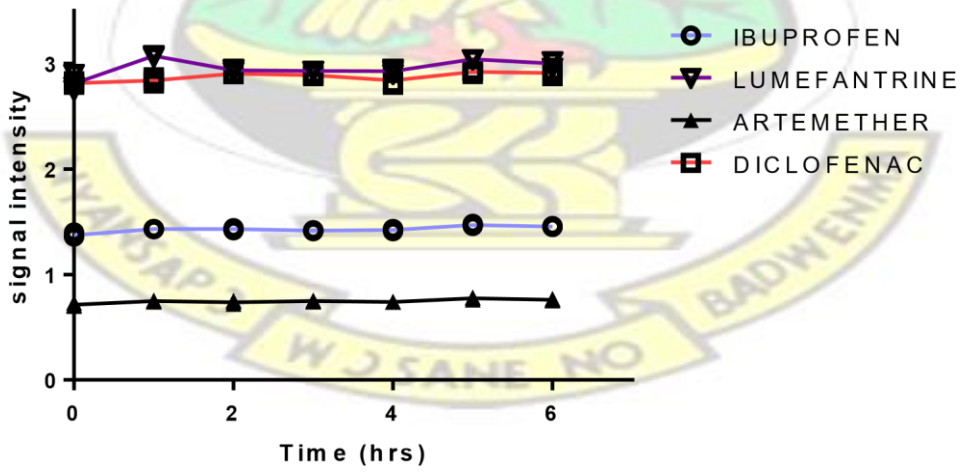


Figure 4.10 Stability profiles of surrogates and analytes

4.5 DETERMINATION OF SURROGATE CONSTANT (K) FOR EACH ANALTYE

Table 4.11 Determination of surrogate constant (K) for Ibuprofen when used as a surrogate for Artemether

A(analyte)	C(standard)	A(standard)	C(analyte)	K
3.0850	0.20	5.9775	4.00	0.025805
1.4900	0.10	2.9500	2.00	0.025254
0.7825	0.05	1.6125	1.00	0.024264
0.6175	0.04	1.2625	0.80	0.024455
0.3175	0.02	0.6600	0.40	0.024053
				Mean K = 0.02477 ± 0.00074

Table 4.12 Determination of surrogate constant (K) for Ibuprofen when used as a surrogate for Lumefantrine

A(analyte)	C(standard)	A(standard)	C(analyte)	K
13.0575	0.20	5.9775	0.040	10.92221
6.2625	0.10	2.9500	0.020	10.61441
3.3725	0.05	1.6125	0.010	10.45736
2.6500	0.04	1.2625	0.008	10.49505
1.3300	0.02	0.6600	0.004	10.07576
				Mean K = 10.513 ± 0.3051

Table 4.13 Determination of surrogate constant (K) for Diclofenac when used as a surrogate for Artemether

A(analyte)	C(standard)	A(standard)	C(analyte)	K
3.1875	0.100	9.4550	4.00	0.008428

1.5925	0.050	5.4100	2.00	0.007359
0.8550	0.025	2.8275	1.00	0.007560
0.6500	0.020	2.2075	0.80	0.007361
0.3275	0.010	1.0950	0.40	0.007477

Mean K = 0.007637 ± 0.00045

Table 4.14 Determination of surrogate constant (K) for Diclofenac when used as a surrogate for Lumefantrine

A(analyte)	C(standard)	A(standard)	C(analyte)	K
12.3060	0.100	9.4700	0.040	3.248680
6.1160	0.050	5.3740	0.020	2.845180
3.2680	0.025	2.8620	0.010	2.854647
2.4760	0.020	2.2060	0.008	2.805984
1.2520	0.010	1.1000	0.004	2.845455

Mean K = 2.919989 ± 0.1847

Table 4.15 Effect of Changing Concentration On Surrogate Constant

Concentration Effect	Limit of Concentration Ratio values	Inference
Diclofenac concentration constant, Lumefantrine concentration varied	**1 – 25**	Not significant
Diclofenac concentration varied, Lumefantrine concentration constant	**1-25**	Not significant
Diclofenac concentration constant, Artemether concentration varied	**8-400**	Not significant
Diclofenac concentration varied, Artemether concentration constant	**8-400**	Not significant

