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FORMULATION AND MONITORING THE STABILITY OF ARTESUNATE IN ARTESUNATE – AMODIAQUINE COMBINATION TABLETS

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DECLARATION

I hereby declare that this submission is my own work towards the MSc and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

Stephen Danso – Mensah ……. July, 2009.

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(Head of Department)

DEDICATION

I dedicate this work to my wife and my baby girl: Joyce Danso – Mensah and Akosua Twumwaa Danso – Mensah, for their support.

ACKNOWLEDGEMENT

I wish to express my sincere gratitude to Jehovah God for guiding me through this programme.

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ABSTRACT

In this work, a single dosage form of **artesunate** and **amodiaquine** was formulated. Each dosage form contains 100mg of artesunate and 300mg of amodiaquine constituting two tablets daily and a total of six tablets for three days for the full dose of adults in the treatment of malaria.

A simple, accurate and precise isocratic reverse phase HPLC method with a mobile phase comprising 0.3% v/v of glacial acetic acid, acetonitrile and triethylamine in the ratio of 55%: 45%: 0.3% was developed using a C_8 column. The final pH was always in the range of 4.5±0.1.

A flow rate of 2.0ml/min with UV detection set at 254nm was employed at room temperature. Artesunate had a retention time of 4.13 minutes and amodiaquine had a retention time of 5.93 minutes.

A linear calibration curve was obtained with r^2 value of 0.998. Relative standard deviation (RSD) obtained was less than 1% indicating a high precision of the method. The method proved to be robust with variations in the mobile phase and stationary phase compositions. The limit of detection of artesunate and amodiaquine are 1.29×10^{-4} $\%^{w}/_{v}$ and 3.3×10^{-5} $\%^{w}/_{v}$ respectively. The limit of quantification of artesunate and amodiaquine are 6.45×10^{-4} $\%^{w}/_{v}$ 1.65×10^{-4} $\%^{w}/_{v}$ respectively.

The shelf – life of the artesunate in the artesunate – amodiaquine combination tablets is 0.41 years or 4.8 months at 28^{o}C and 0.1 years or 1.2 months at 40^{o}C.

Artesunate – amodiaquine combination tablets were found to have a short stability period. Artesunate and amodiaquine are incompatible, and there is severe degradation of the drugs in presence of each other.

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1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The World Health Organisation (WHO) Consultation on the Use of Antimalarial Drugs was held in November 2000 in Geneva, Switzerland. The participants reflected a broad range of expertise in the development and use of antimalarial drugs and in the implementation and adaptation of antimalarial treatment policies.(1)

Early diagnosis and prompt treatment are fundamental components of the WHO global strategy for malarial control. Correct use of an effective antimalarial drug not only shortens the duration of malaria illness but also reduces the incidence of complication and risk of death. Antimalarial drug resistance has spread and intensified over the last 15-20 years, leading to dramatic decline in the efficacy of the most affordable antimalarial drugs. Development of new drugs is not keeping pace, and problems related to the distribution and use of these drugs has compounded the situation. In many malaria endemic areas, a majority of the population does not have ready access to antimalarial drugs and to reliable and consistent information about malaria treatment and prevention.(1)

Malaria treatment has undergone drastic changes in the past. Strains of *Plasmodium falciparum* resistance to chloroquine were first detected in 1957 in Thailand. Since then, resistance to this drug has spread widely and there is now high level resistance to chloroquine in South Asia, South-East Asia, Oceania and South America. In Africa, Chloroquine resistance was first documented in Tanzania in 1979 and has spread and intensified in the last 20 years. In most countries in West Africa, more than 50% of patients experience recurrence parasitaemia with symptoms by day 14 after treatment.(1)

Although, amodiaquine is generally more effective than chloroquine against chloroquine-resistance strains of *Plasmodium falciparum*, there is cross-resistance and moderate-to-high levels of amodiaquine resistance have been reported from East Africa and West Africa. This drug cannot therefore be used as a single dose, and in some countries, it is used in combination with sulfadoxine-pyrimethamine or artemisinin derivatives.[1,2]

High levels of resistance to sulfadoxine-pyrimethamine are found in South-East Asia. In West Africa, resistance rates are variable, ranging from 10-50% in 14-day therapeutic efficacy trials.[1]

Decreasing sensitivity to quinine has been detected in areas of West Africa and South-East Asia where it has been extensively used as the first-line treatment in the past. Patience compliance to 7-day dose regimen as a single drug or with other drugs such as tetracycline is low, leading to incomplete treatment and outbreak of parasite resistant strains of *Plasmodium falciparum.* There is some cross-resistance between quinine and mefloquine, suggesting that the wide use of quinine might have led to the resistance of mefloquine in some countries.[1]

Recurrences of parasitaemia in over 50% of the patients treated with mefloquine alone have been reported in some parts of the world. Existing data indicate that, in some endemic areas, mefloquine-resistance may be found prior to the introduction of the drug. For example, a survey of people with reduced sensitivity to mefloquine has been reported from several sites in West and Central Africa, although the drug has never been widely used there. In such cases, there is the potential for resistance to spread if mefloquine monotherapy is used on a large scale.[2,3]

The decomposition rate of artemisinin and its derivatives is high when these drugs are used in monotherapy, depending on the dose administered, the duration of treatment and the severity of the disease on parasite resistance. Treatment regimens of less than 7 days gave unacceptably high recrudescence rate. In spite of reports of decreasing in vitro

susceptibility so far, there is no confirmed in vitro evidence of resistance of ***Plasmodium falciparum*** to artemisinin and its derivatives.[1]

Other antimalarials such as **halofantrine, primaquine, atovaquone-proguanil** and many others are becoming less effective in the treatment of malaria. Many malaria endemic countries are therefore beginning to face a situation in which there are no affordable, effective antimalarial drugs available. Combination therapy offers hope for preserving the efficacy of antimalarial drugs and prolonging their useful therapeutic life. The development of artemisinin and its derivatives – the most rapidly acting of all the current antimalarial drugs – and recognition of their potential role as a combination therapy have led to several trials aimed at assessing different combinations of existing drugs.[2]

Since *Plasmodium falciparum* has developed resistance to almost all antimalarial drugs, the use of combination therapies of effective antimalarial with different mechanisms of action is required to improve cure rate and delay drug resistance. The World Health Organisation (WHO) now recommends treatment of uncomplicated falciparum malaria with one of several artemisinin–based combination therapies (ACTs), including artesunate plus amodiaquine. To date at least 15 African countries including Ghana have adopted artesunate plus amodiaquine as first line malaria treatment policy. Other ACTs include artemether – lumefantrine, artesunate – mefloquine and artesunate – SP (sulphadoxine / pyrimethamine). [2,3]

1.1.2 Objectives of the research

1. To develop a simple isocratic HPLC method that can elute both artesunate and amodiaquine in combination dosage forms.

2. To formulate single dose combination tablets of artesunate and amodiaquine.

3. To monitor the stability of artesunate in the formulated artesunate - amodiaquine tablets using the method that has been developed.

4. To determine the shelf-life of the formulated tablets.

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1.2 LITERATURE REVIEW

1.2.1 Malaria Treatment.

Malaria, a parasitic protozoan disease, transmitted by the bite of female Anopheles mosquito, is one of the most serious complex and myriads of problems facing humanity this century. The adverse impact of the disease upon tropical countries and upon the world in general cannot be imagined. An estimated 300-500 million persons are infected annually and 1.5 to 2.7 million deaths yearly (WHO). Indeed, it remains the world's most devastating human parasitic infection.[1]

Malaria control requires an integrated approach comprising prevention, including vector control and treatment with effective antimalarials. The affordable and widely available antimalarial chloroquine that has been the main stay of malaria control is now ineffective in most falciparum malaria endemic areas and resistance to sulphadoxine-pyrimethamine is increasing rapidly.[2,3]

In 2002, Ghana adopted artesunate - amodiaquine (AS+AQ) as first – line treatment of malaria based on the evidence of its efficacy and safety observed elsewhere in Africa and on account of its potential production by local manufacturers. However, on Saturday, December 17, 2005, the Daily Graphic reported a directive of the Ministry of Health to withdraw single tablets of the new malaria drug, artesunate-amodiaquine, which contain 600mg of amodiaquine and 200mg of artesunate formulations from the market, until additional safety tests are done. Members of the Ghanaian public have expressed great concern about the developing issue, posing legitimate questions as to whether health authorities conducted due diligence before the adoption of the new malaria policy. [4, 5]

Although the Ministry of Health contended that the reported side effects were caused by the high strengths, it is also very important to establish the stability of the single doses of the artesunate – amodiaquine tablets. This is so because there was no documented information on the stability of the two drugs in a combination dosage form.

1.2.2 BACKGROUND

Several studies and anecdotal reports in the country were said to have thrown doubts on the efficacy of chloroquine in the management of malaria. As a result of the rising resistance to chloroquine, the National Malarial Control Programme (NMCP) established a task force of experts in various aspects of malaria control to give evidence on the efficacy of chloroquine in the treatment of malaria. This task force included Public Health physicians, epidemiologists, social scientists, clinicians, pharmacists, policy makers and Drug Regulatory Bodies.

In 1998, the NMCP in collaboration with Noguchi Memorial Institute for Medical Research initiated a study on the response of *Plasmodium falciparum* to chloroquine in the treatment of uncomplicated malaria. Centered in 6 district hospitals the study showed;

Treatment failure rates of 8.6% to 26%

Adequate clinical response of 75%

Parasitological failure rate of 21.7% to 49 %.[4,5]

Based on a WHO response to antimalarial drug resistance which calls for a period of change when treatment failure exceeds 25%, Ghana adopted a new antimalarial drug policy with **artesunate – amodiaquine** being chosen as the most cost-effective compared to all other alternatives. But with the new policy, for a patient with 70kg weight or more to be adequately treated using the 50mg artesunate and 150mg amodiaquine formulation; he/she requires a total of 24 tablets in a three day treatment regimen programme. With such a treatment programme, many were those who expressed apprehension about the efficacy with respect to drug compliance and adherence.[5]

Simply defined, compliance refers to taking the right dose for the full duration as prescribed. The objective of formulating a single dosage form is to improve patient adherence and compliance resulting in decreased possibility of recurrence or developing resistance. [5]

For example, the effectiveness of three daily doses of artesunate plus amodiaquine combination given unsupervised (n = 32), compared with the efficacy when given under full supervision (n = 29) to children with falciparum malaria were assessed in an unrandomized study. The results showed 61 patients analysed achieved a day 28 parasitological cure rate of 86% (25 out of 26 patients) in the supervised group and 63% (20 out of 32 patients) in the unsupervised group. The difference in outcome between the two groups was statistically significant (p = 0.04). This shows that the effectiveness of the treatment of malaria could be augmented by increased adherence through drug formulation.[6,7]

It was to address this concern that local manufacturers showed initiative and produced single compressed dose of artesunate - amodiaquine with their efforts being lauded by the NMCP.

However, combination of two active pharmaceutical ingredients like artesunate - amodiaquine also poses problem to stability due to possible interaction which may lead to dangerous degradation products.

1.2.3 Profile of Artesunate and Amodiaquine:

1.2.3.1 Artesunate

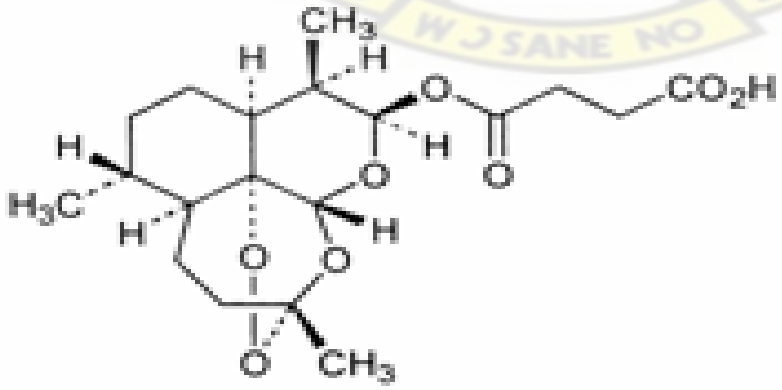

Fig 1a: Structure of artesunate.

Chemical name: (3R,5aS,6R,8aS,9R,10S,12R,12aR)-Decahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin-10-ol, hydrogen succinate.

Molecular formula: $C_{19}H_{28}O_8$

Molecular weight: 384.4

Description: Artesunate is a fine, white crystalline powder. It is a water soluble hemisuccinate derivative of artemisinin. Artemisinin is a sequiterpene lactone isolated from Artemisia annua, a herb that has traditionally been used in china for the treatment of malaria.

Solubility: Very slightly soluble in water; very soluble in dichloromethane; freely soluble in ethanol and acetone.

pH value: pH of an aqueous suspension containing 10mg/l is 3.5-4.5

Storage: Artesunate should be kept in a well – closed container, protected from light and stored in a cool dry place.

1.2.3.1 Therapeutic Indications and Adverse Effects.

Artesunate is an antimalarial agent. It is ideal for the treatment of severe malaria. It is also active against chloroquine and mefloquine resistant strains of plasmodium falciparum.

Artesunate and other related derivatives have been widely used in China, with no reports of any serious adverse reactions. Drug induced fever can occur. Neurotoxicity has been observed in animal studies but not in humans. In view of the uncertainty about toxic effects, caution should be exercised when more that one 3 day treatment is given. Cardio toxicity has been observed following administration of high doses.[8,9]

Possible drug related adverse effects include dizziness, itching, vomiting, abdominal pain, headache, diarrhea, reduction in neutrophil counts and convulsions. However, it is likely that many of these effects are disease – related rather that drug-induced.[9]

1.2.3.1.2 Clinical Pharmacology.

Artesunate and its active metabolite dihydroartemisinin are potent blood schizonticides, active against the ring stage of the parasite. Artesunate binds tightly to parasitized erythrocyte membranes. The functional group responsible for the antimalarial activity of artesunate is the endoperoxide bond. Release of an active oxygen species from this bond kills the parasite if accumulated in the erythrocytic cells. It also suppresses the production or activity of antioxidant enzymes in the erythrocytes, causing lysis of the parasitic cell due to the highly reactive free oxygen radicals. It reduces gametocyte carriage rate.[9,10]

1.2.3.1.3 Pharmacokinetic Properties.

Pharmacokinetic data for artesunate in humans are sparse, with no data demonstrating the rate or extent of absorption or the systemic distribution of artesunate. Artesunate is rapidly hydrolysed to the active metabolite dihydroartemisinin. The oral formulation is probably hydrolysed completely before entering the systemic circulation. Peak serum levels occur within an hour of an oral dose of artesunate and persist up to four hours. Dihdroartemisinin has a plasma elimination half- life of less that two hours, which may slow the development of resistance to artesunate.[11,12]

1.2.3.2 Amodiaquine Hydrochloride.

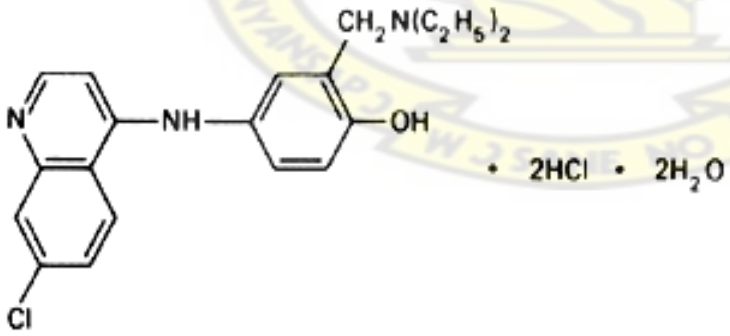

Fig 2b: Structure of amodiaquine hydrochloride.

Chemical name: 4-[(7-Chloro-4-quinolyl) amino]-α-(diethylamino)-o-cresol dihydrochloride dihydrate; 4-[(7-chloro-4-quinolinyl) amino]-2-[(diethylamino)-methyl] phenol dihydrochloride dehydrate.

Molecular formula: $C_{20}H_{22}ClN_3O$, 2HCl, 2H2O

Molecular weight: 464.8

Description: Amodiaquine is yellow crystalline and odourless powder. It is 4-aminoquinoline antimalaraial agent with a similar mode of action to chloroquine.

Solubility: Soluble in about 22 parts of water, sparingly soluble in ethanol, practically insoluble in ether.

pH value: pH of 20mg/l ml solution is 4.0-4.8

Storage: Amodiaquine hydrochloride should be kept in a tightly closed container.

1.2.3.2.1 Therapeutic Indications and side Effects.

Amodiaquine is indicated for treatment of uncomplicated cases of malaria caused by plasmodium falciparum. It is an antimalaraial with schizonticidal activity. It is effective against the erythrocytic stages of all four species of plasmodium falciparum. It is also effective as chloroquine against chloroquine sensitive strains of plasmodium falciparum and chlroquine – resistant strains.

Agranulocytosis and other blood dyscrasias, hepatitis and peripheral neuropathy have been reported occasionally after amodiaquine usage alone. Administration of quinoline type drugs has been associated with haemolytic anaemia. In therapeutic doses used for malaria, amodiaquine may occasionally cause nausea, vomiting, diarrhoea and lethargy. Abdominal pain, headache and photosensitivity have also been reported with amodiaquine. The drug can also cause irregular heartbeats and tremors.[11,12]

1.2.3.2.2 Clinical Pharmacology

Amodiaquine accumulates in the lysosomes and brings about loss of function. The parasite is unable to digest haemoglobin on which it depends for its energy. In general, 4-aminoquinoline derivatives appear to bind to nucleoproteins and inhibit DNA and RNA

polymerase. High drug concentrations are found in the malaria parasites digestive vacuoles.[11,12]

1.2.3.2.3 Pharmacokinetic Properties.

Amodiaquine hydrochloride is readily absorbed from the gastrointestinal track. Amodiaquine is rapidly converted in the liver to the active metabolite desethylamodiaquine; only a negligible amount of amodiaquine is excreted unchanged in the urine. The plasma elimination half – life of desethylamodiaquine has been found to vary from 1 to 10 days or more. About 5% of the total administered dose is recovered in the urine while the rest is metabolised in the body. Amodiaquine and desethyl-amodiaquine have been detected in the urine several months after administration.[13]

1.2.4 Stability of Pharmaceuticals.

The stability of a drug product is the ability of a particular drug formulation in a specific container to retain its physical, chemical, and microbiological and biopharmaceutical properties within specified limits throughout its shelf – life. Such a product must:

- Exhibit not less than 90% of its therapeutic activity.
- Contain at least 90% of its stated concentration.
- Contain an effective concentration of added preservatives.
- Exhibit no visible change, that is, discolouration, precipitation and development of off-odours.
- Develop no toxicity or irritancy.[14]

Quality assurance programmes are targeted at implementing procedures and systems that provide a high probability that each package or dose of a pharmaceutical product will have the same characteristics and properties, within reasonable acceptable limits, to ensure both clinical safety and efficacy of the formulation. Stability poses serious problems for many manufactured products, especially those distributed in territories with adverse climatic conditions.[14]

The period of stability of a pharmaceutical product is the time from the date of manufacture of the formulation until its chemical or bioactivity is not less than 90% of labelled potency and its physical characteristics have not changed appreciably or deleteriously.[14]

The expiry date given on the individual container of pharmaceutical product designates the date up to, and including which the product is expected to remain within specification if stored correctly. It is established for every batch by adding the shelf-life period to the date of manufacture.

The shelf-life or validity period is the period of time during which a drug product is expected, if stored correctly, to comply with specifications, as determined by stability studies on a number of batches of the product. The shelf-life is used to establish the expiry date of each batch. [14]

The stability of artesunate is pH dependent. This explains its rapid conversion to dihydroartemisinin in the stomach as compared to its greater stability in other compartments at higher pH and in the plasma. As a result, in the stomach at pH 1.2, artesunate is short-lived ($t^1/_2$ = 10.3 minutes), but at neutral pH its half-life is significantly longer ($t^1/_2$ = 7.3 hours in plasma).[13,15]

The stability of artesunate is also temperature dependant. The transformation of artesunate to dihydroatemisinin follows pseudo first order kinetics at constant temperature.[13,15]

1.2.5 Degradation and Stability of Pharmaceuticals

A systematic study of the decomposition of pharmaceutical products, using stability testing techniques, is very essential in order to:

• Produce safe products for the patient with uniform dose of drug throughout the shelf-life of the product.

• Minimize or, if possible, prevent decomposition of the products.

• Market products according to the relevant legal requirements concerning the identity, strength, purity and quality of the drugs, thereby preventing the economic repercussions of marketing an unstable product.

Pharmaceutical products differ considerably in their compositions, so they are subject to different forms of degradation.

1.2.6 Factors Affecting Stability of Artesunate.

1.2.6.1 Hydrolysis

Hydrolysis is the reaction of a compound with water and it is considered to be the major cause of degradation of drugs. Artesunate contains an ester. As an ester therefore, it undergoes slow hydrolysis in the presence of moisture.

There is also ionic hydrolysis, that is, interaction of salts of weak acids and bases with water to produce alkaline and acidic solutions respectively. Artesunate is sensitive to the acidity brought by the hydrochloride molecules present in amodiaquine salt that is enhanced by humidity.[15] Molecular hydrolysis is a much slower, irreversible process involving cleavage of the drug molecule. Molecular hydrolysis is mainly responsible for the degradation of esters, amides, and nitriles.

