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DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

STABILITY STUDIES OF ARTESUNATE FORMULATIONS ON THE LOCAL MARKET

BY
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STABILITY STUDIES OF ARTESUNATE FORMULATIONS ON THE LOCAL MARKET

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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BY

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MAY, 2012

DECLARATION

I hereby declare that this thesis is a submission of my own work towards the Master of Science (MSc) at the Department of Pharmaceutical Chemistry, KNUST. Any assistance obtained has been duly acknowledged. This work has not been submitted for any other degree.

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ABSTRACT

Malaria is one of the most important parasitic diseases of man. **Artesunate** is a water soluble derivative of Artemisinin employed in malaria treatment and is more unstable due to the presence of a hemi ester linkage which is susceptible to hydrolysis. The focus of the study was to perform stability studies of commercial samples of **Artesunate** on the market. To monitor stability of these drugs, Thin Layer Chromatography (TLC) was performed to give an indication of the presence or absence of breakdown of **Artesunate**. A High Performance Liquid Chromatography (HPLC) method for simultaneous quantification of **Artesunate** and **Dihydroartemisinin** (DHA) as well as Ultra-Violet (UV) Spectrophotometric methods were developed and validated.

The results of the TLC revealed that two of the commercial samples contained **Dihydroartemisinin** in addition to **Artesunate** and one brand did not contain any **Artesunate**. Analysis of **Artesunate** tablets by the standard titrimetric method indicated that tablet T_1 had a percentage content of **125.215 ± 0.54472 % ^{w}/w** whilst tablets T_2, T_3, T_4 and T_5 were **97.64 ± 0.39, 103.31 ± 1.36, 102.63 ± 0.68,** and **99.69 ± 4.40 %^{w}/w** respectively. Tablet T_6, however, did not contain **Artesunate** or **DHA**. In the UV method development, **2ml** of concentrated HCl was added to **1ml** of **Artesunate** solution and the wavelength readings were recorded at 254nm.

A KNAUER Eurospher 100-5 C-18 column, 250 *4.6mm with precolumn was used in the HPLC analysis. The mobile phase system was Methanol and 0.006 % Trifluoroacetic acid (80:20) at a flow rate of 1.5min/ml and a wavelength of 220nm using a **UV detector**. The retention time of **Artesunate** was 3.74 ± 0.06 minutes and that of DHA was 5.13 ± 0.26 minutes. The LOD for both **Artesunate** and DHA were **0.003749113%^{w}/v** and **0.004235491%^{w}/v** respectively. The LOQ was **0.01136095%^{w}/v** and **0.01283482%^{w}/v** for **Artesunate** and DHA respectively. Analysis of commercial samples revealed the presence of DHA in four of the commercial samples. Four brands of **Artesunate** passed whilst one brand failed. All the samples passed the uniformity of weight and disintegration test. Statistical analysis reveals that there is no significant difference between the HPLC method developed and the standard method.

DEDICATION

I dedicate this work to my parents: Martin Boateng and Agnes Boateng and my good friend Frederick Abeiku Wilson.

ACKNOWLEDGEMENT

I give thanks to the Almighty God for the Grace and Wisdom He endowed me to complete this research successfully. I am grateful to the Academic Board of the Graduate School of K.N.U.S.T for offering me the opportunity to pursue the program.

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LIST OF ABBREVIATIONS

ACT: Artemisinin-based Combination Therapy

AS- Artesunate

BP: British Pharmacopoeia

DHA: Dihydroartemisinin

GC-MS: Gas Chromatography –Mass Spectrometry

GMP: Good Manufacturing Processes

HPLC: High Performance Liquid Chromatography

ICH: International Conference on Harmonization

IP: International Pharmacopoeia

LC-MS: liquid chromatography-mass spectrometry

LOD: Limit of Detection

LOQ: Limit of Quantitation

PABA: *p*-aminobenzoic acid

Rf: Retardation factor

ROS- Reactive Oxygen Species

RP-HPLC: Reverse Phase HPLC

RSD: Relative Standard Deviation

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

SD: Standard Deviation

TDR- Tropical Disease Research

TLC: Thin Layer Chromatography

USP: United States Pharmacopoeia

UV: Ultra-Violet

WHO: World Health Organization

VS: Volumetric solution

CHAPTER 1 – INTRODUCTION

1.1 BACKGROUND

Malaria is one of the most important parasitic diseases of man which causes over 1 million deaths and an economic loss of US$ 12 billion annually **(WHO, 2002)**. Historical records suggest that malaria has infected humans since the beginning of mankind. Fossils of mosquitoes up to 30 million years old also show that the vector for malaria was present well before *Homo sapiens* **(WHO TDR, 2001)**.

In 2008, there were 247 million cases of malaria globally killing between one and three million people. It is widespread in tropical and subtropical regions, including parts of Americas, Asia, and Africa. Malaria contributes extensively to the poor health situation in Africa. Ninety percent of malaria-related deaths occur in Sub-Saharan Africa ranking second after HIV/AIDS and accounting for 10.6% of the disease burden. [In Africa, a child dies every 45 seconds of Malaria and the disease accounts for 20% of all childhood deaths **(WHO, 2008)**.]

In Ghana, the disease accounts for an average of 13.2% of all mortality cases and 22% of all mortalities in children under 5 years old. In the case of pregnant women, out of the total number reporting at the health institutions, 13.8% suffer from malaria and 9.4% of all deaths in pregnant women is caused by malaria **(Asante, Asenso-Okyere et al., 2003)**.

1.1.1 THE MALARIA PARASITE

Malaria is a mosquito-borne infectious disease caused by a eukaryotic protist of the genus *Plasmodium* which infects the red blood cells. The parasites are spread to people through the bites of infected *Anopheles* mosquitoes. There are approximately 380 anopheline species, but only about 60 transmit malaria **(Ursos and Roepe, 2002)**. Four of these species primarily infect human which are namely:

1. *Plasmodium falciparum*; It is responsible for malignant tertian malaria and can be fatal if untreated.
2. *Plasmodium vivax*; this causes benign tertian malaria and produces mild clinical attacks.
3. *Plasmodium ovale;* it causes tertian fever and produces mild clinical attacks.
4. *Plasmodium malariae*; it causes quartian malaria.

Plasmodium falciparum and *Plasmodium vivax* are the most common species. Severe malaria and malaria-related deaths are mostly attributed to *falciparum* malaria. In recent years, some human cases of malaria have also occurred with *Plasmodium knowlesi*; monkey malaria that occurs in certain forested areas of South-East Asia **(WHO fact sheet, 2008)**.

Epidemiological analysis in Ghana has revealed that only three species of the Plasmodium are present; *Plasmodium falciparum* (80%-90%), *Plasmodium malariae* (20%-36%) and *Plasmodium ovale* (0.15%). The *Plasmodium falciparum* is thus the predominant parasite species carried by a combination of vectors. The principal vectors are the *Anopheles gambiae* complex, which is most widespread and difficult to control,

and the *Anopheles funestus* accounting for 95% of all catches **(Asante, Asenso-Okyere et. al., 2003)**.

1.1.2 MODE OF TRANSMISSION

The parasite is transmitted to humans when an infected mosquito bites and inoculates *Plasmodium* sporozoites into the human host at the time of blood feeding occurring through a sexual and an asexual stage. In *falciparum* malaria, the sexual cycle is delayed with respect to the asexual cycle. Asymptomatic pre-erythrocytic maturation occurs in the liver during the asexual stage and causes each infected hepatic schizont to rupture releasing merozoites which then invade red blood cells within 48 hours. This asexual blood stage causes the illness malaria and has historically been the focus of antimalarial treatment. The parasite burden expands logarithmically by approximately ten-fold per cycle **(Simpson et al., 2002)**.

This sexual stage is responsible for infecting the *Anopholine* mosquito and thus transmission of the infection. After several asexual cycles, some merozoites invade red cells and there develop into either male or female gametocytes. Gamete fusion and meiosis takes place in the mosquito's mid gut leading eventually to sporozoite development and subsequent inoculation into another human host. Gametocytes are conventionally divided into five stages of development. In the first three stages the sexual parasites are sequestered, and they are potentially susceptible to the drugs used to treat the asexual stage infection. In stage 4 they re-enter the circulation and by stage 5 the gametocytes circulate and are resistant to all drugs except the 8-aminoquinolines **(Magesa et al., 2000)**.