Ibuprofen concentration constant, Lumefantrine concentration varied	**1-10**	Not significant
Ibuprofen concentration varied, Lumefantrine concentration constant	**1-10**	Not significant
Ibuprofen concentration constant, Artemether concentration varied	**5-200**	Not significant
Ibuprofen concentration varied, Artemether concentration constant	**5-200**	Not significant

4.6 ASSAY OF DIFFERENT BRANDS OF ARTEMETHER/ LUMEFANTRINE USING THE SURROGATE REFERNCES STANDARDS

Table 4.16 Assay of Artemether/ Lumefantrine tablets using Diclofenac as surrogate

Brands	Mean Content (%)*		Standard	Inference
	Artemether	**Lumefantrine**		
AL 1	96.76	105.47	90-110	passed
AL 2	97.92	103.45	90-110	passed
AL 3	98.16	102.62	90-110	passed
AL 4	96.26	104.77	90-110	passed

**indicates mean of three determinations*

Table 4.17 Assay of Artemether/Lumefantrine tablets using Ibuprofen as surrogate

Brands	Mean Content (%)*		Standard	Inference
	Artemether	**Lumefantrine**		
AL 1	98.81	104.12	90-110	passed
AL 2	97.83	102.42	90-110	passed
AL 3	98.64	101.99	90-110	passed
AL 4	97.03	103.79	90-110	passed

***indicates mean of three determinations*

4.7 ASSAY OF ARTEMETHER/LUMEFANTRINE USING THE MODIFIED STANDARD METHOD

Table 4.17 Assay of Artemether/Lumefantrine using the standard method

Brands	Mean Content (%)*		Standard	Inference
	Artemether	**Lumefantrine**		
AL 1	97.62	104.93	90-110	passed
AL 2	97.84	102.96	90-110	passed
AL 3	98.43	102.39	90-110	passed
AL 4	96.52	104.45	90-110	passed

**indicates mean of three determinations*

4.8 STATISTICAL COMPARISION OF THE STANDARD METHOD AND THE NEWLY DEVELOPED METHOD

Table 4.18 Comparison of the new method of assay of Artemether using Diclofenac as SRS to the standard method. Data analysed using Holm-Sidak method

Brand	**Mean Content for new method***	**Mean Content of the standard method***	**P value**
AL 1	96.76	97.62	0.113056
AL 2	97.92	97.84	0.872031
AL 3	98.16	98.43	0.588299
AL 4	96.25	96.52	0.709863

**indicates mean content of three determinations*

Table 4.19 Comparison of the new method of assay of Artemether using Ibuprofen as SRS to the standard method. Data analysed using Holm-Sidak method

Brand	**Mean Content for new method***	**Mean Content of the standard method***	**P value**
AL 1	98.81	97.62	0.102086
AL 2	97.83	97.84	0.973332
AL 3	98.64	98.43	0.800816
AL 4	97.03	96.52	0.320874

**indicates mean content of three determinations*

Table 4.20 Comparison of the new method of assay of Lumefantrine using Diclofenac as SRS to the standard method. Data analysed using Holm-Sidak method

Brand	Mean Content for the new method*	Mean Content of the standard method*	P value
AL 1	105.47	104.93	0.227297
AL 2	103.45	102.96	0.370295
AL 3	102.62	102.39	0.606301
AL 4	104.77	104.45	0.342504

**indicates mean content of three determinations*

Table 4.21 Comparison of the new method of assay of Lumefantrine using Ibuprofen as SRS to the standard method. Data analysed using Holm-Sidak method

Brand	Mean Content for the new method*	Mean Content of the standard method*	P value
AL 1	104.12	104.93	0.173629
AL 2	102.42	102.96	0.367128
AL 3	101.99	102.39	0.262209
AL 4	103.79	104.45	0.151192

**indicates mean content of three determinations*

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

5.1.1 Identification and Assay Pure Samples Used

Ibuprofen

The melting point of the sample determined was 75^0C – 77^0C which corresponds to a stated value of about 76^0C in the IP 2015.

The IR spectrum obtained has a broad peak that spans from 3200 cm^{-1} to 2400 cm^{-1} indicating the presence of carboxylic acid functional group. This broad peak overlaps with C=H stretch around 3000 cm^{-1}. There is also a strong absorption at 1700cm^{-1} due to the presence of the carbonyl group present in the carboxylic functional group. The fingerprint region is similar to the standard IR spectrum of Ibuprofen obtained from literature (*Refer to Appendix A2*).