Drugs in solution may be stabilized against hydrolysis by adjusting the pH of the solution to a value at which the compound is found experimentally to have the lowest reaction rate constant. Non - aqueous solvents, for example, alcohol and propylene glycol, have often been used to replace a portion or all of the water in a solution in order to reduce hydrolysis of a drug.

1.2.6.2 Oxidation

The degradation of pharmaceutical products due to oxidation is nearly as prevalent as that due to hydrolysis. In most cases, it is possible to predict the susceptibility of a compound to oxidation from the knowledge of its standard reduction potential Eo.

The stabilization of an oxidizable drug is thus feasible by incorporating a compound of lower standard reduction potential value in the formulation.[16]

1.2.6.3 Temperature / Heat

According to the rule of thumb, the rate of a reaction doubles for every 10^{o} rise in temperature. High temperature fluctuations lead to a condition known as "breathing'' of packaging materials such as plastics, leading to transport of moisture and air into the product to initiate hydrolysis.

Temperature effects can be controlled by keeping artesunate-containing formulations in cool dry conditions of about 25˚C. The stability of artesunate to the HCl molecule brought by amodiaquine is also temperature dependent.[15]

1.2.6.4 Packaging Materials

Faulty packaging of pharmaceutical dosage forms can invalidate the most stable formulation. The materials commonly used as container components include glass, metal, plastic and rubber. Glass has been the container of choice for pharmaceutical dosage forms because of its resistance to decomposition by atmospheric conditions. Glass has two principal faults namely; the release of alkali and of insoluble flakes into liquids stored in the container. By decreasing the soda content in glass or replacing the sodium oxide with other oxides and surface treatment of glass with sulphur dioxide in the presence of water vapour and heat, it has been possible to overcome undesirable properties of glass. Plastics have the disadvantage of high permeation, leaching, sorption, diffusion and chemical reactivity, with samples, and with its environment.

The phenomenon of 'breathing' and container permeability promotes product deterioration during storage in plastic containers. Lining plastic containers with resins or materials that are inert to the product in question and also permit minimal permeability could minimise this. Many artesunate formulations are blister-packed in metal foils, stored in plastic containers with a cover. It is important that plastics and

aluminium foils of high quality are used in order to prevent permeation, chemical interaction and other factors that could eventually lead to hydrolysis.

1.2.7 Accelerated Stability Studies

Accelerated stability studies (ASS) are studies designed to increase the rate of chemical degradation and physical change of the drug product by using exaggerated storage conditions with the purpose of monitoring degradation reactions and predicting the shelf-life under normal storage conditions.

In the past it was common to assess the stability of a product by observing its quality and potency at suitable time intervals for a period corresponding to the normal time that the product is likely to remain in stock or use. Such a method is time consuming and expensive. Therefore, ASS at higher temperatures are employed that enable rapid prediction of the long–term stability of a product.

The potency or quality level below which a product is no longer acceptable varies, depending upon the form of degradation. The level is often taken to be 90% of the initial potency of the product, and the time for the decrease to this level is termed the shelf-life, t_{90} or $t_{0.9}$. Alternatively, the acceptable level could be that at which a toxic degradation product can no longer be tolerated, or at which a certain degree of odour or colour change is evident. Knowing the preparation date and calculating the shelf-life for specified conditions, the expiration date of the product can be determined.

The degradation of artesunate follows a pseudo-first order kinetics [15] process and the shelf-life can be calculated from the corresponding rate constant for the first order kinetics,

$$\ln C = \ln Co - kt$$

$$t_{90} = \frac{\ln (Co/C)}{k}$$

$$t_{90} = \frac{\ln(1/0.9)}{k}$$

$$t_{90} = 0.105/k \quad [16]$$

Therefore knowing the rate constant from the degradation of artesunate, the shelf-life can be calculated at various temperatures.

1.2.7.1 Climatic Zones

The four climatic zones in the world are distinguished by their characteristic, prevalent climatic conditions. This is based on the concept described by W. Grimm (Drugs Made in the Four Climatic Zones - WHO, 28:196-202, 1985 and 29:39-47, 1986).

Based on this concept, the four international climatic zones are described below:

I : Temperate zone, example, UK, Canada, Russia.

II : Mediterranean, Subtropical zone, example, US, Japan, Southern Europe.

III : Hot, Dry zone, example, Iran, Sudan.

IV : Hot, Humid zone, example, Brazil, Ghana, Indonesia.[17]

Zone *IV* is further divided into *IVa* and *IVb*. Ghana falls under *IVb* which is described as very hot and humid. The ICH (International Conference on Harmonization) guideline for accelerated stability study in this region is shown below:

Table1: Conditions for Accelerated Stability Studies in Zone *IVb*

Test programme	**Storage condition**	**Storage period(weeks)**
Accelerated	40^{o}C±2 ^{o}C/ 75% RH±5%RH	0, 2, 4, 6, 8, 3months, 6months

1.2.7.2 International Conference on Harmonization Guidelines (ICH) for Stability Tests.

In the past, the requirements of authorities for stability testing varied greatly. For companies operating globally, this meant an unacceptable amount of expenditure for the registration of an active pharmaceutical ingredient or a drug product. Efforts made to harmonize these requirements were precipitated by the ICH guidelines.

ICH therefore approved a range of additional stability guidelines in 2003 that focus on the statistical analysis and interpretation of data. The approval also covers data for the registration in climatic zones III and IV which was amended to the new 30^{o}C/65%RH conditions. However, several countries and regions (including Ghana) revised their stability testing guidelines, defining up to 30^{o}C/75%RH as the long term storage conditions for hot and humid regions and 40^{o}C±2^{o}C/75% RH±5%RH for accelerated stability studies for a period of six months.[17]

The ICH also states that ''significant changes'' in a drug product is defined as

***** A 5% change in assay from its initial value

* Any degradation product that exceeds the set limits

***** Failure to meet acceptance criteria for appearance, physical attributes or functionality tests such as colour, phase separation, resuspendability, caking, hardness, dose delivery per atuation

***** Depending on the dosage form, the pH value or dissolution rate (DR) for 12 dosage units no longer satisfies the requirements.[17]

Since Ghana falls under climatic zone IV, the stability study will focus on 40^{o}C±2 ^{o}C/ 75% RH±5%RH conditions. However, the product will also be monitored at 30^{o}C/75%RH and 60^{o}C±2 ^{o}C/ 75% RH±5%RH climatic conditions in order to monitor the effects of the conditions on the stability of the artesunate – amodiaquine tablets.

1.2.8 Tablets

The compressed tablet has a number of advantages as dosage form. It enables an accurate dosage form of medicament to be administered simply. It is easy to transport in bulk and carry by the patient. The tablet is uniform final product as regards weight and appearance, and is usually more stable than liquid preparations. Uniformity of weight, odour, texture, active drug, moisture contents, and humidity effect are also studied during a tablet stability test.

1.2.9 Tablet Formulation.

Tablets are composed of the active drug and excipients. Most of the artesunate and amodiaquine tablets in the market contain 50mg and 150mg of artesunate and amodiaquine respectively. These make up a total of 24 tablets for adults in a three day regimen programme. In this work, a single dosage form made up of 100mg artesunate and 300mg amodiaquine will be formulated making a total of 6 tablets for a three day full treatment of malaria in adults.

Various materials are usually added to convert powders into granules for compression into tablets. Indeed granules for compression into tablets must possess two essential properties: fluidity and compressibility.

Fluidity is required so that the material can be transported through the hopper of a tableting machine. If adequate fluidity does not exist, it gives rise to 'arching'; 'bridging' or 'rat-holding'. Fluidity is also essential so that adequate filling of the die occurs in tableting machine to produce tablets of consistent weight. A better method to enhance powder fluidity is to incorporate a glidant in the formulation.

Compressibility is the property of forming a stable, intact compact mass when pressure is applied. Granulation improves compressibility of powders. It is the process of particle size enlargement of powdered ingredients and is carried out to confer fluidity and compressibility to powder systems.

1.2.9.1 Tablet Excipients.

A tablet does not just contain the active ingredient but also include other substances, known as excipients, which have specific functions. The various classes of excipients include diluents, moistening agents, binding agents (adhesives), glidants, lubricants and disintegrating agents.

Diluents or 'bulking' agents are inert substances which are added to the active ingredient in sufficient quantity to make reasonably sized tablet. The principal substance employed

as diluents is lactose. It has a pleasant taste, rapidly dissolves in water, absorbs very little moisture and is freely neutral in reaction.

In wet granulation, a moistening agent is required and water is usually used. In cases where water cannot be used because the drug is hydrolysable, then alcohol is often substituted as moistening agent. However, care must be taken to remove all traces of the solvent during drying otherwise the tablets will possess alcoholic odour.

Binding agents act as adhesives to bind powders together in wet granulation processes. If too little binding agent is included in a formulation, soft granules result. Conversely, too much binding agent produces large hard granules. Common binding agents include starch mucilage, gelatin solution and polyvinylpyrrolidone (PVP). PVP is soluble both in water and alcohol and has been shown to release drugs faster than with other binders. Rubinstein and Rughani (1978).

Glidants and lubricants are materials which are added to tablet formulations in order to improve the flow properties of granulations. They prevent adherence of the granules to the punch faces and dies and also ensure smooth ejection of the tablet from the die. Talc and magnesium stearate appear to be more effective as punch lubricants.

Disintegrants are always added to tablets to promote breakup of the tablets when placed in an aqueous environment. The object of a disintegrant is to cause the tablet to disintegrate rapidly so as to increase the surface area of the tablet fragments and so promote rapid release of the drug.

Disintegrants act by swelling in the presence of water to burst open the tablet. Starch is the most common disintegrant in tablet formulation and is believed to act by swelling. Lowenthal (19731).[18,19]

1.2.9.2 Wet Granulation.

The preparation of tablets can be divided into dry methods and wet methods. Tableting by the wet granulation process is the most widely used method for pharmaceutical

materials. The wet granulation process has a number of advantages over the other granulation methods but it is not readily suitable for hydrolysable or thermolabile drugs.

In wet granulation, the binder is normally incorporated as a solution or mucilage. The choice of the liquid phase will depend upon the properties of the materials to be granulated. Water is the most widely used binder vehicle but non-aqueous granulation using isopropanol, ethanol or methanol may be preferred if the drug is readily hydrolysed. Changes in drug solubility resulting from a change in solvent have been shown by Wells and Walker (1983) to affect granule strength due to solute migration.[18] But in the case of artesunate and amodiaquine, the combination tablets will be formulated using water and alcohol to monitor stability of the artesunate in these two media.

1.2.10 Spectroscopic Methods

Spectroscopy is the study of interactions of electromagnetic radiation with matter. In absorption spectroscopy, the energy to be absorbed is supplied in the form of electromagnetic radiation but in emission spectroscopy, the energy is based on the measurement of the characteristics of the emitted radiation supplied by electromagnetic radiation sources. Among the more common spectroscopic methods used in pharmaceutical analysis is the Fourier transform infrared (FTIR), ultra-violet visible (UV), nuclear magnetic resonance (NMR), infrared (IR) and mass spectrometry.[11]

In FTIR spectrometry, chemical bonds can be detected through their characteristic infrared absorption frequencies or wavelength. UV visible makes use of strong absorption of ultra-violet radiation by a substance. NMR detects hydrogen atoms in specific environments, and complements both infrared and ultra-violet visible spectroscopy. There are also derivative methods such as infrared microscopy which allows very small areas to be analysed in an optical microscopy.([11]

UV and visible light absorption detector based on UV-visible light absorption is the most commonly used in pharmaceutical analysis because of its high sensitivity, reproducibility and ease of operation at fixed, multiple or variable wavelength. Also, many compounds

absorb UV because they possess chromophores. It is usable with gradient elution technique and can detect low concentration of substances.[11, 23]

1.2.10.1 Ultraviolet-visible Absorption Spectrophotometry

This technique is one of the most frequently employed in pharmaceutical analysis. It involves the measurement of the amount of ultraviolet (190-380nm) or visible (380-800nm) radiation absorbed by a substance in solution. The two empirical laws made by Lambert and Beer govern the phenomenon of absorption of light by molecules. A combination of the two laws is known as the Beer-Lambert law. It defines the absorbance of a solution of a substance as being related to the path length of the solution through which the light passes '*b*' and to its concentration '*c*'. Mathematically, the law is represented as shown below:

$A = abc$ [1].

Where, A = Absorbance of an absorbing substance

b = path length

c = concentration of solution

a = absorptivity.

The name and value of *'a'* depend on the units of concentration and the path length. When '*c'* is in moles per litre and *'b'* is in cm, the constant is called molar absorptivity (formerly the molar extinction coefficient) and has the symbol ε.

Equation [1] then becomes:

$A = \varepsilon bc$

Another form of the Beer-Lambert proportionality constant is the specific absorbance, which is the absorbance of a specified concentration in a cell of a specified path length. The most common form in pharmaceutical analysis is the A (1%, 1cm), which is the absorbance of a 1g/100ml (1%w/v) solution in a 1cm cell. The Beer-Lambert equation therefore takes the form:

A = A (1%, 1cm) bc where '*c'* is in %w/v and '*b*' is in cm.

Occasionally, the concentration of liquids in solution is given as %v/v (example, in the British Pharmacopoeial assay of methyl salicylate and diethyl phthalate in surgical spirit)

in which case the specific absorbance is the absorbance of a 1ml/100ml solution in a 1cm cell.

A mathematical relationship between ε and A (1%, 1cm) is shown below:

$$\varepsilon = (A\ (1\%, 1cm) \times \text{molecular weight})/10.$$ [11,23]

1.2.10.1.1 Causes of Deviation from Beer-Lambert Law

True adherence to Beer's law is observed only with truly monochromatic radiation. The use of radiation that is restricted to a single wavelength is seldom practical.

Deviation from the Beer-Lambert law may be due to physical, chemical or instrumental variations. Instrumental errors may be caused by slit-width effects, by stray light or by polychromatic radiation. Apparent failure of the law may be due to change in concentration resulting from solute-solute or solute-solvent interactions due to hydrogen bonding leading to association, dissociation or ionisation of the molecules.[11,23]

1.2.10.1.2 Spectrophotometric Applications in Pharmaceutical Analysis

The majority of applications in which spectrophotometric measurements are made rely on the compliance of the absorbing substance in solution with the Beer-Lambert's law at the wavelength of measurement. It is used for both qualitative and quantitative analysis.

1.2.10.1.2.1 Identification

Most organic molecules that contain a chromophore give rise to a characteristic electronic spectrum. In addition to other physical and chemical data, this provides a method for identifying structural components in such molecules. The characteristic wavelength of maximum absorption (λ_{max}) values and molar absorptivities are as well used in both qualitative (especially in the detection of impurities) and structural applications. This is because this information can sometimes differentiate two chromophores that absorb at the same wavelength. Artesunate and amodiaquine contain chromophores and they give characteristic absorption spectrum (Refer to Appendix).

1.2.10.1.2.2 Quantitative Analysis

Absorption spectroscopy is one of the most useful and widely used techniques for quantitative analysis. It is used not only for finished pharmaceutical products (such as tablets and capsules) but also for raw materials and intermediate products (such as granules). Tablet dissolution tests and multi-component preparation analysis are no exception. Most drugs in current use contain chromophoric systems, which make them suitable for absorption spectrophotometric analysis.

1.2.10.1.2.2.1 Analysis of raw materials, intermediate and finished products.

Preparing a solution in a transparent solvent and measuring its absorbance at a suitable wavelength is used to carry out the assay of an absorbing substance. The wavelength normally selected is a wavelength of maximum absorption (λ_{max}), where small errors in setting the wavelength scale have little effect on the measured absorbance.

Ideally, the concentration should be adjusted to give an absorbance of approximately 0.9 around which the accuracy and precision of the measurement are optimal. The preferred method is to read the absorbance from the instrument display under non-scanning conditions, that is, with the monochromator set at the analytical wavelength. Alternatively, the absorbance may be read from a recording of the spectrum obtained by using a recording double-beam spectrophotometer. The latter procedure is particularly useful for qualitative purposes and in certain assays in which absorbance at more than one wavelength is required. The concentration of the absorbing substance is then calculated from the measured absorbance using one of the following principal procedures:

Use of a standard absorptivity value: This procedure is adopted by official compendia (example, British Pharmacopoeia) for stable substances such as methyltestosterone that have reasonably broad absorption bands and which are practically unaffected by variation of instrumental parameters such as slit width and scan speed. The use of standard A (1%, 1cm) or ε values avoids the need to prepare a standard solution of the reference substance

in order to determine its absorptivity, and is of advantage in situations where it is difficult or expensive to obtain a sample of the reference substance.

Use of a calibration graph: In this procedure, the absorbances of a number of standard solutions (usually 4-6) of the reference substance at concentrations encompassing the sample concentrations are measured and a calibration graph constructed. Such calibration curves are drawn based upon the Beer-Lambert law that absorption is proportional to concentration. A plot of absorbance against concentration theoretically, gives a straight line passing through the origin. The concentration of the analyte in the sample solution is read from the graph as the concentration corresponding to the absorbance of the solution.

1.2.10.1.2.2.2 Analysis based on formation of (coloured) derivatives

Some drug molecules may absorb UV-visible radiations at specific wavelengths where irrelevant absorption due to the impurities also occurs. In such circumstances, it is better to prepare a derivative, which absorbs at a higher wavelength different from that of the parent compound, and compare the absorptivity with that prepared from a reference standard.

The BP and other pharmacopoeias make use of such derivatives in the analysis of drugs like steroids (use of triphenyltetrazonium chloride or 2, 4-dinitrophenylhydrazine), alkaloids (use of 4-dimethylamino benzaldehyde for ergotamine/ergometrine, alkaloids or sodium nitrite for reserpine-type alkaloids), and penicillins (use of imidazole-mercury reagent or sorbitol reagent).

1.2.10.2 High Performance Liquid Chromatography and Pharmaceutical Analysis.

High performance liquid chromatography (HPLC) is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under pressure and makes it faster. It also allows the use of smaller particle size for column packing materials which gives a much greater

surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture.

(HPLC) is a versatile technology widely used for the analysis of pharmaceuticals, biomolecules, and polymers, organic and inorganic compounds. HPLC is based on mechanisms of adsorption, partition, ion exchange or size exclusion, depending on the type of stationary phase used. HPLC involves a solid stationary phase, normally packed inside a stainless-steel column, and a liquid mobile phase. Separation of the components of a solution results from the difference in the relative distribution ratios of the solutes between the two phases.[20,21]

HPLC has the advantage of rapid precise quantitative analysis, automated separation, high sensitivity detection, quantitative sample recovery and it is amenable to diverse samples. It can be used to assess the purity and/or determine the content of many pharmaceutical substances. It can also be used to determine enantiomeric composition, using suitably modified mobile phases or chiral stationary phases. Individual separation mechanisms of adsorption, partition, ion exchange and size exclusion rarely occur in isolation, since several principles act to together.[20,21]

1.2.10.2.1 HPLC Apparatus

The apparatus consists of a pumping system, an injector, a chromatographic column, stationary and mobile phases, connecting tubing and fittings, a detector, and a data collection device (computer, integrator or recorder).

HPLC pumping systems are required to deliver metered amounts of mobile phase at a constant flow rate. Pumping systems that deliver solvent from one or more reservoirs are available. Pressure fluctuations should be minimized, e.g. by passing the pressurized solvent through a pulse-dampening device. Tubing and connections should be capable of withstanding the pressures developed by the pumping system. Many HPLC pumps are fitted with a facility for "bleeding" the system of entrapped air bubbles.[20,21]

Modern computer- or microprocessor-controlled pumping systems are capable of accurately delivering a mobile phase of either constant (isocratic elution) or varying (gradient elution) composition, according to a defined programme. In the case of gradient elution, solvent mixing can be achieved on either the low or high-pressure side of the pump(s). Depending on the flow rate and composition of the mobile phase, operating pressures of up to 42000kPa (6000 psi) can be generated during routine analysis.[20,21]

The sample solution is usually introduced into the flowing mobile phase at or near the head of the column using an injection system based on an injection valve design which can operate at high pressure. Such an injection system has a fixed-loop or a variable volume device which can be operated manually or by an auto-sampler. Partial filling of loops may lead to poorer injection volume precision.