1.1.3 SYMPTOMS OF MALARIA

The first symptoms which are **fever**, **headache**, **chills**, **arthralgia** and **vomiting** may be mild and difficult to recognize as malaria. If not treated within 24 hours, *P. falciparum* malaria can progress to severe illness often leading to death. Children in endemic areas with severe malaria frequently develop symptoms like severe anaemia, respiratory distress in relation to metabolic acidosis, or cerebral malaria. In adults, multi-organ involvement is also frequent **(WHO, 2008)**.

1.1.4 MALARIA TREATMENT AND PREVENTION

The first effective treatment for malaria came from the bark of **cinchona tree** which contains quinine **(Kaufman and Rúveda, 2005)**. Elimination of the vector, drug therapy and vaccination are three practical ways to control the vector. Antimalarial chemotherapy has been the primary option in the fight against malaria and over the years many drugs have been developed and used in the treatment of the disease. However, the burden of this disease is still very heavy, partly due to the development of multi-drug resistant *Plasmodium falciparum* strains **(WHO, 1993)**.

1.1.5 ANTIMALARIAL DRUGS AND DRUG COMBINATIONS

There are a number of classes of antimalarials, each of which may have an effect on a different stage of the parasite and different species. These include:

1. **Cinchona alkaloids (e.g. quinine, quinidine)**
2. **4-Aminoquinolines (e.g. chloroquine, amodiaquine)**
3. **Diaminopyrimidines (e.g. pyrimethamine)**

4. **Sulphonamides and sulphonea (e.g. sulfadoxine, sulfamethoxypyridazine, dapsone)**
5. **Tetracyclines (e.g. tetracycline, minocycline, doxycycline)**
6. **Quinoline methanols (e.g. mefloquine,)**
7. **Sesquiterpene lactones (e.g. Artemisinins, Artemisinin derivatives)**
8. **Phenanthrene methanols (e.g. halofantrine, lumefantrine, pyronaridine)**
9. **8-Aminoquinolines (e.g. primaquine, tafenoquine)**
10. **Biguanides (e.g. proguanil, chlorproguanil. Cycloguanil) (Phillips and Solomon, 1990)**

1.1.6 ANTIMALARIAL DRUG RESISTANCE

Resistance to antimalarials has occurred over the years. Chloroquine resistance was found in 1959 in both Asia and South America and has spread fairly rapidly **(Ursos and Roepe, 2002)**. The de novo emergence of resistance can be prevented by the use of antimalarial drug combinations. It is based on the synergistic or additive potential of two or more drugs to improve therapeutic efficacy. This has been proposed to delay the emergence and spread of drug resistance **(White, 2004)**.

1.1.7 ANTIMALARIAL COMBINATION THERAPY

Antimalarial combination therapy is the simultaneous use of two or more blood schizontocidal medicines with independent modes of action and thus, different biochemical targets in the parasite. The aim of combination therapy is to improve efficacy against malaria and delay the emergence of parasite resistance to the individual components of the combination **(White, 2004)**.

This mutual protection is thought to prevent or to delay the emergence of resistance. To realize the two advantages, the partner medicines in a combination must independently be sufficiently efficacious in treating malaria. The combination therapies include: non-Artemisinin-based combinations and Artemisinin based combinations or Artemisinin combination therapy (ACT) **(White, 2004)**.

1.1.8 ARTEMISNIN-BASED COMBINATION THERAPIES (ACTS)

The World Health Organisation currently recommends that Artemisinin combination therapies (ACT) should be first-line therapy for *P. falciparum* malaria worldwide **(WHO, 2006)**. Fixed-dose combinations work such that the partner drug is present to eradicate the last parasites while the Artemisinin component removes majority of the parasites at the start of treatment **(White, 2004)**. The Artemisinin derivatives include Artesunate, Dihydroartemisinin, Artemether and Arteether derived from the herb, qinghaosu, sweet wormwood or Artemisia annua L (Asteraceae) **(Ioset and Kaur, 2009)**.

The main drugs to be used in combination with the **Artemisinin** are:

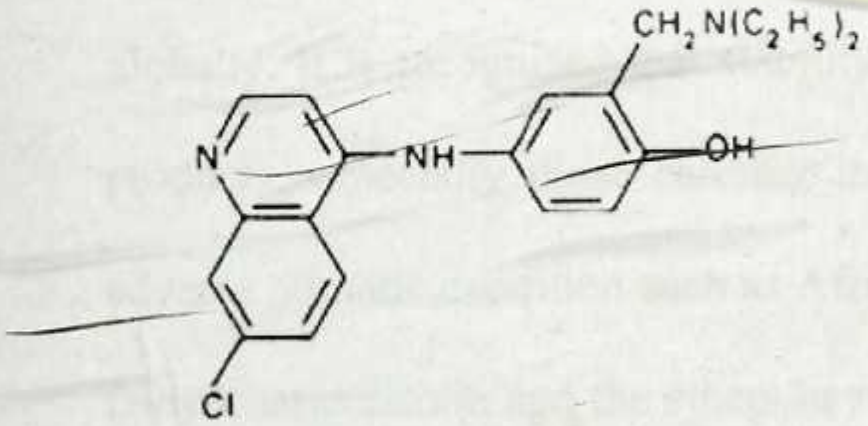
- Amodiaquine: A 4-aminoquinoline, structurally similar to Chloroquine but it is more effective even against some Chloroquine-resistant strains. The mode of action of Amodiaquine is similar to that of Chloroquine **(Krogstad, et. al, 1987)**. Amodiaquine is recommended for the treatment of the uncomplicated malaria and as prophylaxis **(Olliaro and Mussano, 2003)**. It is used in combination with Artesunate.

- Mefloquine: It is a fluorinated 4-quinoline and a 2-aryl substituted chemical structural analogue of Quinine. Mefloquine acts as a blood schizonticide **(Eastman and Fidock, 2009)**. It is effective against most species that cause malaria. It is also used in combination with Artesunate.

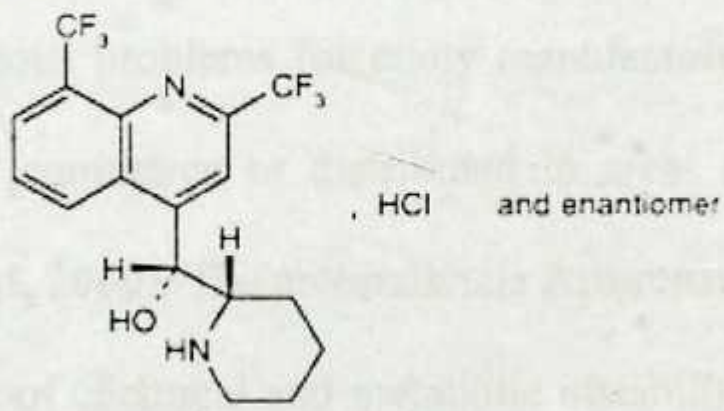
- Lumefantrine: It is also referred to as benflumetol and is structurally related to the hydrophobic arylamino alcohol antimalarials. Its mode of action is similar to mefloquine, quinine and halofantrine. It is used in combination with Artemether.

- Piperaquine: It is a bisquinoline and is also structurally related to Chloroquine. It is used in combination with Dihydroartemisinin **(Eastman and Fidock, 2009)**.

- Sulfadoxine–Pyrimethamine: Sulfadoxine is a structural analog of *p*-aminobenzoic acid (PABA) and a specific inhibitor of the enzyme dihydropteroate synthetase in the tetrahydrofolate synthesis pathway of malaria parasites. Sulfonamides act on the schizont stages of the erythrocytic (asexual) cycle. Sulfadoxine is co-administered with antifolate pyrimethamine, most commonly as fixed-dose sulfadoxine-pyrimethamine. Sulfadoxine- pyrimethamine is usually combined with Artesunate.