Percentage purity determined for Ibuprofen powder was 98.90% and this falls within the range of 98.5 – 101.0% a stated in the BP 2013.

Diclofenac

A blue coloured precipitate was formed after 15 minutes upon addition of Hydrochloric acid to a mixture of the sample, potassium ferricyanide and ferric chloride indicating diclofenac may be present.

The melting point determined was 280^0C – 281^0C with decomposition. This corresponds to the literature value of about 280^0C with decomposition stated in the IP 2015 indicating that the sample is diclofenac powder and may be of high purity. Percentage purity of the powder determined was 99.40 %. This falls within the range as specified by the BP 2103 to be 99.0% - 101.0 %.

Artemether

A yellow coloured solution was produced upon heating a mixture of the sample and potassium iodide indicating Artemether may be present. A pink colouration was produced when a drop of vanillin/sulfuric acid indicating Artemether may be present.

The melting point determined for the sample was 87^0C – 90^0C which corresponds to the IP value of 87^0C – 90^0C. Percentage purity of the sample

determined was 99.50%. This falls within the range stated in the IP of 98.0 – 102%.

Lumefantrine

The melting point of the sample determined was 128^0C – 130^0C and this corresponds to the melting point value of 128^0C – 132^0C of Lumefantrine stated in the IP 2015. This indicates the sample may be Lumefantrine of a high purity. The percentage purity determination was determined to be 99.20 % and this falls within the range of 98.5 – 101.0% stated in the IP.

5.1.2 HPLC Method Development and Validation

Linearity

Linearity was done to determine whether the peak areas produced by the developed method were proportional to the concentrations of surrogates or analytes used within a chosen range. Linearity for both surrogates and the analytes was determined simultaneously. The correlation coefficients calculated for all the samples used were within an r^2 range of 0.9943 to 0.9998. This gives a clear indication of a good correlation between the concentration of samples used and the peak areas obtained.

Accuracy

The accuracy of the developed method was determined by recovery studies. The recovery of the samples used were determined at three concentration levels 80%, 100% and 120% of the working concentrations. The percentage recovery calculated for surrogates and analytes used in the study range from 99.14 % to 104.30%. The method showed good recoveries for all samples used.

Precision

Intra-Day Precision (Repeatability)

Repeatability of the developed method was determined by analyzing three different concentrations of the samples used at different times within the day giving a maximum of nine determinations per sample. The relative standard deviation (RSD) calculated for surrogates and analytes used were all below 2.0%. This meets the acceptance criteria of RSD ≤ 2.0%

Inter-day Precision (Intermediate Precision)

Inter-day precision was assessed by analyzing the working concentration of the samples used on three (3) consecutive days. The relative standard deviation (RSD) calculated for the working concentrations of surrogates and analytes used in the study were all below 2.0%. This falls in line with the acceptance criteria of RSD ≤ 2.0%.

Robustness

The performance of the developed method was assessed when some method parameters are deliberately varied. The parameters varied were using a different ODS column and a different analyst. The varied conditions were used to analyze the working concentration of both surrogates and analytes. The RSD calculated for all samples used were below 2.0%. This falls in line with the acceptance criteria of RSD ≤ 2.0. This indicates that the method is rugged and reliable when some parameters are slightly varied.

Stability

The stability of all compounds in solution were monitored at room temperature by analyzing the solution at specific time intervals for 6 hours. This was done to investigate the influence of the surrogate on the stability profile of the analyte and vice versa as well as how long the solution can be kept after preparation.

Stability of surrogates and analytes were studied simultaneously in the same solution. The compounds studied were stable from time of preparation to 6 hours. The surrogates had no influence on the stability profiles of the analytes and vice versa. This also indicates that solution of surrogate and analyte prepared can be reliably used within 6 hours of preparation.

5.1.3 Effect of Concentration Ratio on the Surrogate Constant

The effect of concentration on the surrogate constant was investigated. This was done by maintaining the concentration of the surrogate while varying the concentration of the analyte and vice versa. The surrogate constant was calculated for the modified concentration ratios and statistically compared to the surrogate constant of concentration ratios used in the developed method.