Chromatographic columns are usually made of polished stainless steel, are between 50mm and 300mm long, and have an internal diameter of between 2mm and 5mm. They are commonly filled with a stationary phase with a particle size of 5µm - 10µm. Columns with internal diameters of less than 2mm are often referred to as microbore columns. Ideally, the temperature of the mobile phase and the column should be kept constant during an analysis. Most separations are performed at ambient temperature but columns may be heated using, for instance, a water-bath, a heating block or a column oven in order to achieve better efficiency. Normally, columns should not be heated above 60°C because of the potential for stationary phase degradation or changes occurring to the composition of the mobile phases.[20,21]

Separation of pharmaceuticals is usually achieved by partition of compounds in the test solution between the mobile and the stationary phases. HPLC systems consisting of polar stationary phases and non-polar mobile phases are described as normal-phase chromatography; those with non-polar stationary phases and polar mobile phases are called reversed-phase chromatography.[20,21]

1.2.10.2.2 Stationary Phase

There are many types of stationary phases used in HPLC including:

- unmodified silica, alumina, or porous graphite, used in normal-phase chromatography, where separation is based on differences in adsorption;

- a variety of chemically modified supports prepared from polymers, silica, or porous graphite, used in reverse-phase HPLC, where separation is based principally on partition of the molecules between the mobile phase and the stationary phase;

- resins or polymers with acid or basic groups, used in ion-exchange chromatography, where separation is based on competition between the ions to be separated and those in the mobile phase;

- porous silica or polymers, used in size-exclusion chromatography, where separation is based on the relative molecular mass of the molecules.[20,21]

Most separations are based on partition mechanisms using chemically modified silica as the stationary phase and polar solvents as the mobile phase (reverse-phase HPLC). The surface of the support, for example, the silanol groups of silica, is reacted with various silane reagents to produce covalently bonded silyl derivatives covering a varying number of active sites on the surface of the support. The nature of the bonded phase is an important parameter for determining the separation properties of the chromatographic system.[20]

Commonly used bonded phases are octadecyl (Si-$(CH_2)_{17}$-CH_3; C_{18}), phenyl (Si-$(CH_2)_3$-C_6H_5; C_6H_5), cyanopropyl (Si-$(CH_2)_3$-CN; CN), aminopropyl (Si-(CH2)3-NH_2; NH_2) and diol (Si-$(CH_2)_3$-OCH(OH)-CH_2-OH). For the separation of enantiomers, special chemically modified stationary phases (chiral chromatography) are available and examples are cyclodextrins and albumins [20, 21]

As a guide, silica-based reverse-phase columns are generally considered to be stable in mobile phases with an apparent pH in the range 2.0 - 8.0, but the column manufacturer's instructions should be consulted before using the column. Columns containing particles of polymeric materials such as styrene divinylbenzene copolymer are stable over a wider pH range.[20]

Analysis using normal-phase HPLC with unmodified silica, porous graphite or polar chemically modified silica (e.g. cyanopropyl or diol) as the stationary phase and a non-polar mobile phase is still employed in certain cases.[20].

For analytical separations the particle size of the most commonly used stationary phases varies between 3μm and 10μm. The particles may be spherical or irregular, of different porosities and specific surface area. In the case of reversed-phase, the extent of bonding of the stationary phase is expressed as the carbon-loading. Furthermore, stationary phases may be "end-capped" by reducing the number of residual silanol groups by methylation. These parameters contribute to the chromatographic behaviour of a particular stationary phase. Tailing of peaks, particularly for basic substances, can occur when residual silanol groups are present.[20,21]

In the case of artesunate – amodiaquine combination separation, if C_{18} column which is more polar is used, amodiaquine elutes before artesunate but the reverse occurs if C_8, which is less polar, is used. In artesunate – amodiaquine tablets, the concentration of amodiaquine is higher than that of artesunate hence if C_{18} is used, amodiaquine elutes and truncates before artesunate. The C_8 column is therefore preferred and it was the one used in this work.[20,21]

1.2.10.2.3 Mobile Phase.

The choice of mobile phases is based on the desired retention behaviour and the physicochemical properties of the analyte.[21]

For normal-phase HPLC using unmodified stationary phases, lipophilic solvents should be employed. The presence of water in the mobile phase must be avoided as this will reduce the efficiency of the stationary phase. In reverse-phase HPLC, aqueous mobile phases with or without organic modifiers, are used.[21]

The mobile phase should be filtered through suitable membrane-type filters with a porosity of 0.45μm to remove mechanical particles. Multicomponent mobile phases should be prepared by measuring the required volumes (unless masses are specified) of

the individual components, followed by manual or mechanical mixing. Alternatively, the solvents may be delivered by the individual pumps or proportioning valves of the liquid chromatograph and mixed according to the desired proportion. Solvents are normally degassed by sparging with helium or by sonification before pumping to avoid the creation of gas bubbles in the detector cell.[21]

If an ultraviolet detector is employed, the solvents used for the preparation of the mobile phase should be free of stabilizers and transparent at the wavelength of detection. Adjustment of the pH, if necessary, should be made using the aqueous component of the mobile phase and not the mixture. Buffers of high molarity should be avoided in the preparation of mobile phases. If buffers are used, the system should be rinsed with an adequate mixture of water and the organic modifier of the mobile phase to prevent crystallization of salts.[21]

Mobile phases may contain other components, example, a counter-ion for ion-pair chromatography or a chiral selector for chiral chromatography using an achiral stationary phase.[21]

1.2.10.2.4 Connecting Tubes and Fittings

The potential efficiency of an analytical column may never be achieved because of the design limitations of injectors and detectors. The connections between injector/column, column/detector, and/or detector/detector may compromise the overall efficiency of the system and any fittings should be of the "zero dead volume" (ZDV) type. It is recommended that minimum lengths of capillary tubing with a maximum internal diameter of 0.25 mm be used for these fittings to minimize band spreading.[21]

1.2.10.2.5 Detectors

Ultraviolet/visible (UV/vis) absorption spectrometers are the most commonly used detectors for pharmaceutical analysis. In specific cases, fluorescence spectrophotometers, differential refractometers, electrochemical detectors, light-scattering detectors, mass spectrometers, or other special detectors may be used. Where an analyte possesses a chromophoric group that absorbs UV/visible radiation, the UV/visible detector is the

most appropriate because of its sensitivity and stability. Such a detector is not suitable for detecting analytes with very weak chromophores.[21]

A variant on the UV/vis type of detector, which is becoming increasingly popular because of its ability to furnish detailed spectral information, is the diode array spectrophotometer. This type of detector acquires absorbance data over a certain UV/vis range and can provide chromatograms at multiple, selectable wavelengths, together with spectra for the eluted peaks. In addition, the detector and accompanying computer programmes can be used to assess the spectral homogeneity of peaks, which may provide information on the chromatographic purity of the peaks. This can be especially useful in method development and validation.[21]

1.2.10.2.6 Data Collection Devices

Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store, and reprocess chromatographic data. The data storage capacity of these devices is usually limited.

Modern data stations are computer based and have a large storage capacity to collect, process, and store data for possible subsequent reprocessing. Analytical reports can often be made according to the needs of the analyst.[21]

Integration of peak areas and the setting of threshold levels are not normally problematic in an assay since the peak of the substance to be analysed should be free of interference. However, in a test for impurities, the selection of the peak area integrator parameters becomes very important, particularly when baseline separations are not always attainable. If baseline separations cannot be obtained, valley-to-valley integration should be employed.(21)

HPLC allows limits to be set for individual impurities and for the sum of impurities, but there is a level at which peaks should not be integrated. This "disregard level" is set in relation to the area of the peak in the chromatogram of the prescribed reference solution is and usually equivalent to 0.05% of the substance being examined.[21]

The limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The limit of quantification (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions.[21]

Though the United States Pharmacopoeia expresses detection limit and quantification limit in terms of 2 or 3, and 10 times noise level respectively, this concept is not very practical. Noise level on a detector during the method development phase may be different when samples are assayed on different detectors. The use of standards in the test method at the quantification limit level is an assurance that the impurity can be observed and quantitated.[21] A method of determining the LOD or LOQ is the use of standard deviation of the response and slope of the calibration curve.

1.2.10.2.7 System Suitability

The system suitability test represents an integral part of the method and is used to ensure the adequate performance of the chosen chromatographic system.[21]

Efficiency, capacity factor, resolution factor, and symmetry factor are the parameters that are normally used in assessing the column performance. Factors that can affect chromatographic behaviour include mobile phase composition, temperature, ionic strength and apparent pH, flow rate, and column length, and stationary phase characteristics such as porosity, particle size and type, specific surface area, and, in the case of reversed-phase supports, the type of chemical modification, carbon loading, and end-capping.[21]

1.2.10.2.7.1 Efficiency (N)

The efficiency of a chromatographic column is defined in terms of the number of theoretical plates (*N*) and can be calculated using the following formula:

$$N = 5.54 \frac{t_R^2}{W_h^2}$$

where

t_R = retention time or the baseline distance between the point of injection and the perpendicular dropped from the maximum of the peak of interest.[21]

W_h = the width of the peak of interest determined at half peak height, measured in the same units as tR.

The number of theoretical plates can be expressed per metre (N'):

$$N' = \frac{N}{l}$$

where

l = length of column in metres.

1.2.10.2.7.2 Capacity Factor (Mass Distribution Ratio, Dm)

The capacity factor or mass distribution ratio is defined as amount of solute in stationary phase to amount of solute in mobile phase.[21]

This factor determines the retention of a solute and can be calculated from the chromatogram using the following formula:

$$D_m = \frac{(t_R - t_M)}{t_M}$$

where

t_R = retention time of the solute

t_M = retention time of an unretained component.[21]

A low D_m value indicates that the peak elutes close to the solvent front, which may compromise selectivity. A minimum D_m value of 1 is recommended for the peak of interest.[21].

The retention time of the test substance can be varied, if necessary, by changing the relative proportion or composition of solvents in the mobile phase. Generally, an increase in the proportion of a more polar solvent will lead to a shorter retention time on a normal-phase column and a longer retention time on a reversed-phase column.[21]

1.2.10.2.7.3 Resolution Factor (R_s)

The resolution between two peaks of similar height in a chromatogram can be calculated using the following formula:

$$R_s = \frac{1.18(t_{R2} - t_{R1})}{(W_{b1} + W_{b2})}$$

where

t_{R1} and t_{R2} = retention times or baseline distances between the point of injection and the perpendicular dropped from the maximum of each of the two peaks.

W_{b1} and W_{b2} = the respective peak widths determined at half peak height, measured in the same units as t_{R1} and t_{R2}.

The value of Rs for a baseline separation between peaks of similar height should be at least 1.5.[21]

1.2.10.2.7.4 Relative retention

The relative retention (r) is calculated as an estimate using the following formula:

$$r = \frac{t_{R2} - t_M}{t_{R1} - t_M}$$

where

t_{R2} = retention time of the peak of interest

t_{R1} = retention time of the reference peak

t_M = retention time of an unretained component

The unadjusted relative retention (r_G) is calculated from the expression:

$r_G = t_{R2}/t_{R1}$

Unless otherwise indicated, values for relative retention stated in the monographs correspond to unadjusted relative retention.[21]

1.2.10.2.7.5 Symmetry Factor (A_s)

The symmetry factor for a peak can be calculated using the following formula:

$$A_s = \frac{W_x}{2d}$$

where

W_x = peak width at 5% of peak height, measured from the baseline.

d = baseline distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 5% of the peak height, measured in the same units as W_x.

Values of A_s which are greater than 2 may lead to incorrect integration, resulting in erroneous quantitation. The main factors that influence peak symmetry depend upon retention, solvent effects, incompatibility of the solute with the mobile phase, or development of an excessive void at the inlet of the column. In reversed-phase chromatography, adsorption phenomena due to the presence of residual silanol groups in the stationary phase may lead to tailing (poor peak symmetry).[21]

1.2.10.2.7.6 Repeatability

Unless otherwise stated in the "Assay" of the individual monograph, the relative standard deviation of peak areas or peak heights for a series of injections of reference solutions bracketing groups of test solutions should not exceed 2.0%.

In a "Related substances" test the relative standard deviation of peak areas or peak heights for three replicate injections of the reference solution should not exceed 5.0%, unless otherwise stated in the individual monograph.

In a series of injections the relative standard deviation of the retention time of the principal peak should not exceed 1.0%.[21]

1.2.11 Review of Analytical Methods

Artesunate

The BP gives no analytical method for determining the levels of artesunate alone or artesunate-containing formulations. The USP (2003 edition) also gives no analytical considerations. However, the IP gives an analytical method for analyzing and assaying artesunate.

1.2.11.1 Artesunate only: IP methodology

Titration: 0.25g of artesunate is dissolved in 25ml of ethanol TS and titrated with 0.05M

sodium hydroxide, using 2 drops of phenolphthalein as indicator. A blank determination is done. The equation of the reaction is: [30]

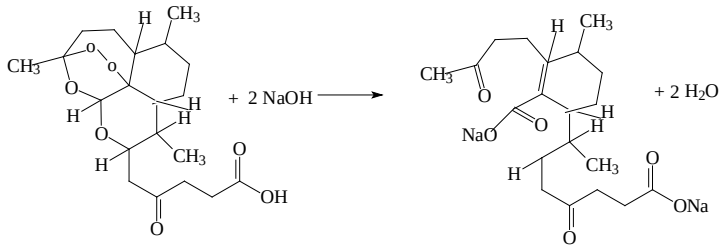

Figure 3a: Reaction between artesunate and sodium hydroxide.

1ml of 0.05M Na0H = 19.22mg of artesunate.

Chromatographic system: An HPLC method using a stainless steel column (12.5cm x 3.5mm) packed with particles of silica gel, the surface of which has been modified with chemically bonded octadecyl groups (5µm). As the mobile phase, use a mixture of equal volumes of acetonitrile R and buffer pH 3.0 (dissolve 1.36g of potassium dihydrogen phosphate R in 1000ml of water and adjust the pH to 3.0 with phosphoric acid).[21]

Solution A of 4.0mg of artesunate per ml is prepared in acetonitrile and solution B of 4.0mg artesunate also in acetonitrile, and for solution C, dilute solution A to obtain a concentration equivalent to 0.04 mg of artesunate per ml.

Operate with a flow rate of 0.6 ml per minute. Maintain a column temperature at 30˚C and use an ultraviolet spectrophotometer at a wavelength of about 216nm. Inject alternately 20µl each of solutions A and B.

Measure the areas of the peak response obtained in the chromatograms from solutions A and B, and calculate the percentage content of artesunate with reference to anhydrous substance.

Identity tests: Thin - layer chromatography using silica gel as the coating substance and a mixture of 5 volumes of ethyl acetate and 95 volumes of toulene as the mobile phase .

Apply separately to the plate 2µl of the following two solutions in toluene containing (A) 0.10mg of artesunate per ml, and (B) 0.10mg of artesunate per ml. After removing the

plate from the chromatographic chamber, allow it to dry in air, spray with methanol, and heat the plate to 120˚C for 5 minutes. Examine the chromatogram in ultraviolet light (254nm).The principal spot obtained with solution A corresponds in position, appearance and intensity with that obtained with solution B.

Colour test: Dissolve 0.1g of artesunate in 40ml of dehydrated ethanol, shake, and filter. To half of the filtrate add 0.5ml of hydroxylamine hydrochloride and 0.25ml of sodium hydroxide. Heat the mixture in water-bath to boiling, cool and add 2 drops of hydrochloric acid and 2 drops of ferric chloride; a light red-violet colour is produced.
Evaporate the remaining filtrate on a water-bath to a volume of about 5ml. Place a few drops of the mixture on a white porcelain dish, add 1 drop of vanillin/sulphuric acid; a reddish-brown colour is produced. [21]

1.2.11.2 Amodiaquine: USP methodology.

UV method: Transfer about 300mg of amodiaquine hydrochloride, accurately weighed to a 200ml volumetric flask; add dilute hydrochloric acid to volume and mix. Pipette 10ml of solution into a 1000ml volumetric flask; add dilute hydrochloric acid to volume and mix. Concomitantly determine the absorbance of this solution and a solution of USP amodiaquine hydrochloride RS in the same medium having a known concentration of about 15µg per ml , in 1 cm cells at the wavelength of maximum absorbance at about 342nm, with a suitable spectrophotometer, using dilute HCl as the blank. Calculate the quantity in mg, of $C_{20}H_{22}ClN_3O.2$ HCl in the portion of amodiaquine hydrochloride taken by the formula:

$$20C\ (Au/As)$$

in which C is the concentration in µg per ml, of USP Amodiaquine Hydrochloride RS in the standard solution and Au and As are absorbance of the solution of amodiaquine hydrochloride and the standard solution respectively.[22]

1.2.12 Choice of Analytical Methods

Neither of the pharmacopoeias gives an HPLC method for analyzing artesunate and amodiaquine tablets in combination. A new HPLC method that elutes both artesunate and

amodiaquine was developed by going through series of stages to improve upon its dependability and reproducibility.

1.2.13 Statistics in Pharmaceutical Analysis

The results of various analyses should be subjected to statistical methods of analysis in order to establish whether the differences are due to experimental errors or due to actual amount of substance in the drug products or the effectiveness of the methodology.

Statistics deals with the collection, organization, analysis, interpretation and presentation of information that can be stated numerically.

In most statistical work, analysis is done in a sub-group of the target population. For the information to be relevant, specific sampling methods are necessary. During drug analysis, two types of errors can be encountered; random and systematic errors.
Also known as indeterminate errors, random error causes individual results to be evenly scattered around the mean. It is impossible to eliminate them entirely, but they can be minimized by careful experimental design and control. Systematic or determinate error results to a bias in measured values.

In drug analysis, mean and the standard deviation are two main parameters used to estimate errors. The mean (μ) is the sum of all the measured properties of all members of the sample ($\sum x_i$) by the total number (n) of all the samples.

$\mu = \sum x_i/n$

where μ is the mean and n is the sample size.

The mean gives a measure of the tendency of the set-off data to center around the numerical value. It is affected by the value of each observation of distribution. The standard deviation is a measure of the spread of the individual variables about the mean. It is a measure of precision.

$$S = \sqrt{\sum(x-\mu)^2/(n-l)}$$

Where (x-μ) is the difference between the observation (x) and the mean (μ) and (n-l) is the number of degree of freedom.

Quantitative data are accepted if they are known to be free from determinate errors and there is no significant difference between the data obtained. This can be tested using the null hypothesis. It suggests that there is no significant difference between two measured statistical variables and any difference resulting is due to random error.

Comparison is based on probability and the range is the confidence interval. The limits of the intervals are confidence limits. The t-test can be used to compare two means.

Mathematically, $t = (\mu_1-\mu_2)/ S\sqrt{(1/n_1 + l/n_2}$. Where μ_1 and μ_2 are the sample means.

The t-test can be used to evaluate population means, difference of sample mean from population means and difference between mean of paired samples. Of much interest is the difference between means of two samples.

F-test

It is a single test of the null hypothesis and it is used to determine the validity of the simple t-test and also to determine if two variances are statistically different. To determine whether the variation amongst different samples from the sample variation is homogenous, the f-test is used. The variances are arranged such that $F \geq 1$. If the F value exceeds a critical value, the null-hypothesis is rejected.

Mathematically, $F = S^2_1/ S^2_1$

where S_1 and S_2 are the standard deviations of the different samples.

1.2.13.1 Analysis of variance (ANOVA)

Analysis of variance is an extremely powerful statistical technique, which can be used separate and estimate causes of variation.

This is used to analyse differences amongst groups. The method compares means of samples from sample population. The use of the t-test to compare means of more than two groups is cumbersome and often confusing and therefore the versatility of

ANOVA is of great importance to the analyst when multiple samples are under consideration. The F-test is used as the sample distribution. ANOVA can be considered as a combination of the principles of the t and F tests.[14]

CHAPTER TWO

MATERIALS, APPARATUS AND METHODOLOGY

2.1 Materials

2.1.1 Equipment

- Libror AEG – 220 Schimadzu Electronic Balance
- Glass and quartz cuvettes.
- Refluxing Apparatus
- **Pump**: LC – 10AT Schimadzu Liquid Chromatograph Pump..
- **Sample injector:** syringe loading sample injector fitted with external 20µl loop.
- **Detector**: Applied Biosystems 783A Programmable Absorbance UV Detector.
- **Syringe**: SGE 100µl
- **Integrator**: Schimadzu CR 501 Chromatopac
- Gallenkamp Melting Point Apparatus.
- **Stationary phase**: Anachem HPLC column, containing silica bonded to Octadecylsilyl groups. (S5C8-3554 250mm x 4.6mm internal diameter).
- 20 mesh sieve (Max equipment).
- Neutronic Humidity Chamber.
- Caplet-Shared Hand Punch Device.

2.1.2 Glassware

- Supertek 50ml Burettes
- Pipettes: Volac BS 700B Pyrex Pipettes - 1ml, 2ml, 3ml, 4ml, 5ml, 10ml, 20ml, 25ml.
- Volumetric flask: Pyrex A - 10ml, 25ml,50ml,100ml,200ml, 250ml,500ml.

2.1.3 Reagents and chemicals.

- Glacial acetic acid VS(BDH Laboratory Reagents)
- Triethylamine(Fisons Scientific Equipment)
- Sodium Hydroxide pellets(BDH Laboratory Reagents)
- Ethanol(BDH Laboratory Reagents)
- Phosphoric acid(ALDRICH Chemicals)
- Hydrochloric acid(36% $^{w}/_{v}$)(BDH Laboratory Reagents)
- Silica(ALDRICH Chemicals)
- Methanol(Fisons Scientific Equipment)
- Acetonitrile(BDH Laboratory Reagents)
- Phenolphthalein indicator
- Pure Artesunate powder RS (ICPA Laboratories Ltd)
- Pure Amodiaquine powder RS (MICRO ORGO-CHEM)
- Excipients (ABHISLEK ORG PVT LTD)

Table 2: Drug samples and code names

Name	Code Name	Date of Manufacture	Date of Expiry.
Artesunate-Amodiaquine combination tablets	ASA+AQA	15-12-08	_
Artesunate-amodiaquine combination tablets	ASW+AQW	15-12-08	_
Artesunate tablets	ASA	28-02-08	_
Artesunate tablets	AM	11/2008	10/2011

*ASA+AQA = Artesunate - Amodiaquine Combination tablets (alcohol used for granulation).