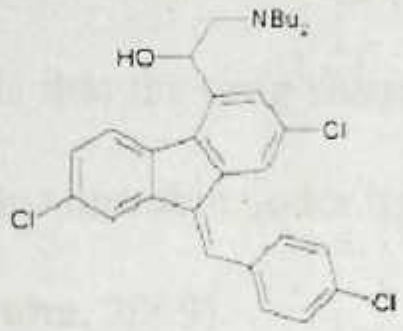


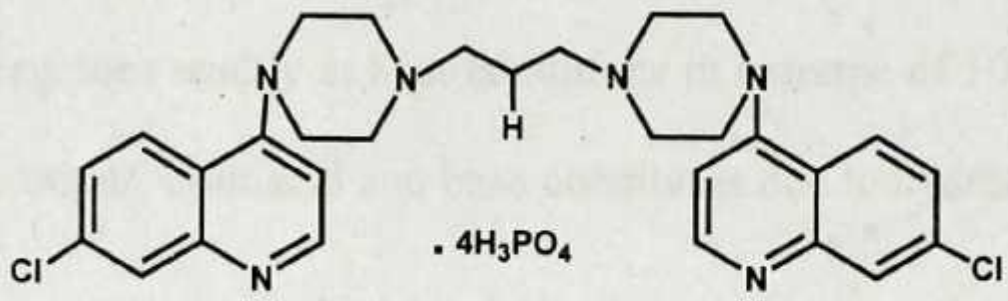
Amodiaquine



, HCl and enantiomer

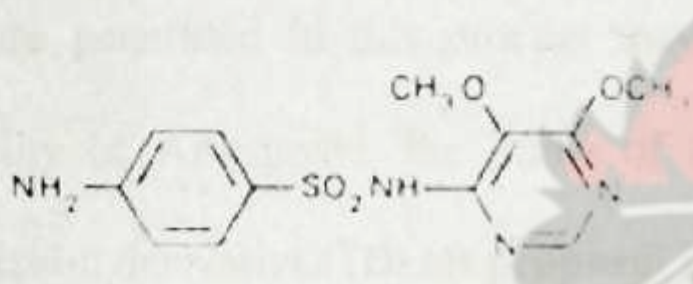
Mefloquine

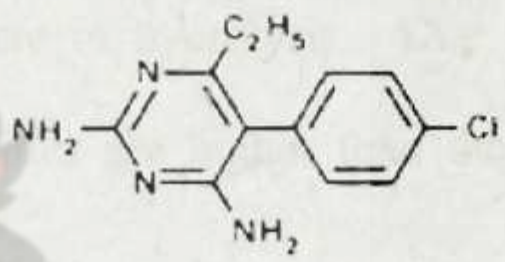




Lumefantrine

Piperaquine phosphate





Sulfadoxine

Pyrimethamine

Fig. 1 Structures of some antimalarials used in combination with Artesunate

1.2 STATEMENT OF PROBLEM

Stability of pharmaceuticals has in recent times become an issue of great concern globally. It is recognized that stability poses serious problems for many manufactured products, especially those entering international commerce or distributed in areas of adverse climatic condition such as Africa **(Olaniyi, 2000)**. The antimalarials Artesunate, Dihydroartemisinin and the ethers have problems of chemical and metabolic instability. The presence of a hemi-ester linkage causes hydrolysis to occur slowly in the presence of moisture. Artesunate is greater than 99% ionized at pH 7.2. The stability of

Artesunate is also temperature dependant **(Bunnag, et.al, 1991)**. Stress testing also reveals that the drug substance degrades readily at heat conditions in extreme of 100°C, it is also unstable under light conditions, both acid and base conditions due to hydrolysis **(Kuwana, 2009)**.

Artesunate is synthesized by esterifying Dihydroartemisinin with succinic anhydride under alkaline conditions **(Presser and Buzzi, 2009)**. Industrial conversion to tablets involves dry or wet granulation and compressing the resulting granules with other materials in the form of binders, disintegrants, lubricants, colorants and preservatives. Moisture generated in this process may contribute to hydrolysis. Due to the high instability of Artesunate, the limits of its impurities are higher than those of other Artemisinin derivatives **(Draft proposal IP, 2006)**.

Artesunate and the other Artemisinin also lack chromophore which is responsible for ultraviolet and fluorescent absorption. This makes their analytical procedures difficult and limits their detection range.

1.3 GENERAL OBJECTIVE

The aim of this work is to perform stability studies on different brands of Artesunate on the local market.

The specific objectives of this project are to:

1. **Develop and validate simple UV/Visible Spectrophotometric method for the assay of Artesunate tablets.**

2. **Develop and validate an HPLC method to quantify Dihydroartemisinin and Artesunate simultaneously.**

3. **Employ the method developed to quantitatively determine extent of breakdown of Artesunate in combination tablets on the Ghanaian market.**

1.4 JUSTIFICATION OF WORK

The WHO recommends Artesunate –amodiaquine as the first line treatment of malaria in sub Saharan Africa. This therefore necessitates the development of reliable and cost effective analytical methods for Artesunate to quantify and hence ensure the quality of drugs formulated. A previous work done described the simultaneous analysis of Artesunate and Dihydroartemisinin in human plasma: using HPLC - mass spectrometer (Thuy, et.al, 2008). Methods for quantification of Artesunate and Dihydroartemisinin in pharmaceutical products like tablets are very few. These methods require expensive reagents and equipment.

As indicated, instability of Artesunate poses issues of great concern and methods to quantify simultaneously Artesunate and its breakdown products are unavailable. This demands that analytical methods be developed to quantify Artesunate and Dihydroartemisinin which is the major breakdown products of Artesunate simultaneously to ensure the quality of drug produced and determine the extent of breakdown.

There is no UV method for the assay of Artesunate in the official monographs. It is therefore necessary to develop a method which is fast, sensitive and requires cheaper reagents for the analysis of Artesunate since UV machine is more readily available.

1.5 SCOPE OF THE STUDY

Artesunate -amodiaquine tablets would be the only Artesunate combination drug considered in this research. This is because Artesunate- Mefloquine is not approved by the World Health Organization as the first-line treatment for malaria in Africa whereas resistance to Artesunate- Sulfadoxine/pyrimethamine limits its usage. The research would take into account at least six brands of Artesunate tablets available on the local market in Kumasi.

1.6 LIMITATION

The major limitation to this work is the low wavelength of detection of Artesunate and Dihydroartemisinin.

1.7 ORGANIZATION OF STUDY

Six brands of Artesunate combination tablets would be obtained from various pharmacies in the Kumasi metropolis. Artesunate and Dihydroartemisinin pure samples would be obtained and identification tests would be conducted based on the international pharmacopoeia. Thin layer chromatography would be performed on the tablets obtained to detect the presence or absence of breakdown products in the tablets. A UV method would be developed by derivatization with acid to a longer wavelength and validated.

Development of an HPLC method for Artesunate and Dihydroartemisinin would then be done and calibration graphs for Artesunate and Dihyroartemisinin would be drawn. The drugs obtained would be assayed using the method developed. Validation of the analytical procedure would then be done.

CHAPTER 2 –LITERATURE REVIEW

2.0 ARTEMISININ-BASED ANTIMALARIAL DRUGS

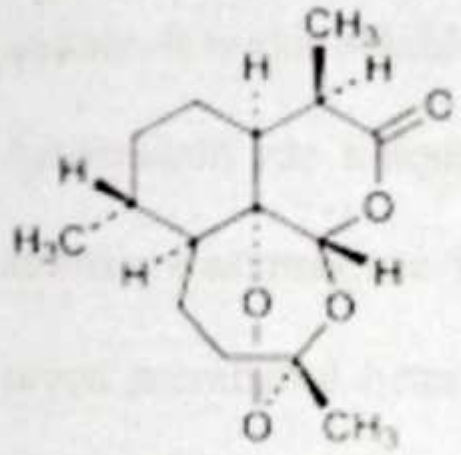


Fig. 2 Structure of Artemisinin

Artemisinin, also known as **qinghaosu**, is a sesquiterpene lactone endoperoxide isolated from the sweet wormwood plant, *Artemisia annua* that Chinese herbalists traditionally used to treat malaria **(Klayman, 1985)**. Artemisinins are currently the most important class of antimalarials available, particularly because they are effective against parasites resistant to almost all the other classes of antimalarials **(Krishna, Uhlemann et al. 2004)**. Artemisinin and its derivatives also possess the most rapid action of all current drugs against falciparum malaria **(White, 1997)**.

In 1971, scientists demonstrated that the plant extracts had antimalarial activity in primate models and in 1972 the active ingredient, Artemisinin was isolated and its chemical structure described. Artemisinin is found in the glandular trichomes of leaves, stems, and inflorescences, and it is concentrated in the upper portions of the plant within new growth **(Duke and Paul, 1993)**.

The Endoperoxide Bridge of Artemisinin is essential for antimalarial activity **(Posner, *et.al*, 2004)**. Artemisinin is chemically represented as (3*R*,5a*S*,6*R*,8a*S*,9*R*,12*S*,12a*R*)-Octahydro-3,6,9-trimethyl-3,12-epoxy-12*H*-pyrano[4.3-*j*]-1,2-benzodioxepin-10(3*H*)-

one. Artemisinin is a colourless needles or white, crystalline powder which is practically insoluble in water, very soluble in dichloromethane, freely soluble in acetone and ethyl acetate. It is also soluble in glacial acetic acid, methanol and ethanol **(IP, 2006)**.