At the end of statistical analysis, concentration ratio limits were specified for each variation. When a concentration ratio outside the specified limit is used, the difference in the calculated surrogate constant and the surrogate constant of the developed method will be statistically significant. This indicates that when the calculated surrogate constant is used further to assay Artemether/ Lumefantrine tablets, the percentage contents obtained will be different from the percentage contents estimated in this study.

However, when concentration ratios chosen happen to be within the specified limits, statistically there is no significant difference between the calculated surrogate constant and the surrogate constant used in the study. Thus when the calculated surrogate constant is used further to assay Artemether/Lumefantrine tablets, the percentage contents obtained will be comparable to the percentage content estimated in this study.

5.1.4 Assay of Artemether /Lumefantrine Tablets Using the Surrogate Constant (K)

Four different brands of Artemether/Lumefantrine tablets were sampled from the market and assayed using the newly developed method. The international Pharmacopeia specifies the percentage content for Artemether and Lumefantrine to be between 90 % to 110 %.

All brands of Artemether/Lumefantrine tablets assayed using Diclofenac and Ibuprofen as surrogate reference standards (SRS) had their percentage contents falling within the range specified in the pharmacopeia. Thus all tablets assayed using the developed method passed the percentage content determination. This also indicates that either Diclofenac or Ibuprofen can be chosen as a SRS for the assay of Artemether/ Lumefantrine in formulations.

5.1.5 Comparison of the Developed Method to the Standard Method

This study sought to develop a cheaper alternative method to the standard method (International Pharmacopeia) that can be used for routine analysis.

The developed method uses isocratic elution whiles the standard method uses gradient elution which is sometimes very complex and requires very expensive instruments. The developed method uses cheap reagents like methanol and acetate buffer whiles the standard method employs the use of expensive reagents like acetonitrile, sodium hexane sulfonate which is sometimes very difficult to access. Sample preparation in the standard method requires the use of acetonitrile, 1-propanonol, distilled water and sodium hexane sulfonate as diluent and sonication for about 20 minutes. This is quite tedious and time consuming compared to the developed method which employs methanol and

acetate buffer with sonication for 10 mins. For the standard method, the retention times for Artemether and Lumefantrine are 19 and 34 minutes respectively with a total run of about 55 minutes. However, the retention times for Artemether and Lumefantrine are 7.48 ± 0.056 mins and 8.63 ± 0.028 mins with a run time of 10 mins.

Four brands of Artemether/Lumefantrine tablets on the market were assayed using both methods and compared statistically. After statistical analysis using the Holm-Sidak method, there was no significant difference at 95% confidence interval between the developed method and the standard method. P- value obtained for the brands AL 1, AL 2, AL 3 and AL 4 were 0.102086, 0.973332, 0.800816 and 0.320874 respectively.

Considering all the factors stated above, the developed method is relatively cheaper and less time consuming with readily available reagents thus can be used for routine analysis

5.2 CONCLUSION

- A quantitative RP-HPLC method was successfully developed for the assay of Artemether/Lumefantrine in formulation using Diclofenac and Ibuprofen as surrogate reference standards.
- The method was validated according to the ICH guidelines and demonstrated acceptable levels of accuracy, precision, robustness, linearity over a given concentration range and sensitivity for Artemether and Lumefantrine.
- The surrogate constant (K) for Diclofenac and Ibuprofen when used as SRS for Artemether were 0.007637 ± 0.00045 and 0.02477 ± 0.00074 respectively. The surrogate constant for Diclofenac and Ibuprofen when

used as SRS for Lumefantrine were 2.919989 ± 0.8147 and 10.513 ± 0.3051

- Four commercial brands of Artemether/Lumefantrine assayed using the developed method has percentage contents that fell within the range given by the International Pharmacopeia.
- There is no statistical difference between the developed method and the standard method in the International Pharmacopeia.
- The effect of varying concentrations of the analytes (Artemether/ Lumefantrine) and surrogate reference standard (Diclofenac and Ibuprofen) showed no significant difference in K values obtained within specified concentration ratios.

5.3 RECOMMENDATIONS

- The developed method should be used in the analysis of pediatric Artemether/Lumefantrine formulations on the market.
- Other factors like temperature which affect the surrogate constant (K) should be investigated
- Further studies should be carried out on the use of surrogate reference standards to assay of multicomponent drugs (anti-hypertensives and anti-diabetic drugs) on the market.