*ASW+AQW = Artesunate - Amodiaquine Combination tablets (water used for granulation)

*ASA = Artesunate tablets (alcohol used for granulation)

*AM = Artesunate tablets (market product).

2.2 Methodology

2.2.1 Identification of Samples.

2.2.1.1 Colour Identification Test

Pure artesunate powder:

0.1001g of pure artesunate powder was weighed into a clean beaker and 40ml of dehydrated ethanol was added, shook and filtered. No colour developed.

To half of the filtrate was added 0.5ml of hydroxylamine hydrochloride and 0.25ml of 2M sodium hydroxide. The mixture was heated in a water-bath to boil, cooled and 2 drops of 2M hydrochloric acid and 2 drops of 2M ferric chloride was added. A light red-violet colour developed.

The remaining filtrate was evaporated on a water bath to a volume of about 5ml. A few drops of the mixture were placed on a white porcelain dish and 1 drop of 1M Sulphuric acid was added. A reddish-brown colour developed

Pure Amodiaquine Hydrochloride powder

20.005mg/l solution of pure amodiaquine solution was prepared. 1ml of the solution was transferred into a clean beaker and 0.5ml of cobaltous thiocyanate was added using a graduated pipette. A green precipitate was produced.

2.2.1.2 Melting Point Determination.

Small portions of finely shaped pure artesunate were transferred into dry capillary tubes. The powder was packed by tapping the tube on a hard surface to obtain about 5mm of the drug in the tube. These were heated slowly in a Gallenkamp Melting Point apparatus. The temperature at which melting of the substance occurred was noted and recorded. This same procedure was repeated for the pure amodiaquine powder and the values recorded.

2.2.1.3 Thin Layer Chromatography

Two plates were prepared using silica gel as the coating substance and a mixture of 5 volumes of ethyl acetate and 95 volumes of toluene were used as the mobile phase. The market artesunate products were grinded and 0.1001mg was weighed and dissolved in acetonitrile and acetic acid solution and the mixture was filtered. The same was done for the artesunate pure powder. The plates were spotted with the prepared samples. The chromatograms were then developed in a chromatographic tank. After removing the plates from the chromatographic tank, they were allowed to dry in air and sprayed with anisaldehyde/methanol after which it was heated to 120˚C for 5 minutes. The chromatograms were then examined in ultraviolet light at 254nm.

2.2.2 Formulation of tablets.

The active ingredients and diluents were weighed and blended together and passed through a 24 size sieve. After that the wet mass was sized through suitable sieves, and the solvent was evaporated to granulate by drying at 50^{o}C-55^{o}C to achieve the desired moisture content. The dry granules were reduced by sizing through a 20 mesh sieve and lubricated with talc. In all the formulations, a 20 mesh sieve was used for the granulation and granules were compressed into caplet size tablets using the caplet shared hand punch device. (Refer to Tables 5, 6 and 7 for details of various weights).

2.2.3 Uniformity of Weight test.

Twenty tablets of each drug sample were selected at random and weighed together and individually using a sensitive Libror AEG-220 electronic balance already calibrated internally. The results were then tabulated. The percentage deviations per tablet were also calculated. According to the BP 2006, no single tablet should deviate by 10% or above and not more than two tablets should deviate by 5%. (Refer to tables 45 to 48)

2.2.4 Assay of pure Artesunate Powder Using Titration.

To a quantity of powder containing 0.2501g were added 25ml of distilled ethanol and this was titrated with 0.05M sodium hydroxide using phenolphthalein as indicator. A blank

titration was run and the sodium hydroxide was standardized using sulphamic acid. The difference between the two titrations represents the volume of alkali that reacted with the artesunate.

1ml of 0.05MNaOH is equivalent to 19.22mg of artesunate.

2.2.5 HPLC Method Development

2.2.5.1 Mobile Phase

Various mobile phase compositions which comprise the following were investigated:

1. 60 : 40 mixture of 0.07% $^{v}/_{v}$ of acetic acid and acetonitrile at pH of 3.6±0.1 using a C_8 column.

2. 60 : 40 mixture of 0.05% $^{v}/_{v}$ of acetic acid and acetonitrile at pH of 5.2 ±0.1 using C_8 column.

3. 55 : 45 mixture of 0.06% $^{v}/_{v}$ of acetic acid and acetonitrile at pH of 6.4 ±0.1 using C_{18} column.

4. 55 : 45 mixture of 0.075% $^{v}/_{v}$ of acetic acid and acetonitrile at pH of 6.7 ±0.1 using C_8 column.

5. 60 : 40 mixture of 0.05% $^{v}/_{v}$ of acetic acid and acetonitrile at pH of 7.4 ±0.1 using C_{18} column.

6. 65 : 35 mixture of 0.062% $^{v}/_{v}$ of acetic acid and acetonitrile at pH of 5.4 ±0.1 using C_8 column.

7. 55 : 405 mixture of 0.05% $^{v}/_{v}$ of sodium dihydrogen phosphate and acetonitrile at pH of 5.3 ±0.1 using C_8 column.

8. 60 : 40 mixture of 0.062% $^{v}/_{v}$ of sodium dihydrogen phosphate and acetonitrile at pH of 2.3 ±0.1 using C_{18} column.

9. 60 : 40 mixture of 0.55% $^{v}/_{v}$ of sodium dihydrogen phosphate and acetonitrile at pH of 3.6 ±0.1 using C_{18} column.

After several trials, a final composition made up of triethylamine, acetonitrile and glacial acetic acid was employed in the analysis. A volumetric mixture comprising 0.3% $^{v}/_{v}$ glacial acetic acid, acetonitrile and triehtylamine in a ratio of 55%:45% was used. The pH

of the final solution was always adjusted to 4.5±0.1 using 0.3% $^{v}/_{v}$ triethylamine. This solution was also used as the stock solution for dissolving the drug samples.

2.2.5.2 Preparation of Stock Solutions.

2.2.5.2.1 Artesunate stock solution

33mg of pure artesunate powder was accurately weighed and transferred into a 100ml volumetric flask. More of the mobile phase solution was added and shaken to dissolve after which it was made to the mark to constitute a 0.033% $^{w}/_{v}$ solution.

2.2.5.2.2 Amodiaquine stock solution

33mg of pure amodiaquine hydrochloride powder was weighed and transferred into a 100ml volumetric flask. More of the mobile phase solution was added and shaken to dissolve after which it was made to the mark to constitute a 0.033% $^{w}/_{v}$ solution.

2.2.5.3 Selection of Wavelength using UV

The mobile phase was used as the blank. Stock solutions of artesunate and amodiaquine were scanned and their wavelength of maximum absorptions recorded. Their spectra were also recorded (Refer to Appendix I and II for UV-spectrum of pure artesunate and pure amodiaquine).

2.2.6 HPLC Qualitative Analysis

0.0099% $^{w}/_{v,}$ 0.0066% $^{w}/_{v}$ and 0.02% $^{w}/_{v}$ solutions of pure artesunate powder solution, pure amodiaquine powder solution and the internal standard solution respectively were injected into the column one at a time and their retention times recorded. These solutions were then injected together and their retention times recorded. (Refer to Figs. 4, 5 and 6)

2.2.7 Calibration Curve for Pure Artesunate

Concentrations of 4.9194mg/l, 3.8262mg/l, 2.730mg/l, 1.6398mg/l, and 0.5466mg/l were prepared from the pure artesunate stock solution by picking corresponding volumes of 1.0ml, 3.0ml, 5.0ml, 7.0ml and 9.0ml respectively and diluted to 10ml. These were then

filtered using membrane filters and then made ready for injection. The samples were analysed at 254.0nm. (Refer to Fig. 7)

2.2.8 Calibration Curve for Pure Amodiaquine

Concentrations of 0.0858mg/l, 0.0660mg/l, 0.0462mg/l, 0.0264mg/l, and 0.0066mg/l were prepared from the pure amodiaquine stock solution by picking corresponding volumes 0.2ml, 0.8ml, 1.4ml, 2.0ml and 2.6ml respectively and diluted to 10ml. These were then filtered using membrane filters and then made ready for injection. The samples were analysed at 254.0nm. (Refer to Fig. 8)

2.2.9 Calibration Curve for Pure Artesunate using the IP method

The mobile phase was prepared using equal volumes of acetonitrile and potassium dihydrogen phosphate. The potassium dihydrogen phosphate was prepared by dissolving 1.36g of it in 100ml of water .The buffer was adjusted to pH of 3.0 with phosphoric acid.

200mg of artesunate was dissolved in 50ml (4.0mg/ml) and used as the stock solution. The HPLC was operated with a flow rate of 0.6ml per minute. A stainless steel column (12.5cm x 3.5mm) packed with particles of silica gel the surface of which has been modified with chemically bonded octedecysilyl groups was used. The wavelength of the ultraviolet spectrophotometer was set at 216nm. (Refer to Fig. 9)

Volumes of 0.6ml, 0.8ml, 1.0ml, 1.2ml and 1.4ml of the stock solution were dissolved to 100ml. These correspond to concentrations of 0.0240mg/l, 0.0320mg/l, 0.0400, 0.0480 and 0.0560 respectively. These were then filtered using membrane filters and 20 µl of each was injected.

2.2.10 Analysis of Stability of Artesunate in the Drug Samples.

2.2.10.1 Sample preparations

The various drug samples made up of ASA+AQA, ASW+AQW, ASA and ASM were divided into three sets. The first set was stored at ambient temperature (28^{o}C), the second set was stored in a Neutronic Humidity Chamber at a temperature of 40^{o}C and a relative

humidity of 75% and the third set was stored at a temperature of 60^{o}C and relative humidity of 75%. At the end of each time period, 20 tablets were individually weighed and triturated to obtain a uniform mixture.

2.2.10.2 Determination of Percentage Content of AS in ASA+AQA

0.3174g of ASA+AQA was weighed and dissolved in 25ml of the dissolution medium. The content of AS in ASA+AQA remaining was determined at time intervals of 0 minutes, 2 weeks, 4 weeks (1 month), 6 weeks (1 month, 2 weeks), 12 weeks (3 months) and 24 weeks (6 months) starting at ambient temperature(28^{o}C) using the HPLC method that has been developed (Refer to 2.2.5.1).

The peak areas were recorded and out of that a graph of ln of percentage content versus time was plotted and the slope determined (Refer to section 1.2.4).

The same procedure was repeated for all the other samples at 40˚C and 60^{o}C.
Refer to tables 13, 14 and 15 for the masses weighed at the various times.

2.2.10.3 Determination of Percentage Content of AS in ASW+AQW

0.3364g in ASW+AQW was weighed and dissolved in 25ml of the stock solution. The content of AS in ASW+AQW remaining was determined at time intervals of 0 minutes, 2 weeks, 4 weeks (1 month), 6 weeks (1 month, 2 weeks), 12 weeks (3 months) and 24 weeks (6 months) starting at ambient temperature(28^{o}C) using the HPLC method that has been developed (Refer to 2.2.5.1).
The peak areas were recorded and out of that a graph of ln of percentage content versus time was plotted and the slope determined (Refer to section 1.2.4).

The same procedure was repeated for all the other samples at 40˚C and 60^{o}C.
Refer to tables 16, 17 and 18 for the masses weighed at the various times.

2.2.10.4 Determination of Percentage Content of AS in ASA.

0.1421g of ASA was weighed and dissolved in 25ml of the sock solution. The content of AS in ASA remaining was determined at time intervals of 0 minutes, 2 weeks, 4 weeks (1 month), 6 weeks (1 month, 2 weeks), 12 weeks (3 months) and 24 weeks (6 months) starting at ambient temperature (28^oC) using the HPLC method that has been developed (Refer to 2.2.5.1).

The peak areas were recorded and out of that a graph of ln of percentage content versus time was plotted and the slope determined (Refer to section 1.2.4).

The same procedure was repeated for all the other samples at 40˚C and 60^oC.
Refer to tables 19, 20 and 21 for the masses weighed at the various times.

2.2.10.5 Analysis of Percentage Content of AS in ASM

0.1270g of ASM was weighed and dissolved in 25ml of the stock solution. The content of AS in ASM remaining was determined at time intervals of 0 minutes, 2 weeks, 4 weeks (1 month), 6 weeks (1 month, 2 weeks), 12 weeks (3 months) and 24 weeks (6 months) starting at ambient temperature(28^oC) using the HPLC method that has been developed (Refer to 2.2.5.1).

The peak areas were recorded and out of that a graph of ln of percentage content versus time was plotted and the slope determined (Refer to section 1.2.4).

The same procedure was repeated for all the other samples at 40˚C and 60^oC
Refer to tables 22, 23 and 24 for the masses weighed at the various times.

2.2.10.6 Other Salient Investigations

2.2.10.6.1 Validation of Analytical Method

2.2.10.6.1.1 Repeatability: A 2.733mg/ml solution of reference artesunate was accurately prepared. The solution was then filtered to remove any particles before the

analyte got onto the column. Samples from these solutions were successively run seven times after stabilizing the chromatographic system with the conditions determined.

Each set was run three times and the average peak area ratio taken to represent the set. The various solutes were determined by their retention times.

The peak area ratios were then used to calculate the actual concentration by interpolation from the calibration graph. These values were then tabulated and statistically analysed for the standard deviation of the method.

2.2.10.6.1.2 Reproducibility: Three different analysts were involved and analysis carried out on three different occasions and the results analysed statistically.

A 2.73mg/ml solution of reference artesunate was accurately prepared. The solution was filtered to remove any particles before the analyte got onto the column. Samples from the solution were successively run five times after stabilizing the chromatographic system with the conditions determined.

Each set was run three times and the average peak area ratio taken to represent the set. The various solutes were determined by their retention times.

The results were then paired and statistically analysed for a potential significant difference in their standard deviations.

2.2.10.6.1.3 Calibration graph: This was done to ensure that artesunate and amodiaquine concentrations in any test sample could be obtained by interpolation. A series of artesunate and amodiaquine solutions of nominal concentrations were analysed under the same HPLC conditions as those used for the previous work.

A 0.033%$^{w}/_{v}$ stock artesunate solution was prepared and serial dilutions of portions of this gave a range of diluted artesunate solutions that were chromatographed to generate the necessary data for the construction of a calibration graph. Samples were run in an ascending order of strength to ensure that of a previously run higher one did not overstate the result of a lower concentration. The serial dilution was done till the lowest level of

artesunate that could not be detected but not necessarily determined quantitatively under the stated experimental conditions was obtained.

The range of concentrations used was 4.92mg/ml, 3.83mg/ml, 2.73mg/ml, 1.64mg/ml and 0.55mg/ml. All determinations were done in triplicate and the average peak area ratios for each of the nominal concentration was calculated and tabulated.
A graph of average peak area ratio on the vertical (y) axis was plotted against concentration on the horizontal axis.

Also a 0.33%$^{w}/_{v}$ stock amodiaquine solution was prepared and serial dilutions of portions of this gave a range of diluted amodiaquine solutions that were chromatographed to generate the necessary data for the construction of a calibration graph. Samples were run in an ascending order of strength to ensure that of a previously run higher one did not overstate the result of a lower concentration. The serial dilution was done till the lowest level of amodiaquine that could not be detected but not necessarily determined quantitatively under the stated experimental conditions was obtained.

The range of concentrations used was $6.6*10^{-3}$mg/ml, $2.6*10^{-2}$mg/ml, $4.6*10^{-2}$mg/ml, $6.6*10^{-2}$mg/ml and $8.6*10^{-2}$mg/ml. All determinations were done in triplicate and the average peak area ratios for each of the nominal concentration was calculated and tabulated.

A graph of average peak area ratio on the vertical (y) axis was plotted against concentration on the horizontal axis.

These were then used in assessing the linearity and range of the method.

2.2.7.6.1.3 Precision: This was assessed by replicate injection of required concentration (n=10) and the precision expressed in relative standard deviation (RSD) for the responses.

2.2.7.6.1.4 Accuracy: Recovery test were performed by adding known amounts of standard solution to sample followed by analysis using the new method under investigation and results tabulated as indicated.

2.2.7.6.1.4 Specificity: The robustness of the new method under investigation was studied by intentionally varying amounts of respective components of the mobile phase to investigate the effect it will have on the results. Adulterants such as aspirin, paracetamol and chloroquine were added to the analyte but the mobile phase was still able to pick artesunate and amodiaquine.

Various columns from different sources were used. Chromatographic conditions such as wavelength of absorption, AUF values, and chart speed and pH changes were all investigated.

2.2.7.6.1.5 Stability: A 0.1%$^{w}/_{v}$ standard solution of both artesunate and amodiaquine were accurately prepared and chromatographed using same conditions as in previous work. The sample were eluted when freshly prepared at zero (0) minutes, 5, 10, 15, 20, 25, 30, 40, and 60 minutes and the trend of peak heights monitored and recorded. A graphical representation of this result was then plotted and analysed.

2.2.7.6.1.6 Limit of Detection and Quantification: 0.004%$^{w}/_{v,}$ 0.0013%$^{w}/_{v,}$ of artesunate and amodiaquine standard solutions respectively was injected five times and the average peak heights and base line noise were measured. Refer to section 3.11. 3.

CHAPTER THREE

RESULTS AND CALCULATIONS

3.1 Identification Test

Table3 : **Colour identification test**

Sample	**Observation**	**Inference**
0.1g of pure artesunate with ethanol and filtered.	No colour observed.	Artesunate may be present.
To half of the filtrate was added 0.5ml of hydroxylamine hydrochloride, 0.25ml of 2M sodium hydroxide and heated. To the mixture was added 2 drops of 2M hydrochloric acid and 2 drops of 2M ferric chloride.	A light red - violet colour developed.	Artesunate present.
Remaining filtrate evaporated to 5ml and 2 drops placed on a porcelain dish and 1drop of 1M sulphuric acid added.	A reddish – brown colour developed.	Artesunate present.
Pure amodiaquine plus 5ml of cobaltous thiocyanate	A green precipitate produced	Amodiaquine present

3.1.1 Melting Point

Table19: **Melting Point Determination**

Sample	Number of determinations(^{o}C)		Reference value/range(^{o}C)
	1	2	
Artesunate	131-134	132-135	132-135
Amodiaquine Hydrochloride	158.5	159.0	159.0

TLC Qualitative Analysis

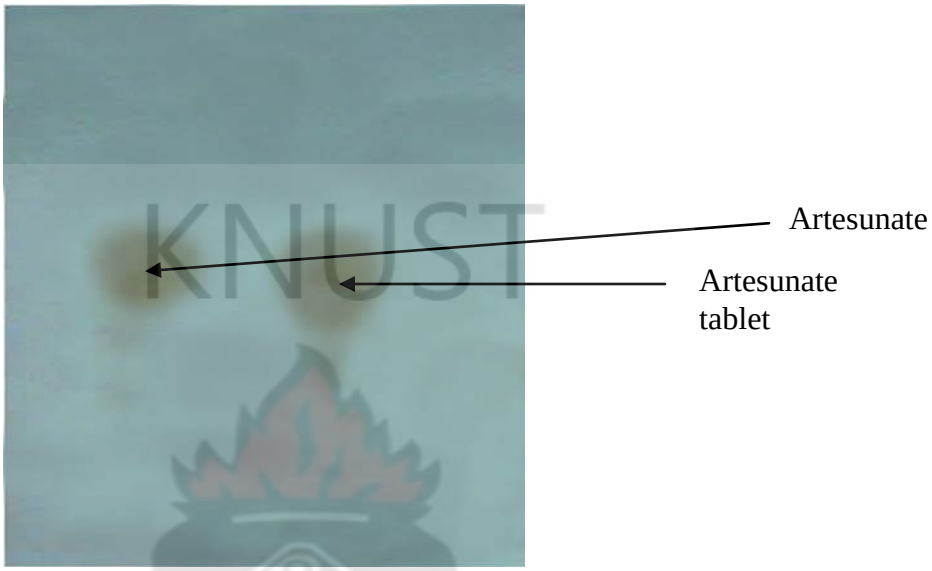

Fig 3: **Chromatograms for pure artesunate powder and artesunate tablets.**

Solute front of pure artesunate = 8.6cm.

Solute front of artesunate tablets (AM) = 8.5cm.

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3.2 Formulation of Tablets: Results showing amount of active drug substance and excipients used in the formulation.