Artemisinin has unique stereochemical features since more than seven of its carbon atoms are asymmetric. It is a three cyclic ring system with a peroxide bridge built over a seven membered heterocyclic (oxygen containing) ring. In this ring, the peroxide is sitting in a configuration called a tri-oxane. In addition, the molecule carries two other oxygens atoms forming a lacton function. The presence of a peroxide bridge on top of oxygen containing seven membered rings is chemically quite remarkable, and these heterocyclic ring systems are known to be chemically rather unstable **(O'Neill, et.al, 2010)**.

2.1 MECHANISM OF ACTION OF ARTEMISININS

The action of Artemisinin derivatives is different from that of the other antimalarial drugs, although both the Artemisinin drugs and the 4-aminoquinolines interact with haem **(Meshnick, *et al,* 1996)**. Most of the antimalarials work at the late trophozoite and schizont stage of the malaria parasite, Artemisinin derivatives however already act at early trophozoite and ring stages **(Price, et. al, 1996)**.

The peroxide within the 1,2,4-trioxane system of Artemisinins is essential for antimalarial activity **(Krishna et. al., 2004)**. Peroxides are generally reactive entities and a link has been drawn between the mechanism of action of Artemisinins and the generation of reactive oxygen species (ROS)—hydroxyl, alkoxyl, (protonated) superoxide or peroxyl radicals—within the parasitized erythrocyte **(Huber et al., 2002)**.

It was found that haem or Fe^{2+} catalyses the opening of the peroxide bridge in Artemisinin, leading to the formation of free radicals **(Meshnick, *et al*, 1993)**. The Artemisinin radical binds subsequently to membrane proteins, and alkylation reactions eventually cause destruction of the parasite.

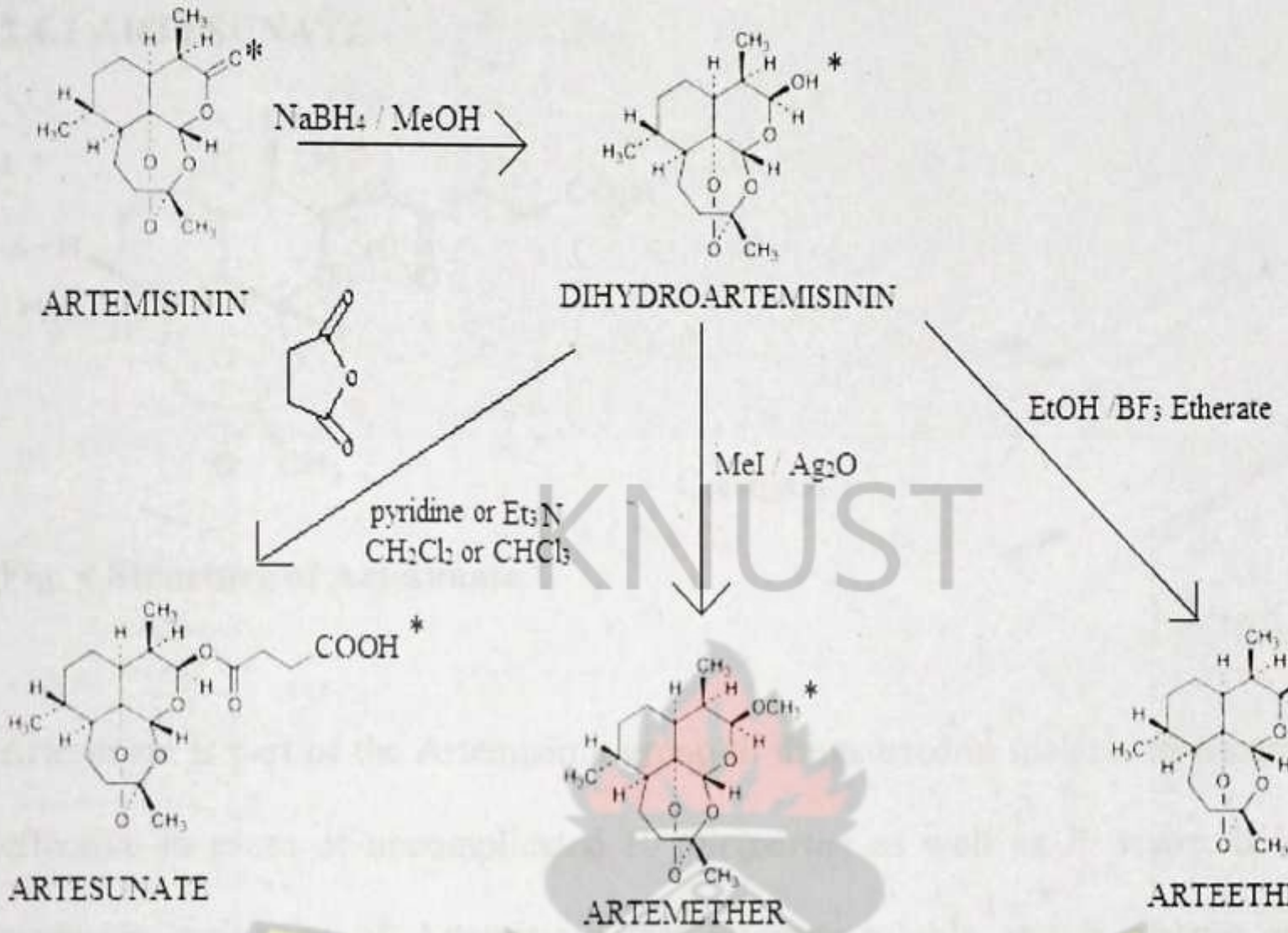
2.2 ARTEMISININ DERIVATIVES

The rationale behind the use of these semisynthetic derivatives of Artemisinin is related to their physical properties. Artemisinin itself is a highly crystalline compound that does not dissolve in oil or water and so can only be given by the enteral route **(Woodrow, Haynes et al. 2005)**. Several Artemisinin derivatives have been synthesised to increase oil or water solubility as well as improved oral bioavailability **(Gary, 1999)**.

Artemisinin derivatives include:

- **Dihydroartemisinin** which is the active metabolite to which artemesinin is reduced.
- **Artemether**: a methyl ether derivative of dihydroartemesinin and is soluble in oil.
- **Arteether**: an ethyl ether derivative of Dihydroartemisinin which is also oil soluble. It is used in combination therapy for cases of uncomplicated resistant P. falciparum.
- **Artesunate**: a hemisuccinate derivative of Dihydroartemisinin which is water soluble and can be given by the oral, intramuscular, intravenous, and even intrarectal routes **(Krishna et al., 2001)**.

2.3 SYNTHESIS OF ARTEMISININ DERIVATIVES



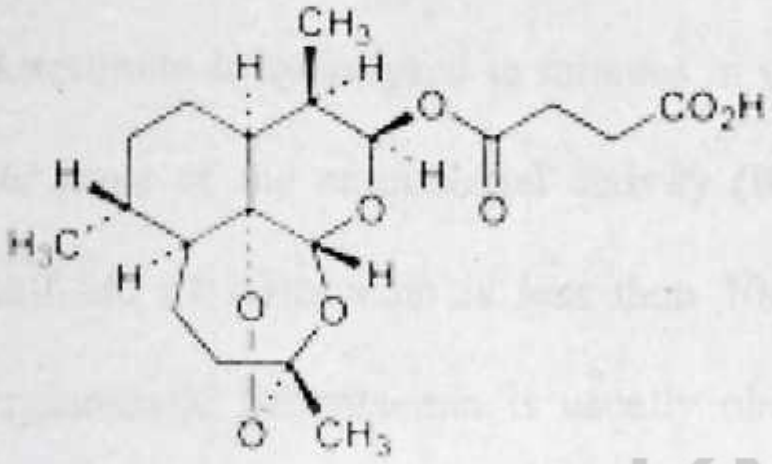
* represents position of chemical modification

Fig. 3 Synthesis of Artemisinin derivatives

In the synthesis of **Artemisinin** derivatives, **Artemisinin** is reduced with sodium borohydride to produce **Dihydroartemisinin** as a mixture of epimers **(Olaniyi A. A., 2005)**. To produce **Artemether**, the mixture is treated with methanol and an acid catalyst **(Haynes and Vonwiller 1994)**. **Arteether** can also be prepared from **Dihydroartemisinin** using boron trifluoride. Artesunate is produced by esterification of **Dihydroartemisinin** using succinic anhydride under basic conditions **(Chekem and Wierucki 2006)**.