REFERENCES

AFOSAH, D. 2010. *Design of Simple UV Spectrophotometric and HPLC Methods for the Assay of Artemether and Lumefantrine in Fixed-Dose Combination Tablets.*

ARUN, R. & SMITH, A. A. 2011. Simultaneous HPLC-UV method for the estimation of artemether and lumefantrine in tablet dosage form. *Int J Pharm Biomed Res,* 2**,** 201-5.

ASARE-NKANSAH, S., KWAKYE, J. & MOHAMMED, S. 2011. Compounds Chemically Related to Analyte as Surrogate Reference Standards in Quantitative HPLC: Preliminary Study and Proof of Hypothesis. *International Journal of Pure and Applied Chemistry, Global Research Publications,* 6**,** 253-264.

BASCO, L. K., BICKII, J. & RINGWALD, P. 1998. In vitro activity of lumefantrine (benflumetol) against clinical isolates of Plasmodium falciparum in Yaounde, Cameroon. *Antimicrobial agents and Chemotherapy,* 42**,** 2347-2351.

BLIESNER, D. M. 2006. *Validating chromatographic methods: a practical guide*, John Wiley & Sons.

BP 2013. British Pharmacopeia. 7th ed.

BUCK, M. L. 2010. Artemether-Lumefantrine for the treatment of Malaria in Infants and Children. *A monthly Newsletter for Health Care Professionals from the University of Virgina Children's Hospital* [Online], 16. [Accessed 10/03/2016].

BUSHRA, R. & ASLAM, N. 2010. An overview of clinical pharmacology of Ibuprofen. *Oman Med J,* 25**,** 155-1661.

DA COSTA CÉSAR, I., NOGUEIRA, F. H. A. & PIANETTI, G. A. 2008. Simultaneous determination of artemether and lumefantrine in fixed dose combination tablets by HPLC with UV detection. *Journal of Pharmaceutical and biomedical analysis,* 48**,** 951-954.

GOODMAN L. , G. A. 2006. Goodman & Gilman's The Pharmacological Basis Of Therapeutics. *In:* LAURENCE L. BRUNTON, J. S. L., KEITH L. PARKER (ed.) 11 ed.: McGraw-Hill, Medical Publishing Division.

HARRIS, D. C. 2010. Quantitative Chemical Analysis. 8th ed.: W. H. Freeman & Company.

HEALTH, U. D. O. H. A. H. S. N. I. I. 2016. *Life cycle of the Malaria Parasite* [Online]. Available: https://www.niaid.nih.gov/topics/Malaria/Pages/lifecycle.aspx [Accessed 13/03/2016 2016].

HOGLUND, R. M., BYAKIKA‐KIBWIKA, P., LAMORDE, M., MERRY, C., ASHTON, M., HANPITHAKPONG, W., DAY, N. P., WHITE, N. J., ÄBELÖ, A. & TARNING, J. 2015. Artemether‐lumefantrine co‐administration with antiretrovirals: population pharmacokinetics and dosing implications. *British journal of clinical pharmacology,* 79**,** 636649.

ICH. Topic Q2 (R1) Validation of analytical procedures: Text and methodology. Int. Conf. Harmon, 2005. 17.

ICH, H. T. G. Validation of Analytical Procedure: Methodology (Q2B). International Conference on Harmonization, 1997.

IP 2015. International Pharmacoepia.

KALRA, K. 2011. Method Development and Validation of Analytical Procedures. *QUALITY CONTROL OF HERBAL MEDICINES AND RELATED AREAS*, 1.

KALYANKAR, T. & KAKDE, R. 2011. Reversed-phase liquid chromatographic method for simultaneous determination of artemether and lumefantrine in pharmaceutical preparation. *Int J ChemTech Res,* 3, 1722-1727.

KENKEL, J. 2010. *Analytical chemistry for technicians*, CRC Press.

MCPOLIN, O. 2009. *Validation of analytical methods for pharmaceutical analysis*, Lulu. com.

NYARKO, E. A. & NETTEY, H. 2013. Quality Assessment of Artemether/ Lumefantrine Tablets Sampled from Pharmacies in Accra, Using the MVHimagePCv8. exe Color Software. *Pharmacology & Pharmacy,* 4, 567.