Table 5: ASA+AQA

Sample	Weight(g)
Amodiaquine	15.0240
Artesunate	5.0046
Lactose	7.5011
PVP (q/s)	1.1118
Starch	3.0095
Talc	0.2091

Table 6: ASW+AQW

Sample	Weight(g)
Amodiaquine	15.1245
Artesunate	5.3455
Lactose	7.4936
PVP (q/s)	1.5004
Starch	3.0081
Talc	0.1480

Table 7: ASA

Sample	Weight (g)
Artesunate	5.1904
Lactose	7.5891
PVP	0.6001
Starch	3.2580
Talc	0.0905

3.3 Uniformity of weight test

Table 8 : **Uniformity of weight test.**

Code name	Total weight of 20 tablets/g	Average weight per tablet/g	Less than 5% Deviation	5% - 10%	Above 10%
ASA+AQA	12.6857	0.6348	20	0	0
ASW+AQW	12.7138	0.6348	20	0	0
ASA	6.5214	0.3250	20	0	0
AM	6.1061	0.2714	20	0	0

3.4 Titration

3.4.1 Assay of pure Artesunate Powder

Preparation of 0.05M NaOH:

40g NaOH ≡ 1000ml = 1M NaOH

2g NaOH ≡ 1000ml = 0.05M NaOH

0.5g NaOH ≡ 250ml = 0.05M NaOH

Purity of NaOH = 98%

(100/ 98)* 0.05g = 0.05102

Preparation of 100ml of standard solution of sulphamic acid;

97.09gH_2NSO_3H≡1000ml of 1MNaOH

4.8545gH_2NSO_3H≡1000ml of 0.05MNaOH

0.00485gH_2NSO_3H≡1ml of 0.05MNaOH

Standardising 0.5 M sodium hydroxide:

Average mass of sulphamic acid used = 4.8545mg

From the milliequivalent, the expected titre value is = 8.20ml

Average actual titre volume = 8.23ml

Factor of 0.05 M NaOH (F_1) = 8.23 / 8.20

F_1= 1.0036 NaOH

1ml 0.05M NaOH ≡ 19.22mg of Artesunate

200.1mg of Artesunate was dissolved in 20ml ethanol and titrated with the NaOH

Blank titration = 0.1ml

The equation of the reaction is:

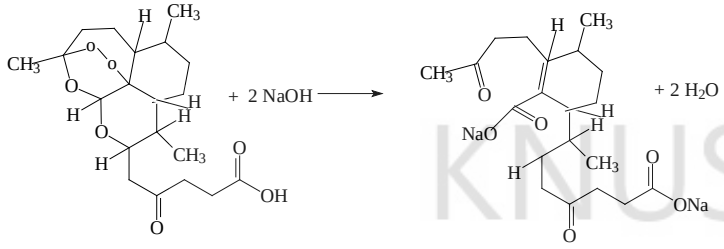


Figure 3b: Equation of the reaction.

Table9**: Titration results**

Mass/mg	200.1	203.0	205.0
Titre values/ml	10.40	10.60	10.80
Actual titre values/ml	10.30	10.50	10.70
F1*Actual/ml	10.33	10.54	10.74

1ml of NaOH = 19.22mg of Artesunate

Therefore 10.33ml of NaOH = (10.33ml/1ml)19.22 = 198.54mg

% purity = (198.54mg/200.1mg)100 = 99.22%

1ml of NaOH = 19.22mg of Artesunate

Therefore 10.54ml = (10.54ml/1ml)19.22 = 202.58mg

% purity = (202.58mg/203.0)100 = 99.79%

1ml 0f NaOH = 19.22mg of Artesunate

Therefore 10.74ml = (10.74/1ml)19.22 = 206.42mg

% purity = (206.42mg/205.0)100 = 100.69%

Average % purity = (99.22 + 99.79 + 100.69)/3 = 99.90%

3.5 Development of HPLC Method of Analysis.

3.5.1 Chromatographic Conditions

Mobile Phase: 0.3%$^{v}/_{v}$ glacial acetic acid: acetonitrile: triethylamine (55% : 45% : 0.3%)

pH : 4.5±0.1

Stationary phase: Anachem HPLC column, containing silica bonded to Octadecylsilyl groups (S5C8-3554 250mm x 4.6mm internal diameter.)

Wavelength of detection: 254.0nm

Flow rate: 2.0ml/minute

AUFS (Sensitivity): 0.500

Chart recorder speed: 5mm/min

Attenuation: 0

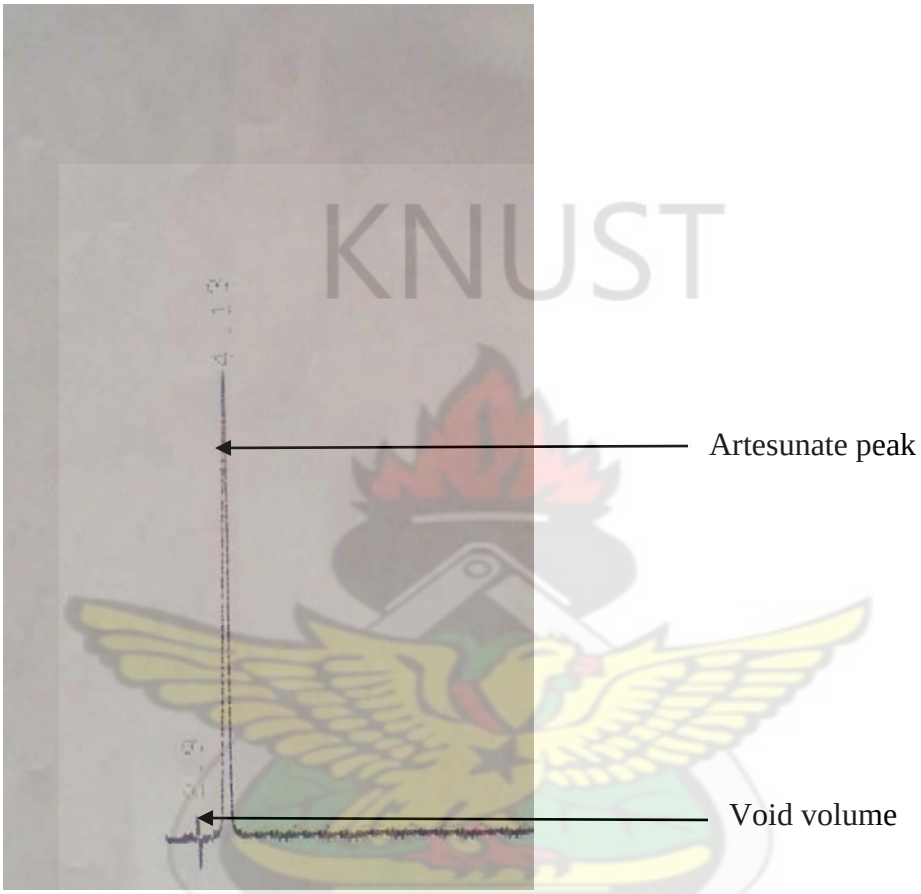
Temperature: Ambient temperature (28^{0}C)

Mean retention time of artesunate: 4.13±0.15mins.

Mean retention time of amodiaquine: 5.933±0.15mins.

3.6 HPLC qualitative Analysis.

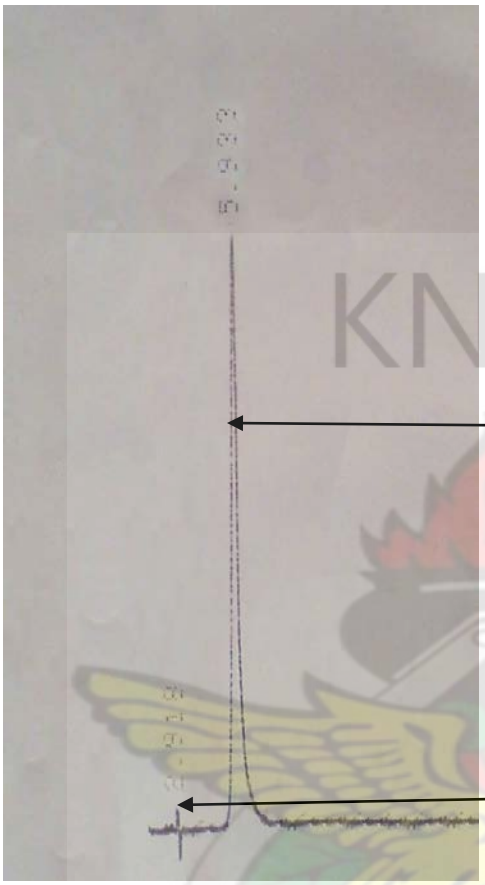
3.6.1 Artesunate.



Retention time = 4.13mins.

Fig 4 : Chromatogram of pure artesunate.

3.6.2 Amodiaquine



Retention time : 5.933mins.

Fig 5 : Chromatogram of pure amodiaquine.

3.6.3 Artesunate, Amodiaquine and Internal Standard.

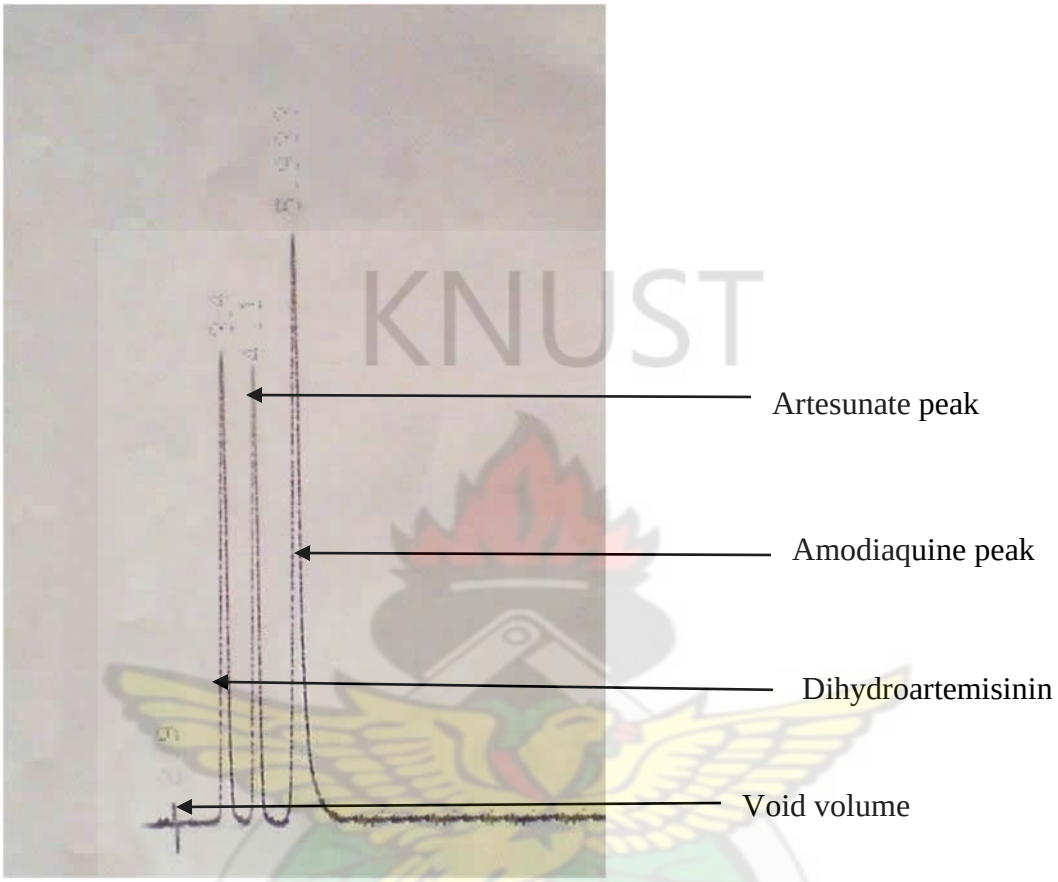


Fig 6 : Chromatograms for pure artesunate and pure amodiaquine showing their retention times.

Retention time of artesunate = 4.1mins.

Retention time of amodiaquine = 5.933mins.

Retention time of dihydroartemisinin = 3.4mins.

3.7 Calibration curves for Pure Samples.

Table10**: Calibration curve for Pure Artesunate powder**

Concentration(mg/ml)	Peak area ratio
4.92	1.78
3.83	1.34
2.73	1.01
1.64	0.65
0.55	0.26

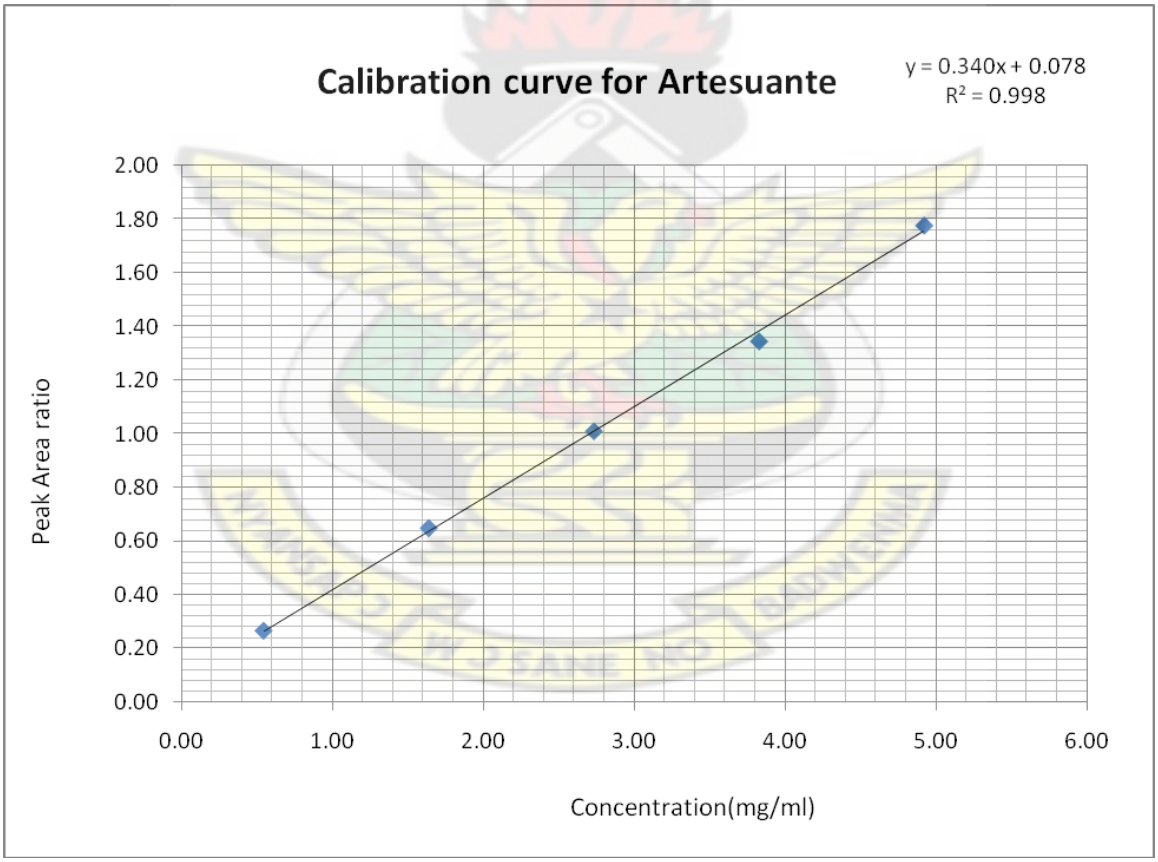


Fig 7: Calibration curve for pure artesunate using developed HPLC method

Table11 **: Calibration curve for Pure Amodiaquine powder**

Concentration in mg/ml	Peak area ratio.
$6.6*10^{-3}$	0.42
$2.64*10^{-2}$	0.55
$4.62*10^{-2}$	0.67
$6.6*10^{-2}$	0.79
$8.58*10^{-2}$	0.92

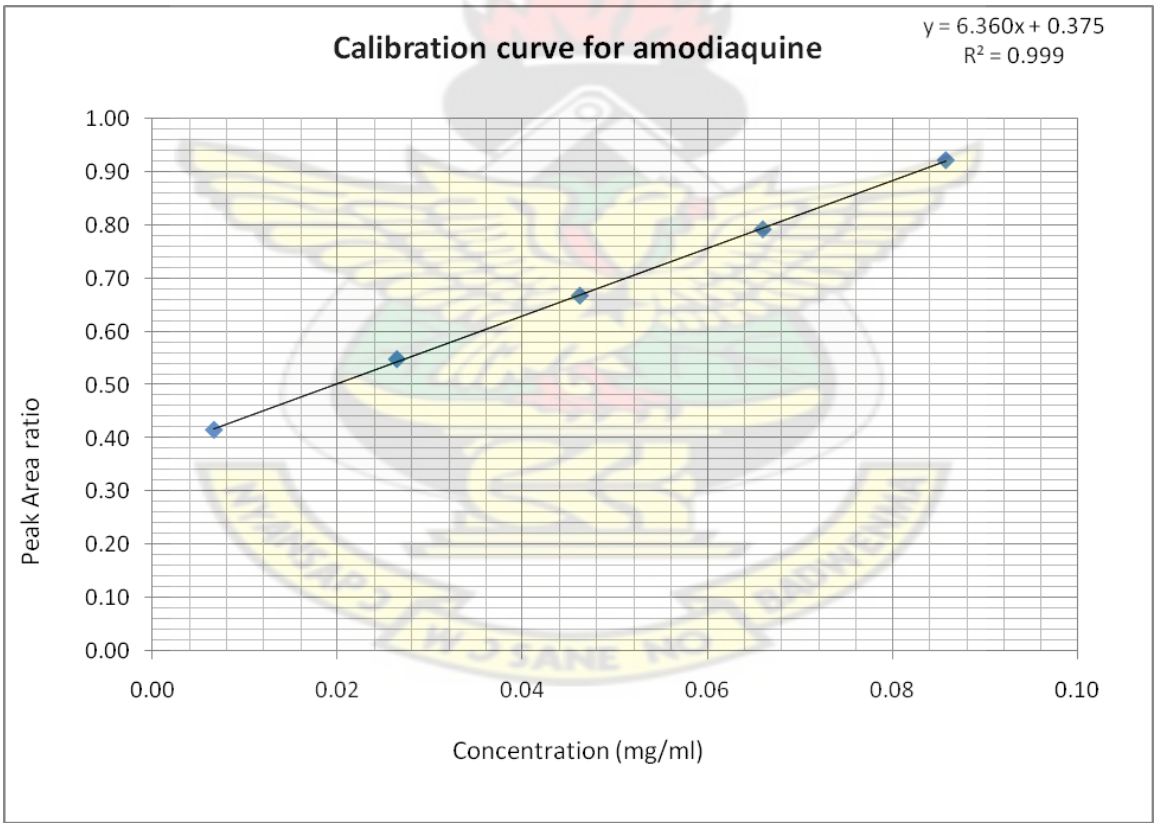


Fig 8: Calibration curve for pure amodiaquine using developed HPLC method

Table12: **Calibration curve for pure artesunate using IP Method**

Concentration in mg/ml	Peak area ratio
0.02	0.42
0.03	0.53
0.04	0.69
0.05	0.81
0.06	0.94

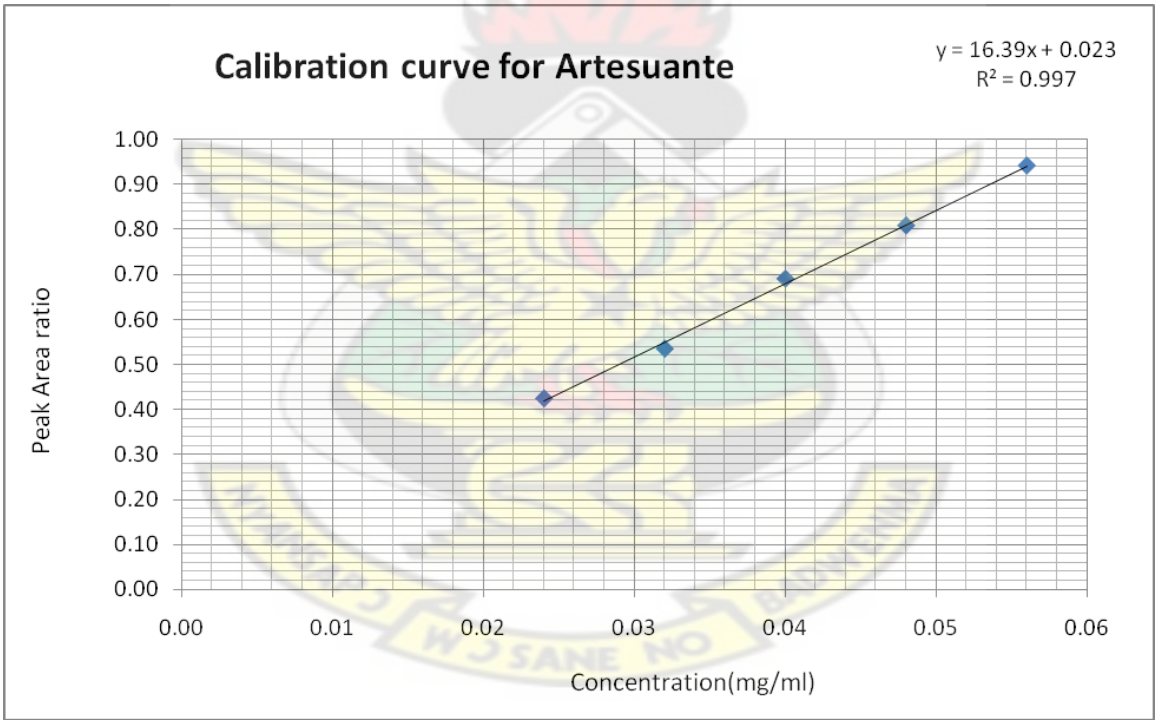


Fig 9: Calibration curve for pure artesunate using HPLC method in IP

3.8 Stability Studies

3.8.1 Tables showing masses of Drug Samples at Various Time Intervals

3.8.1.2 Analysis of AS IN ASA+AQA

Table 13: AS in ASA+AQA at 28^{o}

Time(mins.)	Mass of Product (g)
0.0	0.3174
336.0	0.3745
672.0	0.3491
1008.0	0.3237
1344.0	0.2984
2160.0	0.2730
4320.0	0.2476

AS = Artesunate
ASA+AQA = Artesunate – Amodiaquine combination tablets (alcohol used for granulation). Refer to Tables 34 to 44 for masses of the other drug samples.