2.4 PROFILE OF ACTIVE INGREDIENTS UNDERSTUDY

2.4.1 ARTESUNATE



$C_{19}H_{28}O_8$

Fig. 4 Structure of Artesunate

Artesunate is part of the Artemisinin group of drugs used in malaria treatment and it is effective in cases of uncomplicated *P. falciparum* as well as *P. vivax*. It is a semi-synthetic derivative of Artemisinin that is water-soluble and is unique among the Artemisinin derivatives because it can be administered intramuscularly, intravenously, and even intrarectally (**Krishna et al., 2001**). Oral administration of Artesunate is done in combination with amodiaquine, mefloquine, Sulfadoxine/pyrimethamine.

Artesunate is chemically designated as (3*R*,5a*S*,6*R*,8a*S*,9*R*,*10S*,12*R*,12a*R*)-Decahydro-3,6,9-trimethyl-3,12-epoxy-12*H*-pyrano[4,3-*j*]-1,2-benzodioxepin-10-ol,hydrogen succinate. It is a fine, white crystalline powder which is very slightly soluble in water, very soluble in dichloromethane, freely soluble in ethanol and acetone with a melting point range of 132-135^{0}C **(IP, 2008)**.

Artesunate in its pure form should contain not less than 96.0% and not more than the equivalent of 102.0% of $C_{19}H_{28}O_8$, or not less than 99.0% and not more than the equivalent of 101.0% of $C_{19}H_{28}O_8$ calculated with reference to the anhydrous substance.

Artesunate is assayed by HPLC and titrimetric methods in the official compendia **(IP, 2008)**.

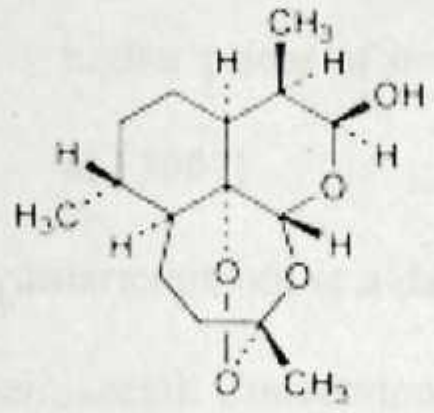
Artesunate is hydrolysed in minutes in vivo to Dihydroartemisinin which then accounts for most of the antimalarial activity **(Barradell and Fitton, 1995)**. The elimination half-life of Artesunate is less than 10mins and ninety percent clearance of asexual erythrocytic parasitaemia is usually observed within 4 hours. Defervescence occurs within 2-3 days after Artesunate administration. Intramuscular and rectal dosing of Artesunate exhibit a slower and a more variable absorption and elimination **(Teja-Isavadharm, *et. al*, 1996)**.

Artesunate is remarkably free of major toxicity. Artesunate bioavailability is acceptable even when it is given by the intramuscular route in severe malaria and there is no associated local toxicity **(Krishna et al., 2001)**.

2.4.1.1 Instability of Artesunate

Artemisinins generally have problems of chemical instability due to the presence of the peroxide bridge in the structure. Artesunate has a greater problem of chemical and metabolic instability due to the susceptibility of hemi-ester linkage to hydrolysis slowly in the presence of moisture. At pH of 1.2, Artesunate conversion to Dihyroartemisinin is rapid, with half-life of 26 min, and at pH 7.4, the half-life is about 10 hours. In addition to a pKa of 4.6, over 99% of Artesunate will be ionized at pH 7.4, and thus uptake by passive diffusion from the intestinal tract will be minimal **(Haynes, 2006)**. Artesunate has a high level of instability and so the limits of its impurities are higher than those of other Artemisinin derivatives **(Nelson, et. al., 2010)**.

2.4.2 DIHYDROARTEMISININ



$C_{15}H_{24}O_5$

Fig. 5 Structure of Dihydroartemisinin

Dihydroartemisinin (also known as **artenimolum** or **artenimol**), is a derivative of Artemisinin and the main active metabolite of a number of Artemisinin derivatives **(O'Neill and Posner, 2004)**. Dihydroartemisinin (DHA) is found to be a more potent antimalarial in vitro than Artemisinin **(Klayman, 1985)**.

Dihyroartemisinin is available as a colourless, needles or a white or almost white, crystalline powder which melts at 158–160^{O}C. Its chemical name is (3*R*,5a*S*,6*R*,8a*S*,9*R*,10*S*,12*R*,12a*R*)-Decahydro-3,6,9-trimethyl-3,12-epoxy-12*H*-pyrano [4,3-*j*]-1,2-benzodioxepin-10-ol and it has a molecular weight of 284.4g/mol. It is practically insoluble in water; slightly soluble in acetonitrile, ethanol and dichloromethane. DHA could be assayed by ultraviolet spectroscopy and should contain not less than 98.0% and not more than the equivalent of 102.0% of $C_{15}H_{24}O_5$, calculated with reference to the dried substance **(IP, 2006)**.

Dihydroartemisinin is synthesized from Artemisinin through two main processes.

1. The first process converts Artemisinin to Dihydroartemisinin by reduction with sodium borohydride in methanol or ethanol at about 0° to 5°C.

2. The second process shows the reduction with DIBAL-H in dichloromethane at -78°C. The disadvantages of DIBAL-H processes are the smaller yield and the higher prices of both the solvent and the means of reduction **(Buzzi, Presser et al., 2007)**.

Dihydroartemisinin is a derivative of Artemisinin with the C-10 lactone group replaced by hemiacetal. Conversion of the lactone carbonyl group at C-10 of Artemisinin into the hydroxyl group in DHA yielded a new stereochemically labile centre in the molecule, which, in turn, provided two interconverting lactol hemiacetal epimers, namely 2α and 2β. The rate of interconversion depends on buffer, pH, and solvent polarity. The 2α epimer has not been isolated in the solid state **(D'Acquarica et. al., 2010)**.

DHA is available only in oral preparations and is usually administered in combination with Piperaquine phosphate. DHA is the most effective Artemisinin compound and the least stable. It has a strong blood schizonticidal action and reduces gametocyte transmission. It is used for therapeutic treatment of cases of resistant and uncomplicated *P. falciparum*. The reported elimination half-life of DHA is 43 min and the pharmacokinetic properties of DHA are affected only by gender and body weight **(McGready et al., 2006)**.

2.5 THEORY OF ANALYTICAL PROCEDURES

2.5.1 IDENTIFICATION TEST

Identification tests are essential to verify the quality and identity of drug or product obtained. It further provides means of ensuring that the materials have been correctly

labelled. Identification is usually achieved by a combination of simple chemical tests and measurement of appropriate physical constants. Identification tests include: infrared absorption spectroscopy (this forms the basis for most identification procedures owing to the high structural specificity of infrared absorption spectroscopy), thin layer chromatography, Gas chromatography, Nuclear magnetic resonance spectroscopy as well as physical constants such as melting point, boiling point, refractive index, and solubility characteristics are distinctive properties that are useful for identification and maintenance of standards of purity **(Beckett and Stenlake, 1997)**.

2.5.1.1 Thin layer Chromatography

Thin-layer chromatography (TLC) is a very commonly used technique for identifying compounds, determining their purity and following the progress of a reaction. TLC can be both a preparative and analytical method in which the mobile phase moves by capillary action across a uniform, thin, layer of a finely divided stationary phase, an adsorbent usually, silica gel, alumina, cellulose, kieselguhr, celite, magnesium trisilicate or florisil mixed with a binding agent to ensure adherence to the plate and a fluorescent indicator **(Olaniyi, 2000)**.

TLC is performed on a sheet of glass, plastic, or aluminum foil, coated with a thin layer of adsorbent material. The separation is based on adsorption, partition, ion-exchange or on combinations of these mechanisms and is carried out by migration of solutes in a solvent or a suitable mixture of solvents through the thin-layer **(BP, 2007)**.

Thin layer chromatography involves dissolving the analyte in an appropriate solvent and spotting on one side of the prepared plate and immersing the prepared plate in a

Chromatographic tank containing a suitable eluting solvent. The solvent gradually moves up the plate via capillary action, and it carries the deposited substances along with it at different rates. The solvent is allowed to rise to about 10-15cm up the plate. Then it is removed and air dried. Spots are located by visual inspection under UV light (254 and 366nm) or sprayed with suitable chromogenic agents which will react with the substances to give characteristic spot colouration. Substances could be identified based on their R_f values. The R_f is defined as:

$$R_f = \frac{\text{Distance travelled by substance}}{\text{Distance travelled by solvent}}$$

R_f varies from zero to one. TLC is usually run with a reference standard or pure sample **(Olaniyi, 2000)**.