SCHMAUSER, B. 2010. Analytical Method development.

SHABIR, G. A. 2004. A practical approach to validation of HPLC methods under current good manufacturing practices. *Journal of validation technology,* 10, 210-218.

SHAH, S. R., BAPNA, M., BRAHMBHATT, K. D., PATEL, R. & PATEL, C. 2013. DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR SIMULTANEOUS ESTIMATION OF ARTEMETHER AND LUMEFANTRINE IN BULK AND MARKETED FIXED DOSE COMBINATION. *Pharma Science Monitor,* 3.

SNYDER, L. R., KIRKLAND, J. J. & GLAJCH, J. L. 2012. *Practical HPLC method development*, John Wiley & Sons.

SRIDHAR, B., RAO, K. H., SRINIVAS, T., MADHURI, V. S., MADHURI, K. & RAO, J. S. 2010. A validated reverse phase HPLC method for the simultaneous estimation of Artemether and Lumefantrine in pharmaceutical dosage forms. *An Int J Adv Pharm Sci,* 1**,** 95.

SRINIVAS. 2016. *Antimalarial Drugs* [Online]. Available: http://www.malariasite.com/malaria-drugs/[Accessed 13/03/2016 2016].

SULEMAN, S., VANDERCRUYSSEN, K., WYNENDAELE, E., D'HONDT, M., BRACKE, N., DUCHATEAU, L., BURVENICH, C., PEREMANS, K. & DE SPIEGELEER, B. 2013. A rapid stabilityindicating, fused-core HPLC method for simultaneous determination of β-artemether and lumefantrine in anti-malarial fixed dose combination products. *Malar J,* 12**,** 10.1186.

SUNIL, J., NATH, M. S. & MOORTHY, U. S. 2010. HPLC method development and validation for simultaneous estimation of Artemether and Lumefantrine in pharmaceutical dosage forms. *Int J Pharm Pharm Sci,* 2**,** 93-6.

WHO 1999. Counterfeit Drugs: Guidelines for the development of measures to combat counterfeit drugs.

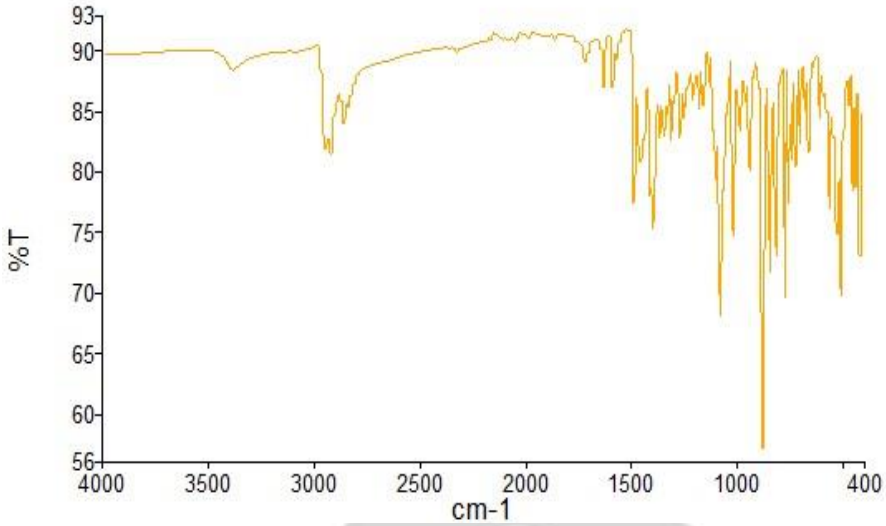
WHO 2015. Guidelines for the Treatment of Malaria. 3rd ed.

WHO. 2016. *Malaria* [Online]. Available: http://www.who.int/mediacentre/factsheets/fs094/en/ [Accessed 11/03/2016 2016].