3.9 % AS and ln of % AS in Drug Samples after Analysis and Corresponding Graphs.

*In all cases, the In was plotted to four decimals but only two decimals are shown in the graphs.

Table 14**: % AS in ASA+AQA at 28^{o}C**

Time(mins)	Percentage Content	ln(% content)
0.00	97.97	4.58
2.02E+04	96.30	4.57
4.03E+04	95.38	4.56
6.05E+04	94.36	4.55
8.06E+04	93.43	4.54
1.30E+05	91.65	4.52
2.59E+05	86.47	4.46

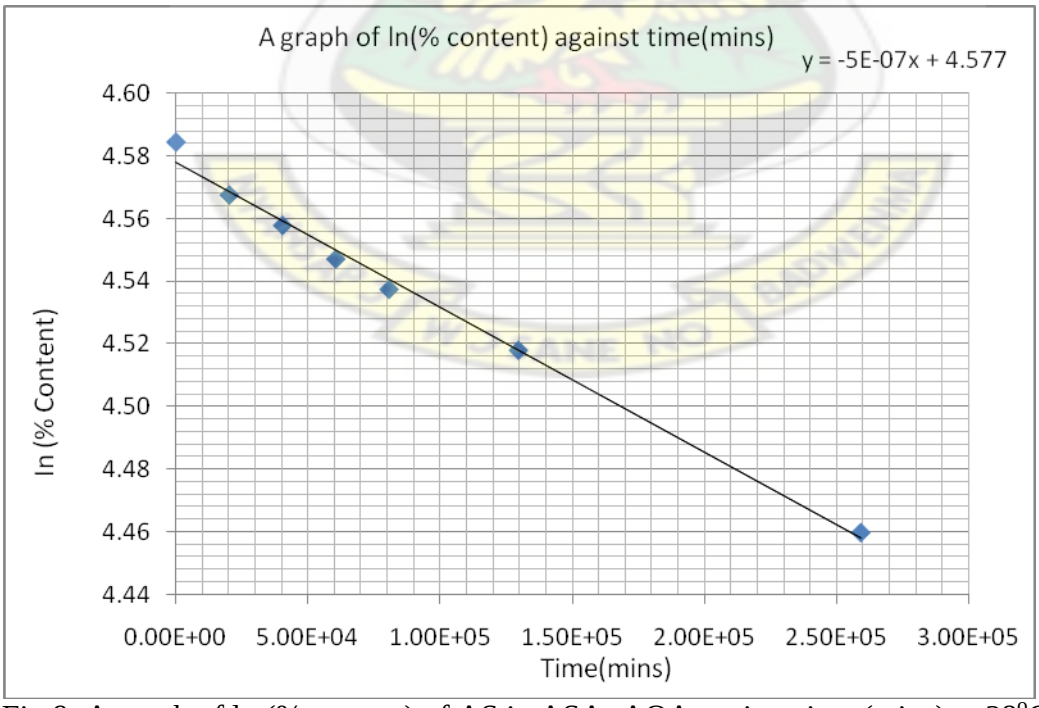


Fig 9: A graph of ln (% content) of AS in ASA+AQA against time (mins) at 28^{o}C

Table 15**: % AS in ASA+AQA at 40^{o}C**

minutes	Percentage Content	ln(% content)
0.00	97.23	4.58
2.02E+04	94.14	4.54
4.03E+04	91.15	4.51
6.05E+04	80.78	4.48
8.06E+04	85.41	4.45
1.30E+05	79.01	4.37
2.59E+05	64.23	4.16

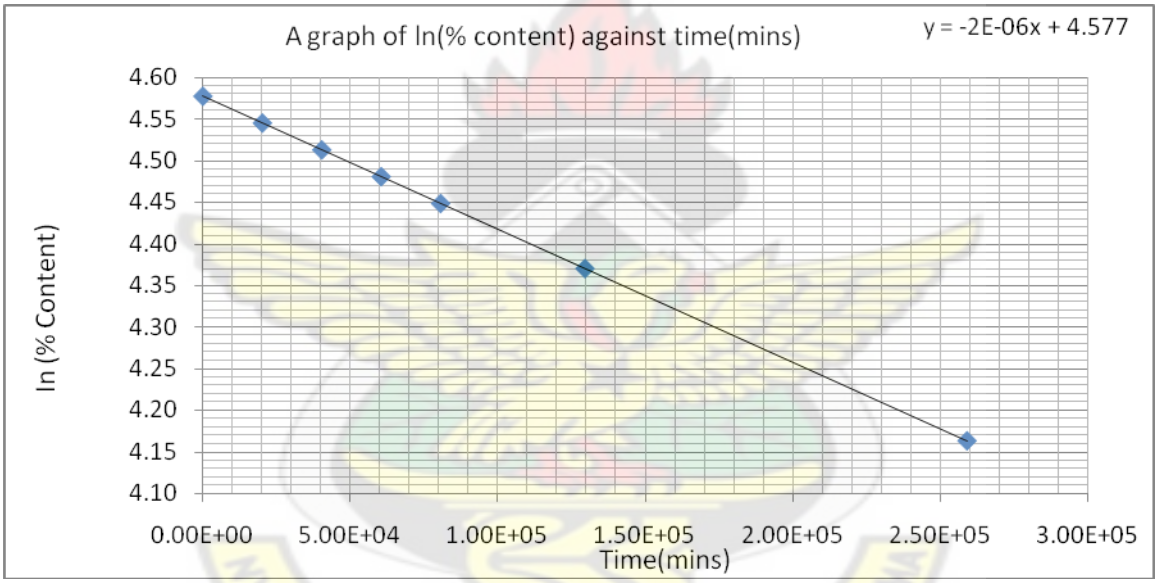


Fig10: A graph of ln (% content) of AS in ASA+AQA against time (mins) at 40^{o}C

Table 16**: % AS in ASA+AQA at 60^{o}C**

minutes	%Content	ln(% content)
0.00	97.23	4.58
2.02E+04	91.36	4.51
4.03E+04	85.39	4.45
6.05E+04	80.78	4.39
8.06E+04	75.23	4.32
1.30E+05	61.29	4.12
2.59E+05	40.27	3.69

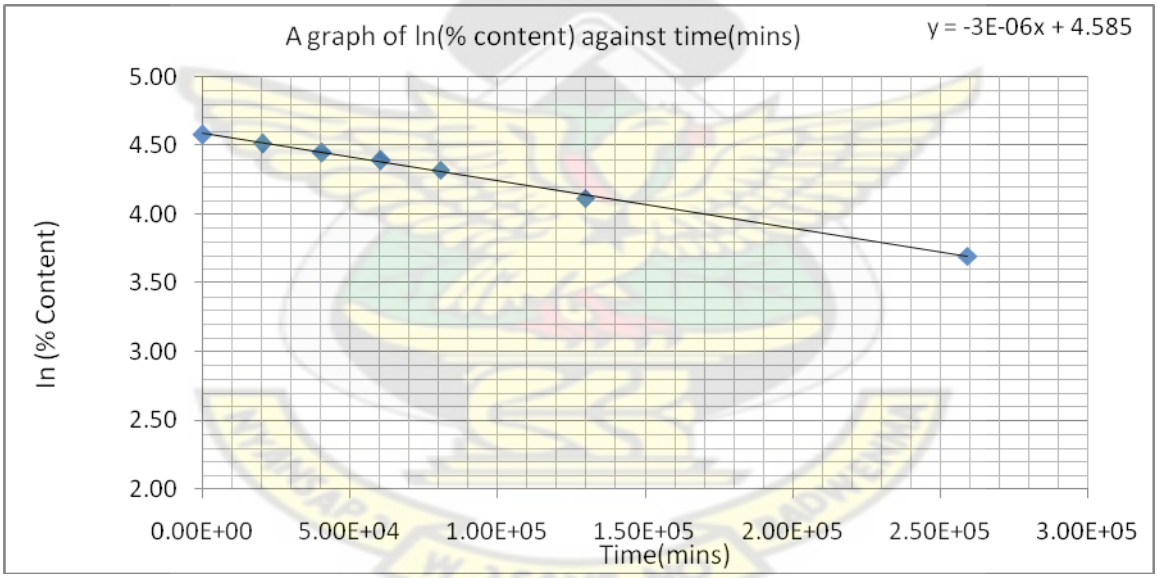


Fig11: A graph of ln (% content) of AS in ASA+AQA against time (mins) at 60^{o}C

Table 17**: % AS in ASW+AQW at 28^{o}C**

minutes	Percentage Content	ln(% content)
0.00	98.61	4.59
2.02E+04	97.24	4.58
4.03E+04	96.07	4.57
6.05E+04	95.35	4.56
8.06E+04	94.48	4.55
1.30E+05	91.84	4.52
2.59E+05	85.63	4.45

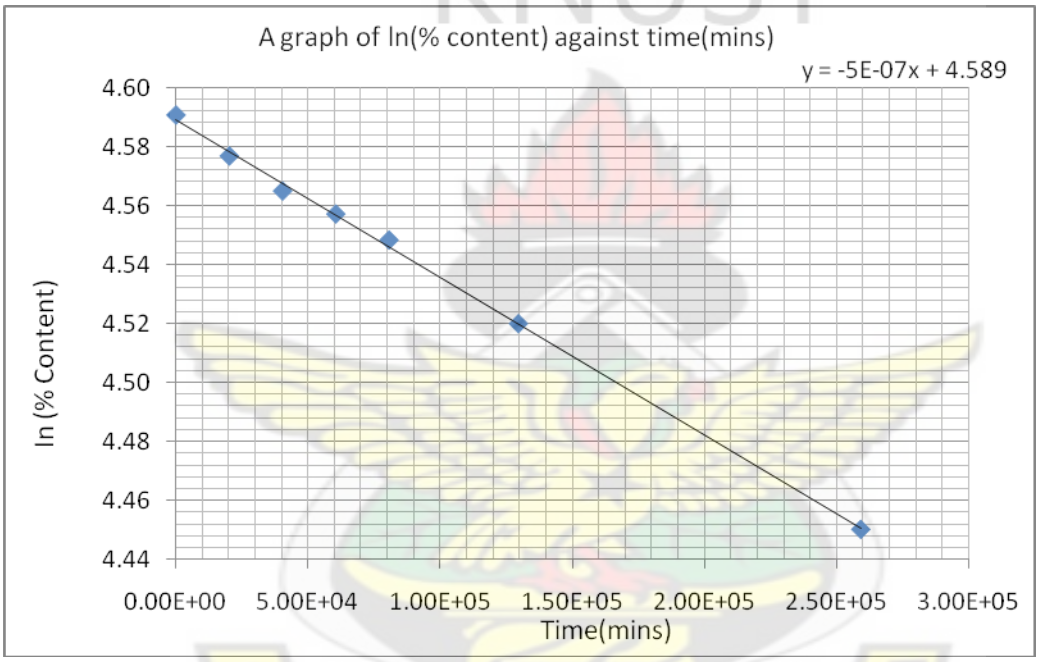


Fig12: A graph of ln (% content) of AS in ASW+AQW against time (mins) at 28^{o}C

Table 18**: % AS in ASW+AQW at 40^{o}C**

minutes	Percentage Content	ln(% content)
0.00	98.17	4.59
2.02E+04	93.49	4.54
4.03E+04	90.31	4.50
6.05E+04	84.33	4.43
8.06E+04	81.17	4.39
1.30E+05	72.17	4.28
2.59E+05	52.89	3.97

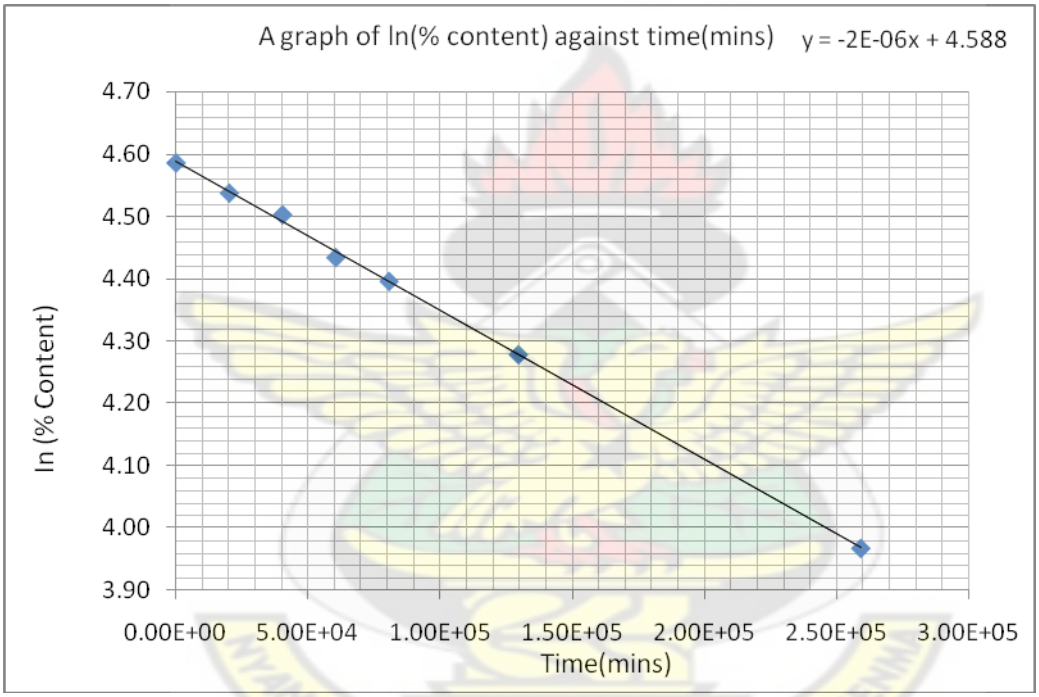


Fig12: A graph of ln (% content) of AS in ASW+AQW against time (mins) at 40^{o}C

Table 19**: % AS in ASW+AQW at 60^{o}C**

minutes	Percentage Content	ln(% content)
0.00	98.50	4.59
2.02E+04	85.88	4.45
4.03E+04	85.88	4.45
6.05E+04	80.19	4.38
8.06E+04	74.88	4.32
1.30E+05	63.39	4.15
2.59E+05	40.79	3.71

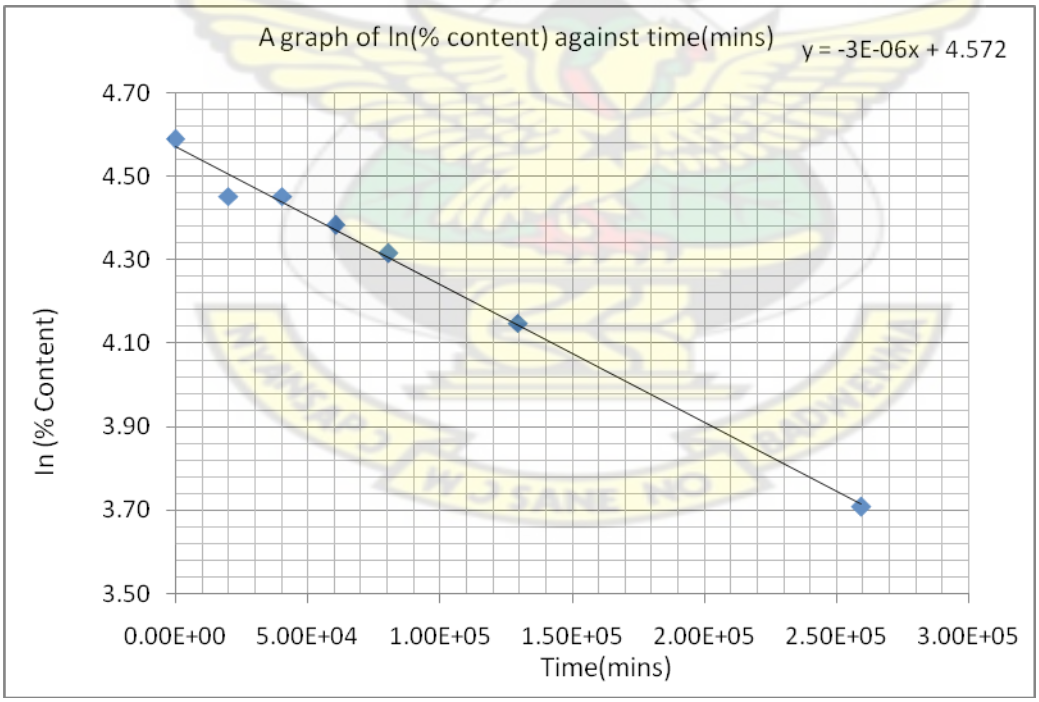


Fig12: A graph of ln(% content) of AS in ASW+AQW against time(mins) at 60^{o}C

Table 20**: % AS in ASA at 28^{o}C**

minutes	Percentage Content	ln(% content)
0.00	95.91	4.56
2.02E+04	95.81	4.56
4.03E+04	95.66	4.56
6.05E+04	95.49	4.55
8.06E+04	95.31	4.55
1.30E+05	94.92	4.55
2.59E+05	93.99	4.54

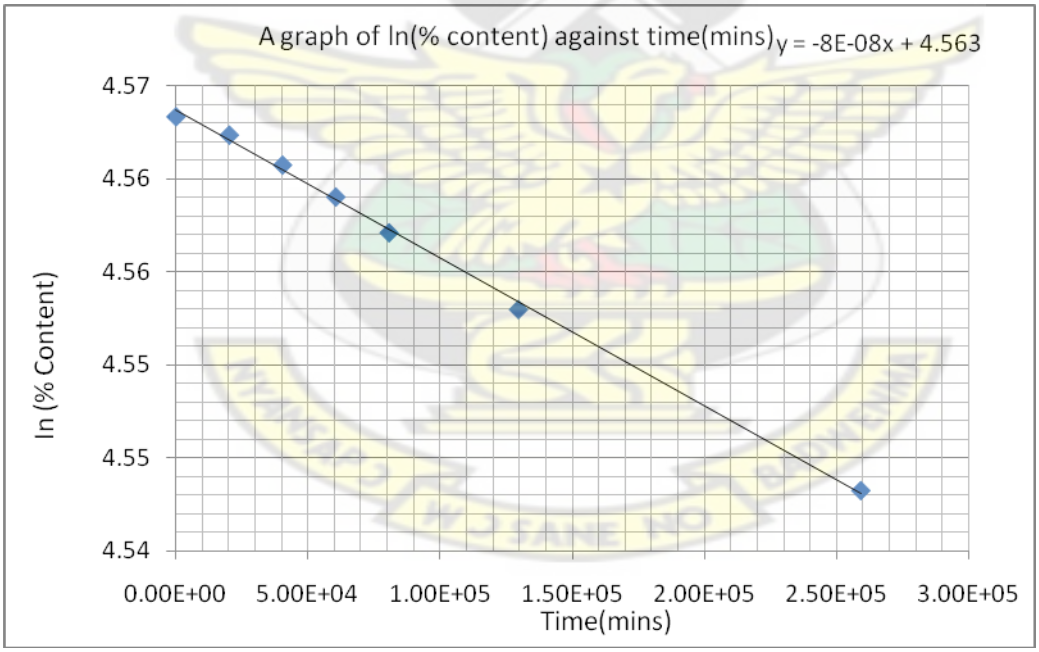


Fig14: A graph of ln (% content) of AS in ASA against time (mins) at 28^{o}C

Table 21**: % AS in ASA at 40°C**

minutes	Percentage Content	ln(% content)
0.00	95.97	4.56
2.02E+04	95.78	4.56
4.03E+04	95.38	4.56
6.05E+04	95.39	4.56
8.06E+04	95.19	4.56
1.30E+05	93.96	4.55
2.59E+05	93.52	4.54