2.5.1.2 Melting Point or Range

Melting point is one of the oldest identification methods and it is the range between the corrected temperatures at which the substance begins to collapse or form droplets on the wall of a capillary tube and the corrected temperature at which it is completely melted as shown by the disappearance of the solid phase **(IP, 2006)**.

The limitations of melting point data owing to factors such as sample size, capillary dimensions, temperature of insertion and rate of temperature rise makes it difficult to ensure reproducibility. Melting range is therefore a more practical criterion of identity and purity than melting point **(Beckett and Stenlake, 1997)**.

Determining the melting range is a simple and fast method used in many diverse areas of chemistry to obtain a first impression of the purity of a substance. This is because even small quantities of miscible impurities would usually produce a marked change in the melting range and cause the commencement of melting to occur at a temperature lower than the melting point of the pure substance or at least clearly enlarge its melting range **(Furniss, et. al, 1989)**.

2.5.2 ASSAY METHODS

2.5.2.1 Ultraviolet- Visible spectrophotometry

Ultraviolet-visible spectrophotometry is one of the frequently used techniques 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 **(Beckett and Stenlake, 1997)**. Absorption of light in both the ultraviolet and visible regions of the electromagnetic spectrum occurs when the energy of light matches that required to induce in the molecule an electronic transition and its associated vibrational and rotational transitions. Ultraviolet- visible spectrophotometers which are employed to measure the intensity of light absorbed normally have both Deuterium and Tungsten lamps and selection of the appropriate lamp is made by moving either lamp mountings or mirrors **(Beckett and Stenlake, 1997)**.

The use of the UV/Visible spectrophotometer for quantitative work follows the Beer-Lambert's law. It states that the proportion of light absorbed by a solute in a transparent solvent is independent of the intensity of the incident light and is proportional to the

number of absorbing molecules in the light path. Mathematically the Beer-Lambert's Law is given by the equation below:

$$\log10\ (Io/I) = A = \varepsilon cl$$

Where; Io = intensity of incident light

I = intensity of transmitted light

ε = molar absorptivity or molar extinction coefficient

c = concentration of solute in moles per litre

l = cell (path) length (cm)

A = absorbance

2.5.2.2 Acid- Base Titrations

Titrimetry is widely used for quantitative analysis and is an absolute method of analysis in which the purity of a substance is determined without a reference standard. Titrimetry is generally simple and inexpensive, involving the use of volumetric flasks, burettes and pipettes. Several titrimetric methods exist but the choice of the method depends on the sensitivity required, presence of interfering substances and alternate methods of analysis **(Olaniyi, 2000)**.

Acid-base titrations may involve a direct titration or a back titration. A direct titration involves the accurate determination of the strength of a solution using a standard solution of known purity and strength. It usually results in the formation of salts which

are not hydrolysed in aqueous solution. A back titration consists of the addition of a definite excess of a standard volumetric solution to a weighed amount of the sample and the determination of the excess not consumed by the sample **(Olaniyi, 2000)**.

2.5.2.3 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography is an analytical technique that was developed in the late 1960's as an improved form of column chromatography to identify, quantify and purify the individual components of a mixture. HPLC is currently the most important and predominantly used separation technique in all areas related to chemistry. HPLC is based on the same modes of separation as classical column chromatography which include adsorption, partition, ion exchange, stereochemical interactions and gel permeation **(Beckett and Stenlake, 1997)**. HPLC technique involves the injection of a small volume of liquid sample onto a column where individual components of the sample are moved down the column by a mobile phase which is forced through the column under high pressure delivered by a pump. The components are separated from one another on the column and detected at the exit of column by a flow through the detector that measures their amount.

HPLC Instrumentation

HPLC instrumentation basically includes a pump, injector, column, detector and data analysing system. The heart of the system is the column where separation occurs. A

mobile phase reservoir is also needed to contain the mobile phase required for the chromatographic process.

Pump

Pumps are required to deliver a constant flow of mobile phase at pressures ranging from 0.1 to 55MPa. Pumping systems that deliver solvent from one or more reservoirs are available. Many HPLC pumps are fitted with a facility for "bleeding" the system of entrapped air bubbles. 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 **(IP, 2006)**. Three types of pumps are available namely: Reciprocating Piston Pumps, Syringe Type Pumps, and Constant Pressure Pumps.

Injector

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 column

HPLC columns are usually made of polished stainless steel which are between 50 and 300mm long and have an internal diameter of between 2 and 5mm. They are commonly filled with a stationary phase of particle size 5 - 10μm. Columns with internal diameters

of less than 2 mm are often referred to as microbore columns. The column may be packed with various packing materials (stationary phase material) depending of the polarity of the compounds for which it may be employed to analyse. For example silica, alumina or porous graphite may be employed in the column packing. Care of the column is usually of key importance.

Detectors

Several detectors may be employed in HPLC work. Ultraviolet-visible (UV-Vis) spectrophotometers, including diode array detectors are the most commonly employed detectors. Fluorescence spectrophotometers, differential refractometers, electrochemical detectors, mass spectrometers, light scattering detectors, radioactivity detectors or other special detectors may also be used.

Data analysing systems

The data handling system primarily interprets the detectors output. 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. Modern data stations are computer based and have a large storage capacity to collect, process and store data for possible subsequent reprocessing **(IP, 2006)**.

Choice of mobile phase

Choice of mobile phase in HPLC analysis is very crucial and mobile phase additives are therefore added to control pH (to be between 2-8 pH units) and ensure reliable and efficient separations. It has also been realised that ionic pharmaceuticals retain better on

columns with a mobile phase whose pH is closer to the p*K*a of the analyte. In addition an acidic compound would be ionized at a pH slightly above its pKa and the reverse would occur in basic compounds.

2.5.3 ANALYTICAL METHOD VALIDATION

Analytical method validation is described as a systematic study which helps to prove that the systems, facilities and processes perform their intended jobs as adequately and consistently as designed. It is a high level review to determine how well a system, process or product conforms to internally established standard **(Olaniyi, 2000)**. Validation studies are an essential part of GMP and should be conducted in accordance with pre-defined protocols. Validation based on the ICH- guidelines include an investigation of parameters such as: Accuracy, Precision, Specificity, Limit of detection (LOD), Limit of quantification (LOQ), Linearity, Range, Robustness and system suitability testing.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Specificity is usually demonstrated by measuring the response of the sample matrix and any expected or known species (for example excipients, impurities or degradation products).

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration of analyte in the sample. Linearity is usually expressed in terms of the variance around the slope of the regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte. R^2 should be between 0.995and 1.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. Accuracy is usually demonstrated by adding known amounts of analyte(s) to the sample matrix and determining the measured result using the analytical procedure. Accuracy may also be demonstrated by the method of standard additions, or by cross-correlation of results with a second, independent procedure. For an analytical method to be accurate percentage recovery should be between 98-102%.

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple samplings of the same homogeneous sample under prescribed conditions. Precision is usually expressed either as the variance, standard deviation or relative standard deviation (co-efficient of variation). For precision

of an analytical method to be established the Relative Standard Deviation (RSD) should not be more than 2%. Precision should be considered at different levels as follows:

Repeatability (Intra-assay precision)

Repeatability expresses the precision under the same operating conditions over a short interval of time. A minimum of three determinations are made for three different concentrations across the intended range, or a minimum of six determinations at the test concentration is recommended.

Intermediate Precision

Intermediate precision expresses within-laboratory variations: different days, different analysts or equipment, etc.

Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardisation of methodology). Reproducibility is usually demonstrated by means of an inter-laboratory trial.

Limit of Detection (LOD)

The limit of detection of an analytical procedure is the lowest concentration of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The detection limit is usually expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample.

Quantitation Limit (LOQ)

The quantitation limit of an analytical procedure is the lowest concentration of analyte in a sample that can be determined with suitable precision and accuracy under the stated experimental conditions. It is usually expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness is demonstrated by making small deliberate changes to one of the operating parameters of the method, analysing samples and comparing the results to those obtained using the prescribed method.

Range

The range of an analytical method is the interval between the upper and lower concentration of analyte for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. Range is usually demonstrated by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range.

System Suitability Testing

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analysed constitute an integral system that can be evaluated as such. Efficiency, capacity factor, resolution factor, and symmetry factor are parameters that are normally used in assessing the column performance **(ICH guidelines on analytical method validation, 1996)**.