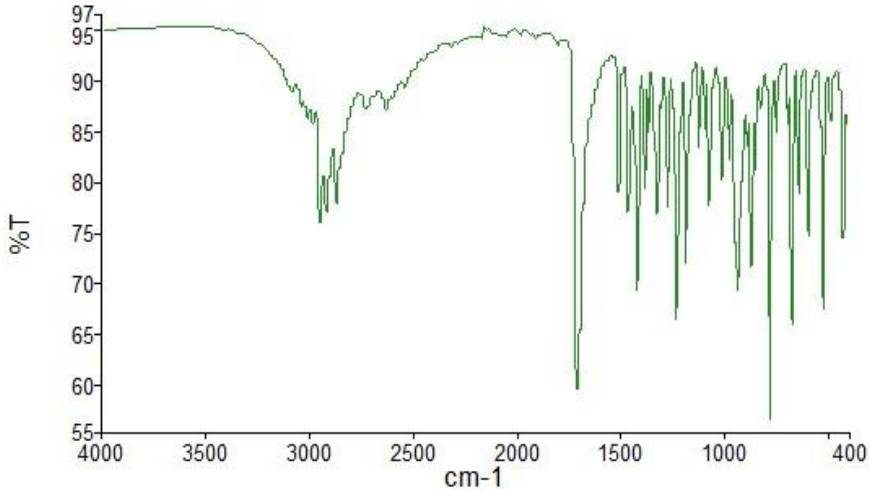
APPENDICES

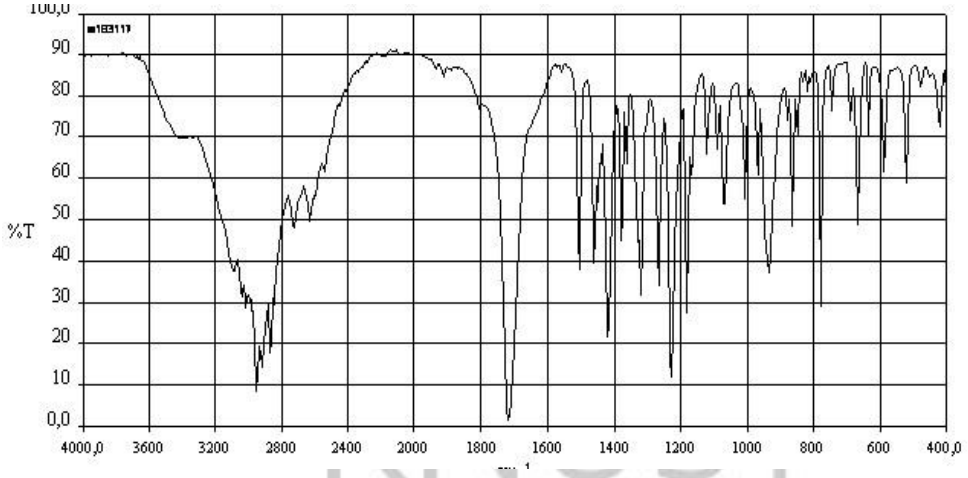
Appendix A IR Spectrums of Samples

Appendix A1: IR spectrum of Lumefantrine



Appendix A2: IR spectrum of Ibuprofen





IR spectrum of Ibuprofen (IP 2015)

Appendix B: Profile of brands of Artemether / Lumefantrine sampled in the study

SAMPLE	Manufacturing Company	Strength (mg)	Batch number	Expiry
AL 1	Bliss Gvs Pharma Ltd	80/480	E1AFM128	08/2017
AL 2	Ernest Chemist Limited	40/240	0106R	06/2017
AL 3	Novartis Pharma	80/480	K0008	05/2017
AL 4	Lupin Ltd	40/240	IBF407	06/2016

Appendix C: Uniformity of weight of A/L 2 tablets

Tablet No.	Weight (g)	Deviation	% Deviation
1	0.550	0.005	0.917
2	0.534	-0.011	-2.018
3	0.544	-0.001	-0.183
4	0.551	0.006	1.101
5	0.560	0.015	2.752
6	0.538	-0.007	-1.284
7	0.538	-0.007	-1.284
8	0.536	-0.009	-1.651
9	0.538	-0.007	-1.284
10	0.528	-0.017	-3.119
11	0.538	-0.007	-1.284
12	0.544	-0.001	-0.183
13	0.542	-0.003	-0.550
14	0.537	-0.008	-1.468
15	0.553	0.008	1.468
16	0.589	0.044	8.073
17	0.537	-0.008	-1.468
18	0.563	0.018	3.303
19	0.549	0.004	0.734
20	0.538	-0.007	-1.284