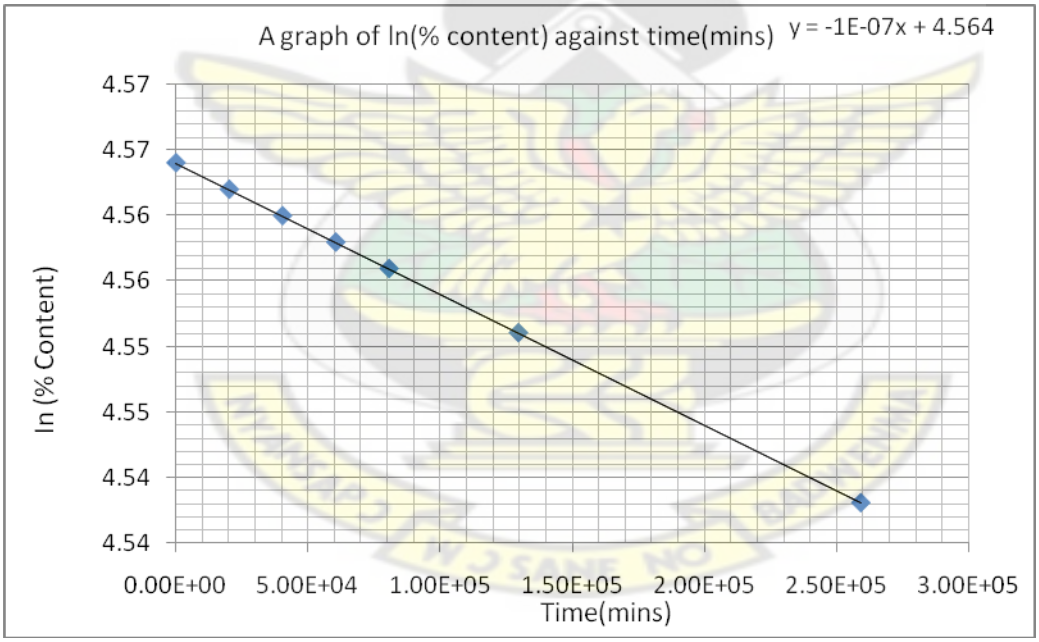


Fig14: A graph of ln (% content) of AS in ASA against time (mins) at 40°C

Table 22**: % AS in ASA at 60^{o}C**

minutes	Percentage Content	ln(% content)
0.00E+00	95.96	4.56
2.02E+04	95.09	4.55
4.03E+04	94.81	4.55
6.05E+04	93.85	4.54
8.06E+04	93.67	4.54
1.30E+05	92.31	4.53
2.59E+05	88.79	4.49

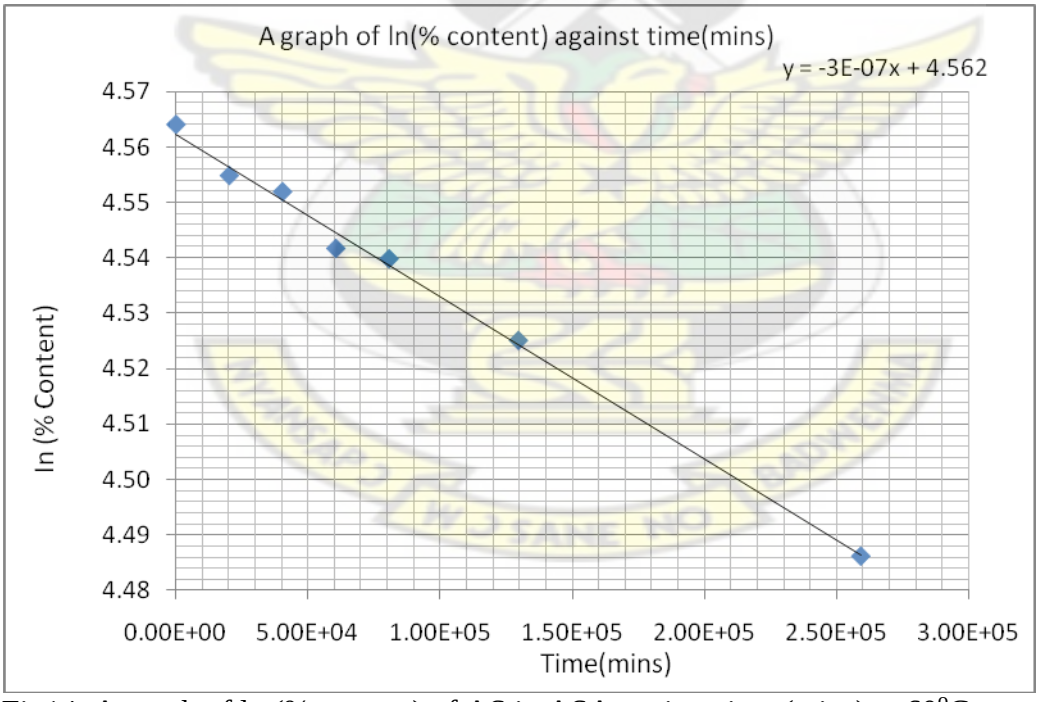


Fig14: A graph of ln (% content) of AS in ASA against time (mins) at 60^{o}C

Table 23**: % AS in AM at 28^{o}C**

minutes	Percentage Content	ln(% content)
0.00	98.00	4.58
2.02E+04	97.80	4.58
4.03E+04	97.40	4.57
6.05E+04	97.50	4.57
8.06E+04	96.97	4.57
1.30E+05	96.98	4.57
2.59E+05	95.99	4.56

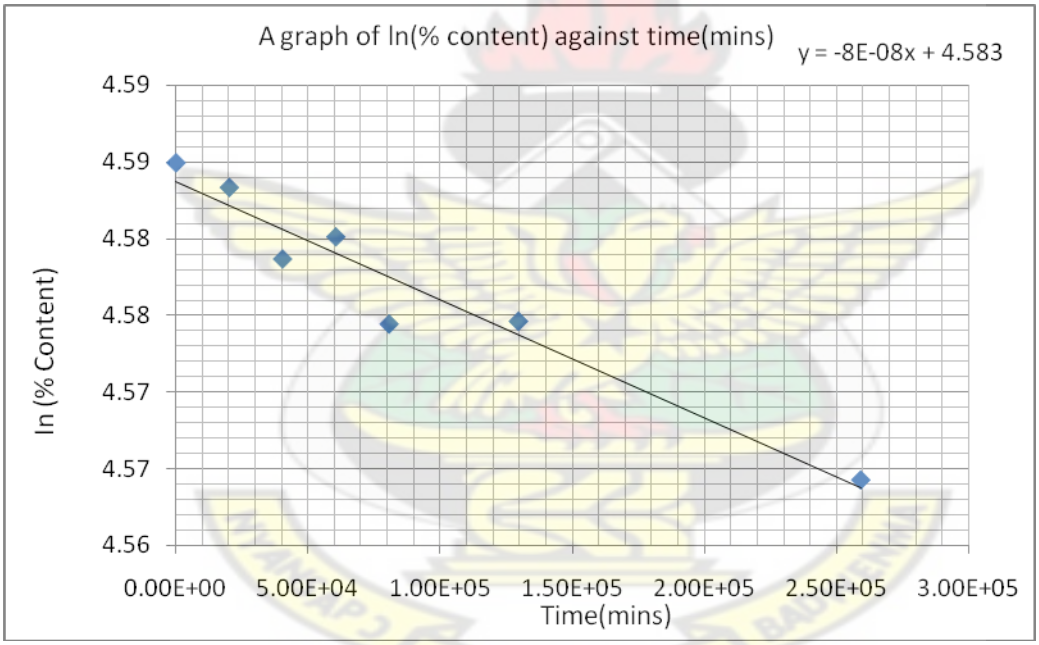


Fig17: A graph of ln (% content) of AS in AM against time (mins) at 28^{o}C

Table 24**: % AS in AM at 40^{o}C**

minutes	Percentage Content	ln(% content)
0.00	98.03	4.58
2.02E+04	97.74	4.58
4.03E+04	97.48	4.57
6.05E+04	97.19	4.57
8.06E+04	96.87	4.57
1.30E+05	96.39	4.56
2.59E+05	94.66	4.54

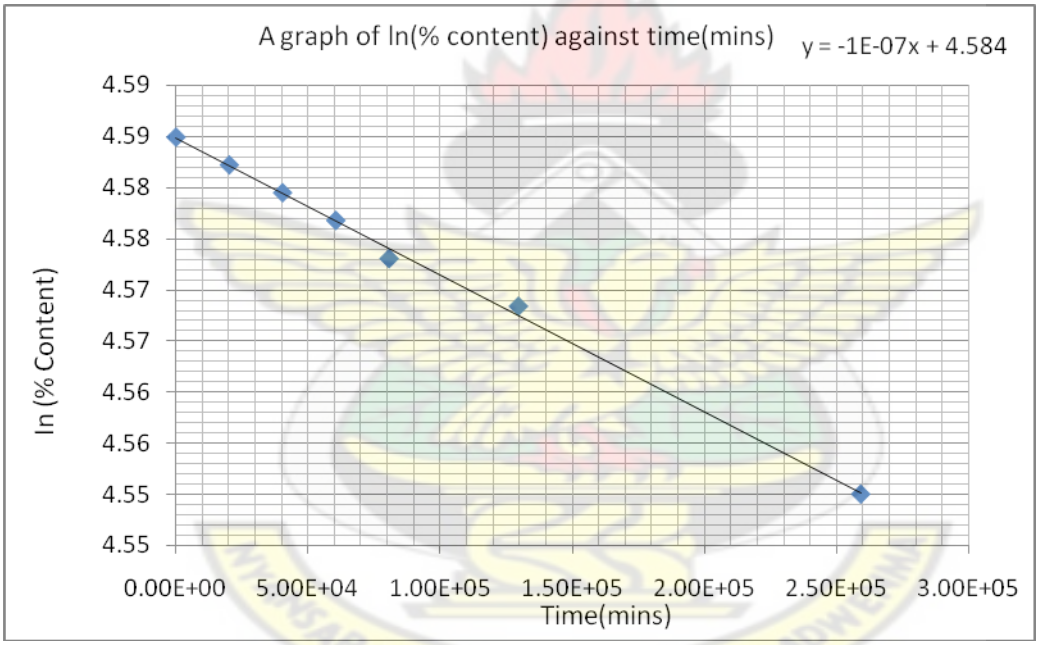


Fig17: A graph of ln (% content) of AS in AM against time (mins) at 40^{o}C

Table 25**: % AS in AM at 60^{o}C**

minutes	Percentage Content	ln(% content)
0.00	98.00	4.58
2.02E+04	97.38	4.57
4.03E+04	96.69	4.57
6.05E+04	95.15	4.56
8.06E+04	95.17	4.55
1.30E+05	94.27	4.54
2.59E+05	90.64	4.51

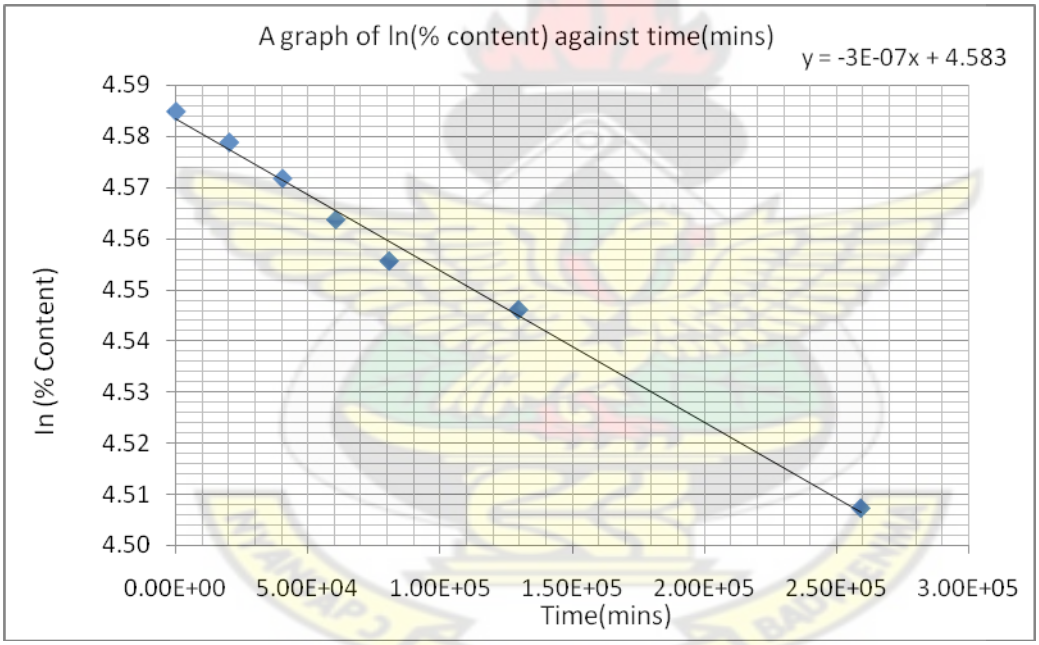


Fig18: A graph of ln (% content) of AS in AM against time (mins) at 60^{o}C

3.9.1 Calculations of % Content.

From the pure artesunate calibration curve, the equation of the line is **y = 0.340x + 0.078**

where y = the peak area and x = concentration.

For ASA+AQA,

At time 0 and at 28^{o}C, the peak area ratio is 0.7442

→ 0.7442 = 0.340x + 0.078

X = 1.9594mg/ml (this was dissolved to 25ml)

Actual mass = 1.9594mg/ml*25ml = 48.9850mg (actual mass after dissolution).

Nominal mass

If 0.6348g = 100ml

→ 0.3174g = (0.3172/0.6348)*100 = 50.0000mg

% content = (48.9850/50.000)*100

= 97.97%

The same calculation was done for all the other drug samples.

3.10 Half – Life of samples

Table 26: Half – life of ASA+AQA

Temperature(^{0}C)	Minutes	Hours	Days	Months	Years	k
28	2.10E+05	3.50E+03	145.83	4.86	0.41	5.0E-07
40	5.25E+04	8.75E+02	36.46	1.22	0.10	2.0E-06
65	2.63E+04	4.38E+02	18.23	0.61	0.05	3.0E-06

Table 27: Half – life of ASW+AQW

Temperature(^{0}C)	Minutes	Hours	Days	Months	Years	k
28	1.75E+05	2.92E+03	121.53	4.05	0.34	5.0E-07
40	5.25E+04	8.75E+02	36.46	1.22	0.10	2.0E-06
65	2.63E+04	4.38E+02	18.223	0.61	0.05	3.0E-06

Table 28: Half – life of ASA

Temperature(^{0}C)	Minutes	Hours	Days	Months	Years	k
28	1.31E+06	2.19E+04	911.46	30.38	2.53	8.0E-08
40	1.05E+06	1.75E+04	729.17	24.31	2.03	1.0E-07
65	3.50E+05	5.83E+03	243.06	8.10	0.68	3.0E-07

Table 29: Half – life of AM

Temperature(^{0}C)	Minutes	Hours	Days	Months	Years	k
28	1.31E+06	2.19E+04	911.46	30.38	2.53	8.0E-08
40	1.05E+06	1.75E+04	729.17	24.31	2.03	1.0E-07
65	3.50E+05	5.83E+03	243.06	8.10	0.68	3.0E-07

3.10 Half – life Calculations.

For ASA+AQA (28^{o}C)

t_{90} = 0.105/k (refer to 1.2.4)
From table 31, k = $5*10^{-7}$

t_{90} = $0.105/5*10^{-7}$

= $2.1*10^{5}$minutes.

= 0.405years.

The same calculation was done for all the other drug samples.

3.11 Method validation.

3.11.1 HPLC data for repeated measurements of 2.733mg/ml solution of pure artesunate.

Table 30 : Repeatability and precision of HPLC method.

Number	Nominal concentration (mg/ ml)	Actual concentration (mg/ml)	Standard Deviation	RSD(%)	% Content
1	2.733	2.7111	0.02	0.92	99.20
2	2.733	2.7219	0.05	1.71	99.59
3	2.733	2.6765	0.02	0.91	97.93
4	2.733	2.7760	0.02	0.76	101.57
5	2.733	2.7147	0.05	1.98	99.33
6	2.733	2.7173	0.02	0.90	99.42

*Average of 6 determinations.

3.11.2 Reproducibility of Method.

Table 31: **HPLC calibration data collected from three (3) different days**

Number	**Day 1**	**Day 2**	**Day 3**
	Mean Concentration mg/ ml		
1	2.7310	2.6970	2.7483
2	2.7100	2.6480	2.7830
3	2.6780	2.6810	2.7602
4	2.7450	2.7090	2.7180
5	2.7630	2.6443	2.7530
6	2.7010	2.6978	2.7588

Table 32: **Analysis of reproducibility data using ANOVA.**

Comparing Artesunnate recovered	Sum of Squares	d_f	Mean Square	F	Sig.
Between Groups	0.02	2	0.02	2.406	0.124
Within Groups	0.07	15	0.07		
Total	0.09	17	0.09		

There is no significant difference between the actual mean and the means from the assay. F(2,15)=2.406,

3.11.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD = Concentration yielding a signal-to-noise ratio of 2:1.

LOQ = Concentration yielding a signal-to-noise ratio of 10:1.

Artesunate:

A concentration of 0.00 4%$^{w}/_{v}$ gave average peak height of 62mm.

LOD = 2mm x {(0.004 %$^{w}/_{v}$)/62 mm}

= $1.29*10^{-4}$ %$^{w}/_{v}$

LOQ = 10mm x {(0.004 %$^{w}/_{v}$) / 62 mm}

= $6.45*10^{-4}$ %$^{w}/_{v}$

Amodiaquine:

A Concentration of of 0.0013 %$^{w}/_{v}$ gave average peak height of 79mm.

LOD = 2mm x {(0.0013 %$^{w}/_{v}$)/ 79mm}

= $3.3*10^{-5}$ %$^{w}/_{v.}$

LOQ = 10mm x {(0.0013 %$^{w}/_{v}$) / 79 mm}

= $1.65*10^{-4}$ %$^{w}/_{v.}$

CHAPTER FOUR

DISCUSSIONS, CONCLUSION AND RECOMMENDATIONS

4.1 DISCUSSIONS

4.1.1 Identification tests

4.1.1.1 Colour Identification test

Artesunate

When ethanol was added to 0.1001g of artesunate, no was colour developed. Upon addition of 0.5ml of hydroxylamine hydrochloride solution and 0.25ml of 2M sodium hydroxide solution followed by 2 drops of 2M hydrochloric acid and 2 drops of 2M ferric chloride solution, a light red colour developed. This shows the presence of pure artesunate powder (IP). Also, a reddish-brown colour developed when half of the filtrate was evaporated to 5ml and 1 drop of 1M sulphuric acid was added. This confirms the presence of pure artesunate powder (IP).

Amodiaquine

When 5ml of cobaltous thiocyanate was added to 1g of pure amodiaquine powder, a green precipitate was produced. This gave a positive test for amodiaquine (IP).

4.1.1.2 Melting point Determination

Artesunate

The reference range for the melting point of pure artesunate is 132ºc to 135ºC (IP).

For both determinations using pure artesunate powder, the melting point obtained were 131^{o}C to 134^{o}C and 132^{o}C to 134^{o}C. The values obtained agree with the reference range.

Amodiaquine

The reference range for the melting point of pure amodiaquine hydrochloride is 159ºC For two determinations using the pure amodiaquine powder; the melting points were 158.5ºC and 159.0ºC. These values agree with the reference value.

4.1.1.3 TLC Identification

The R_f value of pure artesunate obtained is 2.2 and the R_f value the AM is also 2.2. This confirms that artesunate was present in the tablets from the market.

4.1.2 Formulation of Tablets.

Three sets of tablets were formulated. These are ASA+AQA, ASW+AQW and ASA. ASA+AQA and ASA+AQW each contain 100mg of artesunate. During the granulation, water and alcohol were used. This was done in order to monitor the stability of artesunate in these two media.

4.1.3 Uniformity of Weight test.

According to the BP 2005, no single tablet should deviate by 10% or above and not more than two tablets should deviate by 5%. From ASA+AQA, ASW+AQW, AM and ASA, no tablet deviated by this margin. Therefore, by the BP standards, all the four brands passed the uniformity of weight test.

4.1.4 Assay of Artesunate

Titration

According to the IP, artesunate contains not less than 99.0% and not more than 101.0% of C_{19} $H_{28}O_8$ using the assay method described and should be calculated with reference to the anhydrous substance. The percentages purity of the artesunate powder assayed was 99.90% and this meets the requirement in the IP. It also confirms that the pure artesunate powder used for the formulation was 99.90% pure.

4.1.4 HPLC Method

The final conditions were obtained after series of work has been done on details such as wavelength of absorption, auf value, chart speed, mobile phase composition and effects of pH.

The final mobile phase composition comprised 0.3%$^{v}/_{v}$ glacial acetic acid, acetonitrile and triethylamine in the ratio of 55%: 45%: 0.3%. The pH of the final solution was always in the range of 4.5±0.1. It was also observed that as the pH of the mobile phase went below 4.4, the retention time of the amodiaquine was prolonged. Also when C18 ODS column was used, amodiaquine eluted before artesunate but when it was changed to C_8 ODS column, artesunate eluted before amodiaquine. But the C_8 ODS column was employed because at the 254nm wavelength, amodiaquine is highly absorbing and it therefore elutes and truncates when the C_{18} ODS column. This affected the elution of the artesunate and made it impossible for the peak of artesunate to be observed.

The wavelength of the absorption was set at 254.0nm. When artesunate and amodiaquine were scanned in the UV, both gave a spectrum at this wavelength. But it was realised that with the same concentration, amodiaquine gave pronounced spectrum than artesunate. This was employed during the analysis by preparing small concentrations of amodiaquine in order to prevent the peaks from truncating.