2.6 REVIEW OF ANALYTICAL METHODS

There are currently three ACTs used for malaria treatment in Ghana. They are Piperaquine- Dihydroartemisinin, Artesunate- Amodiaquine and Artemether-lumefantrine of which Artesunate- Amodiaquine and Artemether- lumefantrine are widely available and recommended. It is therefore prudent that analytical methods to assess the efficiency and stability of these antimalarials be readily available and economical.

The B.P and the USP in current use have no monographs of Artesunate and Dihydroartemisinin, however, the monographs of Artesunate and Dihydroartemisinin are in the international pharmacopoeia and the USP SALMOUS Standard. The official method for the assay of Artesunate in the IP is by method of titrimetry and HPLC. There is no UV method for the assay of **Artesunate**. However, there are UV Spectrophotometric methods for the analysis of Artemether and Dihydroartemisinin which employs HCl and NaOH respectively to form a decomposition product with possess the ability to absorb at longer wavelengths.

The major limitation of the development of selective analytical methods for determination of concentrations of Artesunate and other Artemisinin derivatives is based on certain physicochemical properties such as:

• Thermal labiality

• Lack of UV or fluorescent chromophores for absorption which causes them to absorb weakly in the low wavelength region where interferences are numerous therefore making quantification difficult.

The HPLC method for the assay of Artesunate and Dihydroartemisinin stated in the international pharmacopoeia and the USP SALMOUS edition employs reverse phase HPLC with UV detection at a wavelength of 216nm. The mobile phase system used in the analysis is acetonitrile due to its low cut-off point.

Instability of Artesunate has been an issue of great concern and has been reported by a number of publications. Ochekpe et.al, in 2010 in a research to characterize some impurities in Artesunate on the Nigerian market by method of GC-MS revealed high levels of succinic acid in the tablet. TLC revealed Dihydroartemisinin (DHA) to be the other degradation product the brands assayed.

An analytical method to simultaneously quantify Artesunate and its active metabolite, Dihydroartemisinin, in human plasma has been designed by Thuy le et al, in 2008. The method however employs the use of liquid chromatography-mass spectrometry (LC-MS) and acetonitrile as the mobile phase.

CHAPTER 3- EXPERIMENTAL

3.1 MATERIALS AND EQUIPMENT

- Stuart Melting point apparatus SMP10
- Eutech instruments pH 510 pH meter
- Erweka Tablet Disintegration Apparatus
- Cecil CE 2041 2000 Series-UV Spectrophotometer
- Clifton Sonicator
- Adam-analytical weighing balance, WA 210 ; 210/0.0001g
- HPLC Chromatograph
    - Kontron instrument HPLC pump 422
    - Applied Biosystems 783 programmable Absorbance Detector
    - Powerchrome 280 software Integrator
    - Hp desktop
    - KNAUER Eurospher 100-5 C-18, 250 *4.6mm with precolumn
- Volumetric flasks (200ml, 1000ml, 50ml, 25ml)
- Conical flasks
- Measuring beakers (25ml)
- Transfer pipettes (0.5ml, 1ml, 2ml, 5ml, 10ml)
- Graduated pipettes (1ml, 5ml, 10ml)
- No. 1 sintered glass crucible
- No. 1 whatman filter paper
- Glass funnel

- Quick-fit test tubes with stoppers
- Melting point capillary tubes
- Pre-coated TLC plates (Gf 254, 0.25mm Merck W.)

3.2 REAGENTS AND SAMPLES

- Sodium hydroxide (BDH)
- Trifluoroacetic acid TFA (98%)
- Glacial acetic acid (BDH)
- Phenolphthalein/ethanol
- Acetone
- Potassium iodide
- Sulphuric acid (98%w/w) (BDH)
- Acetonitrile (BDH)
- Ethyl acetate (BDH)
- Dichloromethane
- Methanol (HPLC Grade)
- Methanol (BDH)
- Toluene
- Ethanol
- Vanillin
- Tablet excipients
- Talc

- Lactose
- Starch
- Acacia

Table 1 Pure Samples Used

SAMPLE	SOURCE	BATCH NO.	MFG. DATE	EXP DATE
ARTESUNATE	Letap Pharmaceuticals Ltd.	9002AS2RI	0ct. 2009	Sept. 2012
DHA	IPCA Labs.	7006DKRI	Sept. 2009	August 2011

Table 2 Brands of Tablets Used

Brand	Code	Dosage	Batch No.	MFG DATE	EXP DATE	Country of origin
Letasunate	T_1	50mg	1100019	April 2009	Mar 2011	Ghana
Arsuamoon	T_2	50mg	LQ101009	Aug. 2010	July 2012	China
Camosunate plus	T_3	100mg	1002012	Feb. 2010	Feb. 2012	Ghana
Artenex	T_4	200mg	002	April 2009	April 2013	Ghana
Gsunate	T_5	100mg	GK-08	Aug. 2009	July 2012	India
Artesunate	T_6	50mg	080504	May 2008	May 2011	China

3.3 METHOD

3.3.1 IDENTIFICATION TESTS

An amount of 0.100g of Artesunate pure sample was weighed and dissolved in 40 ml of dehydrated ethanol R. It was shaken and filtered to obtain a clear solution. The filtrate was divided into two and evaporated to a volume of about 5 ml. A few drops of the

mixture were placed on a white porcelain dish and 1 drop of vanillin/sulfuric acid was added.

3.3.2 MELTING POINT DETERMINATION

Artesunate pure sample was used to fill melting point tubes to obtain a level of about 1cm on the melting point tubes. The melting point tubes were then placed in the melting point apparatus and melting point readings were taken. The procedure was repeated for pure samples of DHA.

3.3.3 SOLUBILITY TESTS

An amount of 0.05g of Artesunate pure sample was dissolved in 5ml of each of the following solvents; **water, dichloromethane, ethanol and acetone** and observation recorded.

An amount of 0.05g of DHA pure sample was dissolved in 5ml of each of the following solvents; **water, acetonitrile, ethanol and dichloromethane** and observation recorded.

3.3.4 IDENTIFICATION OF ARTESUNATE AND DHA IN TABLETS BY TLC

Stationary phase: Pre-coated TLC plates were used.

Mobile phase: The mobile phase system consisted of Ethyl acetate: acetone: glacial acetic acid (18: 4: 0.1).

Developing solvent methanol: sulphuric acid of ratio (95: 5).

Procedure: 50mg of Artesunate pure powder, 50mg of Dihydroartemisinin pure powder and an equivalent of 50mg of Artesunate tablet was dissolved in 10ml of methanol each and each was individually spotted on precoated TLC plates and put into a chromatographic chamber. After removing each plate from the chromatographic chamber, the plate were placed in a development chamber consisting of methanol: sulphuric acid of ratio (95:5) and heated on a hot plate after drying. The shape of each spot was observed and the R_{fs} were calculated. The procedure was repeated with the six brands Artesunate purchased from the market.

3.3.5 PHARMACOPOEIAL TESTS

3.3.5.1 Uniformity of Weight Test

Twenty tablets from each of the six brands of the tablets were selected at random. The tablets were weighed together and the average weight of a tablet determined. The tablets were weighed individually and the deviations of the weights of each tablet from the average weight of a tablet were calculated. The percentage deviation of each tablet from the average tablet weight was calculated and the results compared to the standards in the BP.

3.3.5.2 Tablet disintegration test

Six tablets were taken from each of the six brands and a tablet was placed in each of the cylindrical tubes in the disintegration basket. The bottom of the disintegration basket

was at least 15mm below the surface of the water and the apparatus was made to operate. The time taken for each tablet to disintegrate was recorded.

3.3.6 ASSAY OF ARTESUNATE PURE POWDER BY ACID-BASE TITRATION

3.3.6.1 Preparation of 0.05M NaOH

About 0.5055g of NaOH pellets was accurately weighed. It was then transferred into a 250ml volumetric flask and made up to volume with distilled water.

3.3.6.2 Preparation of 0.05M Sulphamic acid

About 0.4904g of Sulphamic acid was accurately weighed. It was then transferred into a 100ml volumetric flask and made up to volume with distilled water.

3.3.6.3 Standardization of 0.05M NaOH

A volume of 25ml of 0.05M sulphamic acid was pipetted and two drops of phenolphthalein/ethanol indicator was added and titrated against 0.05M NaOH. The volume of NaOH added was recorded and the procedure was repeated.

3.3.6.4 Assay of Artesunate pure powder

A quantity of 0.2504 g of Artesunate was accurately weighed and 25 ml of neutralized ethanol was added and titrated against 0.05M standardized sodium hydroxide solution, using 2 drops of phenolphthalein /ethanol TS as indicator.