Total weight of 20 Tablets = 10.900 g

Average weight of Tablets = $\frac{10.900}{20}$ = 0.545 g

Appendix D: Sample Calculations

SAMPLE CALCULATIONS FOR LUMEFANTRINE

Limit OF Detection and Limit of Quantitation

Standard deviation (σ) = 0.08318

Slope of the calibration curve (S) = 323.72

Limit of Detection (LOD) = $\frac{3.3\sigma}{S}$

Thus LOD = $\frac{3.3x0.08318}{323.72}$ = 8.4794 x 10-4 mg/ml

Limit of Quantitation (LOQ) = $\frac{10\sigma}{S}$

Thus LOQ = $\frac{10x0.08318}{323.72}$ = 2.5695 x 10 -3 mg/ml

Accuracy

Concentration(mg/ml)	**Peak Areas**		
	1	2	3
0.012	3.86	3.88	3.89
0.010	3.31	3.38	3.40
0.008	2.66	2.67	2.65

Concentration (mg/ml)	**% Recovery**			**Mean % Recovery**	**RSD (%)**
	1	**2**	**3**		
0.012	98.71	99.23	99.48	99.14	**0.40**
0.010	101.46	103.63	104.24	103.11	**1.42**
0.008	101.34	102.13	101.73	101.73	**0.39**

Precision

Inter-day Precision

DAY	**Peak Areas**		**Mean Peak Areas**	**RSD (%)**	1	2	3	4	5	6
1	3.41	3.41	3.38	3.36	3.40	3.40	3.39	**0.58**		
2	3.40	3.40	3.38	3.38	3.35	3.38	3.38	**0.54**		
3	3.37	3.37	3.37	3.39	3.40	3.40	3.38	**0.44**		

Surrogate constant (K) for Ibuprofen when used as a surrogate for Lumefantrine

A(analyte) = 13.0575 C(analyte) = 0.04 mg/ml

A(standard) = 5.9775 C(standard) = 0.20 mg/ml

$$K = \frac{A(analyte)xC(standard)}{C(analyte)xA(standard)}$$

Hence , $$K = \frac{13.0575x0.20}{0.04x5.9775} = 10.922$$

EFFECT OF CONCENTRATION ON THE SURROGATE CONSTANT

Ibuprofen concentration varied whiles Lumefantrine concentration is constant

Concentration Ratio	Significant	P value	Mean1	Mean2	RSD
10		0.838713	10.478	10.513	**1.26**
20	*	0.00240268	11.2853	10.513	**1.26**
25		0.0109306	11.0846	10.513	**1.26**
50		0.00782816	11.1264	10.513	**1.26**
100	*	0.00304576	11.2519	10.513	**1.26**
2.5		0.0183103	11.0689	10.513	**1.96**
12.5		0.00558316	11.2321	10.513	**1.96**
1.25		0.0467163	10.1229	10.513	**1.06**
6.25		0.180755	10.2722	10.513	**1.06**
1		0.319295	10.343	10.513	**0.76**
2		0.00726389	9.92122	10.513	**0.76**
4		0.31288	10.6856	10.513	**0.76**
0.5		0.0109347	9.8954	10.513	**2.11**
0.25	*	< 0.0001	8.94867	10.513	**2.82**

Ibuprofen concentration constant whiles Lumefantrine concentration is varied

Concentration Ratio	Significant	P value	Mean1	Mean2	RSD

2.5		0.0126386	11.0657	10.513	**1.24**
1.25		0.0489672	10.1221	10.513	**1.24**
1		0.335367	10.3426	10.513	**1.24**
0.5		0.00681576	9.89205	10.513	**1.24**
0.25	*	< 0.0001	8.94349	10.513	**1.24**
10		0.842825	10.4768	10.513	**1.81**
2		0.0113043	9.92079	10.513	**1.81**
20	*	0.00243195	11.284	10.513	**1.27**
4		0.33338	10.6852	10.513	**1.27**
25		0.00797652	11.0832	10.513	**0.31**
12.5	*	0.00246992	11.2288	10.513	**0.31**
6.25		0.163756	10.2713	10.513	**0.31**
50		0.00972393	11.1251	10.513	**1.62**
100	*	0.00237407	11.2505	10.513	**0.74**