The final method used for analysis was simple, fast, precise, accurate, reproducible and robust under changing conditions. The calibration curves of artesunate and amodiaquine gave a linear correlation of mean peak area ratio to the concentrations of the standard solutions prepared. The aim of checking linearity is to derive a direct proportionality between the detector signal and concentration of a substance in the sample over a certain range. The correlation coefficient must be specified. Under most circumstances, regression coefficient (r) is = ≥ 0.999. The regression coefficient of calibration curve of artesunate is r =0.998 and that of amodiaquine is r = 0.999. Also the statistical values showed the deviation from zero position was significant and this indicates that the range

of concentration used were satisfactory. The limit of detection of artesunate and amodiaquine are $1.29*10^{-4}$ $\%^{w}/_{v}$ and $3.3*10^{-5}$ $\%^{w}/_{v}$ respectively. The limit of quantification of artesunate and amodiaquine are $6.45*10^{-4}$ $\%^{w}/_{v}$ $1.65*10^{-4}$ $^{\%w}/_{v}$ respectively.

The method was also reproducible. As defined by ICH, reproducibility expresses precision between results of different analysts. From Table 43, analysis of three different analysts A, B and C shows $F(2,15) = 2.406$, $P > 0.05$ shows that there is no significant difference between the actual means and the means from the assay.

Again the RSD values obtained were very small and this indicates a good precision and accuracy of the method. The method is also reproducible as the analysis of variance in three different occasions indicates in Table 42.

The method was accurate as the recovery values obtained in the table b shows. The F value (1.10) = 0.3860, P 0.05 shows a good recovery of the method (100.0%).

The robustness of the new method under investigations was studied by intentionally varying amounts of respective components of the mobile phase to investigate the effect it will have on the results.

Various columns from different sources were used. Chromatographic conditions such as wavelength of absorption, AUF values, and chart speed and pH effects were all investigated and the method proved to be robust.

This isocratic method of elution is simple, fast, accurate, and reproducible for the analysis of combination dosage forms of artesunate and amodiaquine. It is worth mentioning that this method is also able to elute combination dosage forms of artemether and lumefantrine.

4.1.5 Stability Studies

The gradient of the stability graphs (3.7.2) shows a pseudo first order reaction. This confirms that the degradation of artesunate follows a pseudo first order reaction at constant temperature (Refer to 1.2.4)

Four drug samples were used for the analysis. These are ASA+AQA, ASW+AQW, ASA and AM. Their stability at 40ºC are shown below:

Table 33: Stability of samples at 40°C±2 °C/ 75% RH±5%RH

Sample	ASA+AQA	ASW+AQW	ASA	AM
Stability at 40°C±2°C/ 75% RH±5%RH/Yyrs.	0.101	0.101	2.025	2.025

The results indicate that ASA+AQA and ASW+AQW have short shelf - lives. The shelf - life of ASA+AQA at 40ºC is 0.101 years and that of ASW+AQW is 0.101 years. But that of the AM under the same condition is 2.025 years.

With the same excipients and active ingredient, the short shelf - life of the ASA+AQA and ASW+AQW could be due to interaction of the artesunate and amodiaquine leading to degradation products.

The results also indicate that as temperature increases, the stability of all the drug samples reduces. This buttresses the point that drug products of artesunate and amodiaquine should not be stored at higher temperatures.

At 28ºC, ASA+AQA is more stable than ASW+AQW. ASA was therefore formulated to compare the stability of artesunate in alcohol and water media (during the granulation process). The shelf – life of ASA at 40ºC was found to be 2.025 years. The results show

that there is possible interaction of AS and AQ when they are in combination and cannot therefore be put together in a formulation.

Both ASA and AM have half – life of 2.025 years. This shows that artesunate alone is stable but when in combination with amodiaquine, it becomes unstable.

Most of the antimalarial in the Ghanaian market have a shelf – life of 2 to 3 years. Comparing this to the results obtained, it is evident that ASA+AQA and ASW+AQW have short shelf – lives. Although, the acceptable level could be that at which a toxic degradation product could no longer be tolerated, or at which a certain degree of odour or colour change is evident, it is imperative that combination products of AS+AQ are incompatible and have short stability period.

Artesunate and amodiaquine are incompatible, wherein severe degradation of the drugs in presence of each other. This is said to be based on the sensitivity of artesunate to the acidity brought by HCl salt of amodiaquine that is enhanced by temperature and humidity.[25]

The two drugs are chemically incompatible and this causes mutual decomposition when they come together and leads to stability problems. This poses a challenge to the formulating pharmacist while presenting the combination of the two drugs in a single dosage form to enhance patient compliance resulting in decreased possibility of recurrence or developing resistance.

However, the HPLC method was not able to pick these interaction products and therefore the possible degradation products could not be determined.

4.2 CONCLUSION

An isocratic method of elution comprising a 0.3%v/v of glacial acetic acid, acetonitrile and triethylamine in the ratio of 55%: 45%: 0.3% was developed.

The method is able to elute both artesunate and amodiaquine.

The method is simple, fast, accurate, and reproducible for the analysis of artesunate and amodiaquine.

Combination tablets of artesunate and amodiaquine were formulated.

ASA+AQA have a shelf – life of 0.10 years or 1.2 months and therefore a short stability period.

ASW+AQW have a shelf – life of 0.10 or 1.2 months.

ASA and AM have a shelf - life of 2.03 years or 24.31 months.

Combination tablets of artesunate and Amodiaquine have a short stability period.

4.3 RECOMMENDATIONS

• Formulation of direct combination products of artesunate and amodiaquine tablets is not practical at the moment because of incompatibility of the two drugs.

Five different ways to solve the problem of incompatibility between the two active ingredients are recommended below:

• Formulations of monolithic and multilayer tablet

• The formulation can also be presented as other solid dosage forms such as capsule, pills, granule, or in a mutually protected environment such as coated granules.

• Use of classical excipients, pH regulators and hydrophobic agents during granulation.

• Use of dry granulation in order to limit the humidity content in the formulation. Impact of controlled temperature and humidity conditions.

• Future work on artesunate and amodiaquine is recommended, this time developing a method that could elute degradation products from the interaction of artesunate and amodiaquine.

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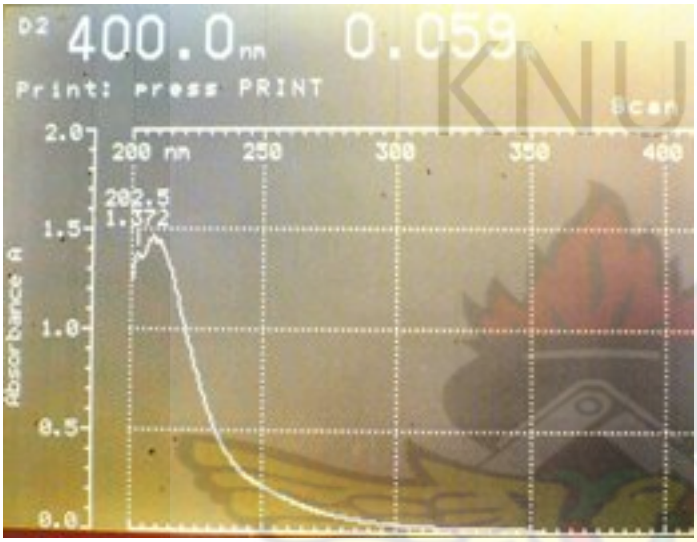
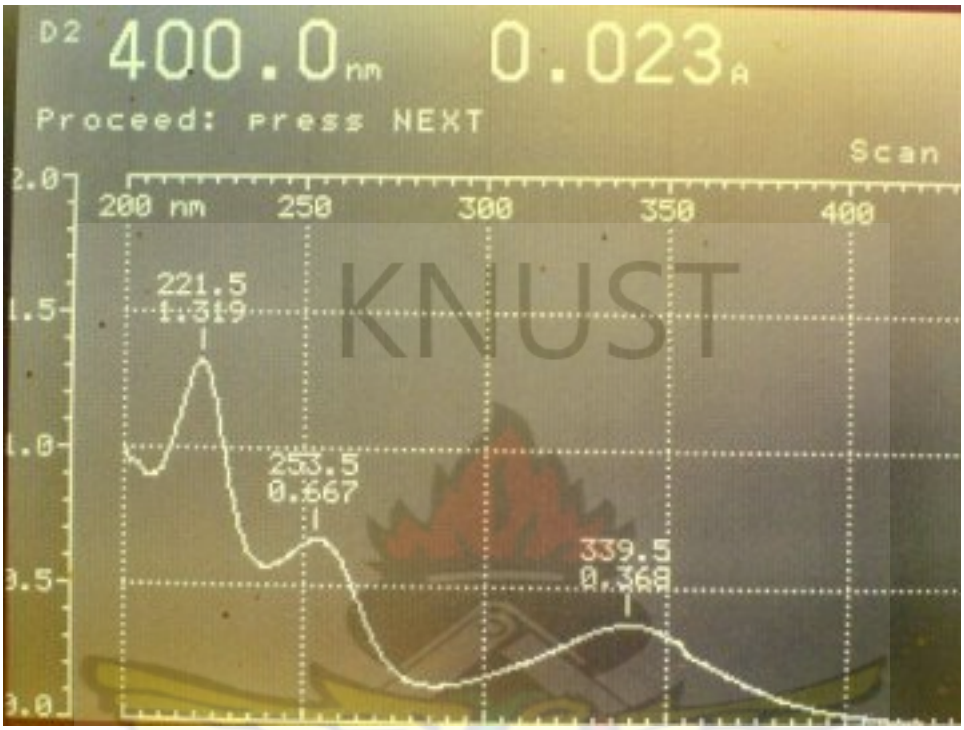
APPENDIX

UV Spectrum of Pure Artesunate.



Appendix I

UV Spectrum of Pure Amodiaquine.



Appendix II

Table34: AS in ASA+AQA at 40^{o}C

Time(mins.)	Mass of Product (g)
0.00	0.3111
2.02E+04	0.2857
4.03E+04	0.3174
6.05E+04	0.2984
8.06E+04	0.2920
1.30E+05	0.2857
2.59E+05	0.2793

Table35: AS in ASA+AQA at 60^{o}C

Time(mins.)	Mass of Product (g)
0.00	0.3428
2.02E+04	0.3491
4.03E+04	0.3555
6.05E+04	0.3174
8.06E+04	0.3111
1.30E+05	0.3047
2.59E+05	0.2984

3.8.1.3 Analysis of AS IN ASW+AQW

Table 36: AS in ASW+AQW at 28^{o}C

Time(mins.)	Mass of Product (g)
0.00	0.3364
2.02E+04	0.3618
4.03E+04	0.3111
6.05E+04	0.3364
8.06E+04	0.3428
1.30E+05	0.3618
2.59E+05	0.3777

Table37: AS in ASW+AQW at 40^{o}C

Time(mins.)	Mass of Product (g)
0.00	0.2984
2.02E+04	0.3267
4.03E+04	0.3302
6.05E+04	0.3491
8.06E+04	0.2750
1.30E+05	0.2629
2.59E+05	0.2353

Table38: AS in ASW+AQW at 60^{o}C

Time(mins.)	Mass of Product (g)
0.00	0.2987
2.02E+04	0.3378
4.03E+04	0.3320
6.05E+04	0.3532
8.06E+04	0.3201
1.30E+05	0.2870
2.59E+05	0.2539

3.8.1.4 Analysis of AS IN ASA

Table39: AS in ASA at 28^{o}C

Time(mins.)	Mass of Product (g)
0.00	0.1421
2.02E+04	0.1410
4.03E+04	0.1431
6.05E+04	0.1471
8.06E+04	0.1398
1.30E+05	0.1325
2.59E+05	0.1252

Table40: AS in ASA at 40^{o}C

Time(mins.)	Mass of Product (g)
0.00	0.1578
2.02E+04	0.1698
4.03E+04	0.1634
6.05E+04	0.1698
8.06E+04	0.1699
1.30E+05	0.1700
2.59E+05	0.1701

Table41: AS in ASA at 60^{o}C

Time(mins.)	Mass of Product (g)
0.00	0.1544
2.02E+04	0.1558
4.03E+04	0.1579
6.05E+04	0.1592
8.06E+04	0.1578
1.30E+05	0.1564
2.59E+05	0.1550

3.8.1.4 Analysis of AS IN ASA

Table42: AS in ASM at 28^{o}C

Time(mins.)	Mass of Product (g)
0.00	0.1270
2.02E+04	0.1244
4.03E+04	0.1349
6.05E+04	0.1244
8.06E+04	0.1376
1.30E+05	0.1244
2.59E+05	0.1376

Table43: AS in ASM at 40^{o}C

Time(mins.)	Mass of Product (g)
0.00	0.1402
2.02E+04	0.1270
4.03E+04	0.1270
6.05E+04	0.1376
8.06E+04	0.1402
1.30E+05	0.1376
2.59E+05	0.1402

Table44: AS in ASM at 60^{o}C

Time(mins.)	Mass of Product (g)
0.00	0.1349
2.02E+04	0.1297
4.03E+04	0.1403
6.05E+04	0.1270
8.06E+04	0.1297
1.30E+05	0.1270
2.59E+05	0.1297

Uniformity of Weight Test Results – Raw Data.

Table 45

ASA+AQA			
	Mass	Deviation	% Deviation
1	0.6201	-0.0142	-2.2387
2	0.6387	0.0044	0.6937
3	0.6122	-0.0221	-3.4842
4	0.6389	0.0046	0.7252
5	0.6231	-0.0112	-1.7657
6	0.6341	-0.0002	-0.0315
7	0.6432	0.0089	1.4031
8	0.6435	0.0092	1.4504
9	0.5976	-0.0367	-5.7859
10	0.6538	0.0195	3.0743
11	0.6219	-0.0124	-1.9549
12	0.6532	0.0189	2.9797
13	0.6433	0.0090	1.4189
14	0.6233	-0.0110	-1.7342
15	0.6433	0.0090	1.4189
16	0.6533	0.0190	2.9954
17	0.6322	-0.0021	-0.3311
18	0.6233	-0.0110	-1.7342
19	0.6433	0.0090	1.4189
20	0.6433	0.0090	1.4189
	Average mass =0.6348		

Table46

ASW+AQW			
	Mass	Deviation	% Deviation
1	0.6351	-0.0006	-0.0944
2	0.6297	-0.0060	-0.9438
3	0.6343	-0.0014	-0.2202
4	0.6201	-0.0156	-2.4540
5	0.6543	0.0186	2.9259
6	0.612	-0.0237	-3.7282
7	0.6498	0.0141	2.2180
8	0.6429	0.0072	1.1326
9	0.6534	0.0177	2.7843
10	0.6432	0.0075	1.1798
11	0.6221	-0.0136	-2.1394
12	0.6222	-0.0135	-2.1236
13	0.6573	0.0216	3.3978
14	0.6122	-0.0235	-3.6967
15	0.6422	0.0065	1.0225
16	0.6376	0.0019	0.2989
17	0.6122	-0.0235	-3.6967
18	0.6359	0.0002	0.0315
19	0.6439	0.0082	1.2899
20	0.6534	0.0177	2.7843
	Average mass =0.6348		

Table 47

ASA			
	Mass	Deviation	% Deviation
1	0.3244	-0.0006	-0.1846
2	0.3286	0.0036	1.1077
3	0.3199	-0.0051	-1.5692
4	0.3211	-0.0039	-1.2000
5	0.3344	0.0094	2.8923
6	0.3387	0.0137	4.2154
7	0.3211	-0.0039	-1.2000
8	0.3122	-0.0128	-3.9385
9	0.3289	0.0039	1.2000
10	0.3287	0.0037	1.1385
11	0.3177	-0.0073	-2.2462
12	0.3165	-0.0085	-2.6154
13	0.3122	-0.0128	-3.9385
14	0.3210	-0.0040	-1.2308
15	0.3244	-0.0006	-0.1846
16	0.3319	0.0069	2.1169
17	0.3298	0.0048	1.4769
18	0.3211	-0.0039	-1.2000
19	0.3244	-0.0006	-0.1846
20	0.3255	0.0005	0.1538
	Average mass =0.3250		

Table 48

AM			
	Mass	Deviation	% Deviation
1	0.2755	0.0041	0.6459
2	0.2744	0.0030	0.4726
3	0.2966	0.0252	3.9698
4	0.2433	-0.0281	-4.4266
5	0.2794	0.0080	1.2602
6	0.2933	0.0219	3.4499
7	0.2789	0.0075	1.1815
8	0.2834	0.0120	1.8904
9	0.2867	0.0153	2.4102
10	0.2633	-0.0081	-1.2760
11	0.2699	-0.0015	-0.2363
12	0.2477	-0.0237	-3.7335
13	0.3055	0.0341	5.3718
14	0.2844	0.0130	2.0479
15	0.2455	-0.0259	-4.0800
16	0.2897	0.0183	2.8828
17	0.2744	0.0030	0.4726
18	0.2744	0.0030	0.4726
19	0.2834	0.0120	1.8904
20	0.2698	-0.0016	-0.2520
	Average mass =0.2714		

Mass and Peak area of Samples – Raw Data

Table 49: ASA+AQA at 28^{o}C

Mass of Product (g)	Peak area ratio
0.3174	0.7442
0.3745	0.8511
0.3491	0.7918
0.3237	0.7328
0.2984	0.6755
0.2809	0.6141
0.2982	0.6307

Table 50: ASA+AQA at 40^{o}C

Mass of Product (g)	Peak area ratio
0.3111	0.7263
0.2857	0.6544
0.3174	0.6981
0.2984	0.6424
0.2920	0.6129
0.2857	0.5617
0.2793	0.4624

Table51: ASA+AQA at 60^{o}C

Mass of Product (g)	Peak area ratio
0.3428	0.7925
0.3491	0.7616
0.3555	0.7286
0.3174	0.6275
0.3111	0.5808
0.3047	0.4781
0.2984	0.3353

Table52: ASW+AQW at 28^{o}C

Mass of Product (g)	Peak area ratio
0.3364	0.7891
0.3618	0.8322
0.31110	0.7186
0.3111	0.7656
0.3428	0.7723
03618	0.7904
0.3777	0.7713

Table53: ASW+AQW at 40^{o}C

Mass of Product (g)	Peak area ratio
0.3039	0.7175
0.3494	0.7783
0.3656	0.7858
0.4140	0.8264
0.3388	0.6675
0.3643	0.6415
0.4449	0.5823

Table54: ASW+AQW at 60^{o}C

Mass of Product (g)	Peak area ratio
0.2987	0.7086
0.3378	0.6998
0.3320	0.6892
0.3532	0.6851
0.3201	0.5917
0.2870	0.4678
0.2539	0.2998

Table55: ASA at 28^{o}C

Mass of Product (g)	Peak area ratio
0.1421	0.6485
0.1410	0.6435
0.1431	0.6511
0.1471	0.6660
0.1398	0.6358
0.1325	0.6045
0.1252	0.57o6

Table56: ASA at 40^{o}C

Mass of Product (g)	Peak area ratio
0.1578	0.7136
0.1698	0.7589
0.1634	0.7318
0.1698	0.7562
0.1699	0.7551
0.1700	0.7522
0.1701	0.744

Table56: ASA at 60^{o}C

Mass of Product (g)	Peak area ratio
0.1544	0.6984
0.1558	0.6983
0.1579	0.7048
0.1592	0.7035
0.1578	0.6969
0.1564	0.68245
0.1550	0.6541

Table57: AM at 28^{o}C

Mass of Product (g)	Peak area ratio
0.1270	0.7020
0.1244	0.6881
0.1349	0.7369
0.1244	0.6861
0.1376	0.7469
0.1244	0.6827
0.1376	0.7402

Table58: AM at 40^{o}C

Mass of Product (g)	Peak area ratio
0.1402	0.7671
0.1270	0.7003
0.1270	0.6987
0.1376	0.7485
0.1402	0.7589
0.1376	0.7429
0.1402	0.7434

Table59: AM at 60^{o}C

Mass of Product (g)	Peak area ratio
0.1349	0.7411
0.1297	0.7112
0.1403	0.7581
0.1270	0.6889
0.1297	0.6966
0.1270	0.6782
0.1297	0.6674

Statistical Analysis

Table60

	Percentage content					
	1	2	3	4	5	6
IP method	98.003	98.001	98.000	98.000	98.020	98.010
New method	98.011	98.000	98.000	98.002	98.003	98.000

Table61

ANOVA					
mass recovered					
	Sum of Squares	d_f	Mean Square	F	Sig.
Between Groups	2.7E-05	1	2.7E-05	0.661	0.435
Within Groups	0.000408667	10	4.08667E-05		
Total	0.000435667	11			

There is no significant difference between the two means. $F(1,10)= 0.661, p>0.05$

Table62

Actual mass= 352 mg

	Mass recovered (mg)					
	1	2	3	4	5	6
IP Method	352.210	352.010	352.143	352.089	352.020	351.860
New method	352.070	352.080	351.680	351.990	352.090	352.102

Table63

ANOVA					
mass recovered					
	Sum of Squares	d_f	Mean Square	F	Sig.
Between Groups	0.00801	1	0.0080	0.3860	0.5483
Within Groups	0.20747	10	0.0207		
Total	0.21548	11			

There is no significant difference between the two means. $F(1,10)= 0.3860, p>0.05$

Table64
Precision of developed method for Artesunate

Mass weighed (mg)	Mean mass	SD	RSD%
352	352.0020	0.1627	0.0462

Table65
Accuracy of developed method for Artesunate

Mass weighed (mg)	% mass recovered	SD	RSD%
352	100.0007	0.0462	0.0462