Each ml of sodium hydroxide (0.05 mol /l) Volumetric Solution is equivalent to 19.22mg of Artesunate.

3.3.7 UV SPECTROPHOTOMETRIC METHOD DEVELOPMENT

3.3.7.1 Assay of Artesunate

Derivatisation of Artesuante with HCl

About 100mg of Artesunate was dissolved in 100ml methanol. 1ml of the solution was pipetted and placed into 10 different test tubes. 1ml of conc HCl was added to each of them and shaken thoroughly. The solution was allowed to stand and the reaction was stopped at 5 mins intervals and topped up to 50ml with methanol. The absorption spectrum was determined between 200.0nm and 400.0nm. The procedure was monitored for about an hour. The procedure was repeated by varying the amount of HCl added and further by varying the amount of Artesunate in methanol solution pipetted.

Calibration curve of Artesunate

About 200mg of Artesunate was accurately weighed and dissolved in methanol to give a 100ml solution. Concentrations of approximately 0.15, 0.1, 0.0075, 0.05, and 0.0375% $^{w}/v$ were prepared by serial dilution from the stock solution. 1ml of each of the resulting solutions was pipetted into a quick-fit test tube and 2ml of concentrated HCl added. The test tubes were stoppered and allowed to stand for 30 minutes. Each of the resulting solutions was diluted with sufficient methanol to 50ml. The absorption spectrum was determined between 200.0nm and 400.0nm and the absorbance readings were taken at a maximum of 254nm against a blank solution made up of 2ml of HCl made up to 50ml with methanol. A calibration curve was plotted with the readings.

Assay of Artesunate in tablets

An amount of the powdered sample containing about 100mg of Artesunate was weighed and dissolved in sufficient methanol to produce 100ml. The resulting solution was filtered using a sintered glass crucible, discarding the first 10mls. 1ml of the resulting solution was pipetted into a quick-fit test tube and 2ml of concentrated HCl added. The test tube was stoppered and allowed to stand for 30 minutes. The resulting solution was diluted with sufficient methanol to 50ml. The absorption spectrum determined between 200.0nm and 400.0nm and the absorbance reading at a maximum of 254nm was taken against a blank solution made up of 2ml of HCl made up to 50ml with methanol. The content of Artesunate was calculated from the calibration curve.

Analytical method validation

To demonstrate linearity, about 200mg of Artesunate was accurately weighed and dissolved in methanol to give a 100ml solution. Concentrations of approximately 0.15, 0.1, 0.0075, 0.05, and 0.0375% $^{w}/v$ were prepared by serial dilution from the stock solution. 1ml of each of the resulting solutions was pipetted into a quick-fit test tube and 2ml of concentrated HCl added. The test tubes were stoppered and allowed to stand for 30 minutes. Each of the resulting solutions was diluted with sufficient methanol to 50ml. The absorption spectrum determined between 200.0nm and 400.0nm and the absorbance readings were taken at a maximum of 254nm against a blank solution made up of 2ml of HCl made up to 50ml with methanol. A graph of absorbance against concentration was plotted with the readings and analyzed.

The LOD and LOQ were also calculated from the graph plotted.

To demonstrate accuracy of the method, about 100mg of Artesunate pure powder was weighed and mixtures of tablet excipients (Talc, Lactose, Starch, Acacia) were added. The mixture was dissolved in sufficient methanol to obtain 100ml. The resulting solution was filtered using a sintered glass crucible, discarding the first 10mls. 1ml of the resulting solution was pipetted into a quick-fit test tube and 2ml of concentrated HCl added. The test tube was stoppered and allowed to stand for 30 minutes. The resulting solution was diluted with sufficient methanol to 50ml. The absorption spectrum determined between 200.0nm and 400.0nm and the absorbance reading at a maximum of 254nm was taken against a blank solution made up of 2ml of HCl made up to 50ml with methanol. The recovery of Artesunate was calculated from the calibration curve.

To demonstrate precision, about 100mg of Artesunate pure powder was weighed and dissolved in sufficient methanol to obtain 100ml. 1ml of the resulting solution was pipetted into a quick-fit test tube and 2ml of concentrated HCl added. The test tube was stoppered and allowed to stand for 30 minutes. The resulting solution was diluted with sufficient methanol to 50ml. The absorption spectrum determined between 200.0nm and 400.0nm and the absorbance reading at a maximum of 254nm was taken against a blank solution made up of 2ml of HCl made up to 50ml with methanol. The percentage content of Artesunate was calculated from the calibration curve. The procedure was repeated six times with the same amount of Artesunate and the content of Artesunate was calculated from the calibration curve to obtain intraday precision and further repeated on three different days to obtain inter day precision.

To demonstrate specificity of the method, a solution containing a mixture of the tablet excipients (lactose, starch, acacia and talc) was prepared using the sample preparation procedure and the UV spectrum of this solution was recorded in the range of 200–400nm for any interferences.

3.3.8 HPLC METHOD DEVELOPMENT AND VALIDATION

3.3.8.1 Chromatographic Conditions

Column: KNAUER Eurospher 100-5 C-18, 250 *4.6mm with precolumn

Mobile phase: Methanol: 0.006% TFA (80:20)

Flow rate: 1.5ml/min

Wavelength of detection: 220nm

AUFS: 0.003

Mode of elution: Isocratic

Mode of HPLC: Reverse Phase Liquid Chromatography (RPLC)

3.3.8.2 Assay of Artesunate

Preparation of mobile phase

To prepare 0.006%^{w}/v TFA, 1%^{v}/v of TFA was prepared by diluting 1ml of the TFA stock solution with distilled water and up to 100ml and 10ml of the resulting solution after vigorous shaking was diluted to 100ml to obtain 0.1% TFA. 6ml of the resulting solution was further diluted to 100ml to obtain 0.006% TFA.

500ml of mobile phase was prepared by measuring 100ml of 0.006% TFA with 400ml of HPLC grade methanol. The solution was further filtered through a membrane filter and sonicated to expel gases.

Calibration curve of Artesunate

Artesunate solution was prepared by weighing 400mg of Artesunate pure powder and dissolving it in methanol. The solution was made up to 100 ml. The solution was sonicated and filtered. Serial dilutions were done with methanol to obtain concentration such as 0.3, 0.2, 0.15, 0.1 and 0.05 % ^{w}/v. The solutions were filtered injected and results recorded.

Assay of Artesunate in tablets

An equivalent of 20mg of Artesunate was weighed and diluted with sufficient methanol to make up to 10ml. The solution was shaken thoroughly and filtered. The solution was filtered injected and results recorded.

Validation of analytical method

To demonstrate to linearity, 400mg of Artesunate pure powder was accurately weighed and dissolved in methanol to give a 100ml solution. Concentrations of approximately **0.05, 0.1, 0.15, 0.2, 0.3** and **0.4%** ^{w}**/v** were prepared by serial dilution from the stock solution. The solutions were filtered and injected and results recorded. A graph of absorbance against concentration was plotted with the readings and analyzed.

The LOD and LOQ were also calculated from the graph plotted.

To demonstrate accuracy of the method, about 20mg of Artesunate pure powder was weighed and a mixture of tablet excipient was added. The mixture was dissolved in sufficient methanol to obtain 10ml of the mixture. The resulting solution was filtered and injected and results recorded.

Precision was determined by weighing 20mg of Artesunate pure powder and dissolved in sufficient methanol to obtain 10ml. The solution was filtered and injected and results recorded. The procedure was repeated with the same concentration for six determinations. For reproducibility, three different concentrations were weighed three times and injected and results recorded.

For robustness, different parameters were varied each at a time whilst keeping all other chromatograph parameters constant. The parameters varied were: mobile phase composition and flow rate.

3.3.8.3 HPLC method development for Dihydroartemisinin

Preparation of mobile phase

To prepare 0.006%^{w}/v TFA, 1%^{v}/v TFA was prepared by diluting 1ml of TFA stock solution with distilled water and up to 100ml and 10ml of the resulting solution after vigorous shaking was diluted to 100ml to obtain 0.1% TFA. 6ml of the resulting solution was further diluted to 100ml to obtain 0.006% TFA.

500ml of mobile phase was prepared by measuring 100ml of 0.006% TFA and 400ml of HPLC grade methanol. The solution was further filtered through a membrane filter and sonicated to expel gases.

Calibration curve of Dihydroartemisinin

Dihydroartemisinin solution was prepared by weighing 400mg of Dihydroartemisinin pure powder and dissolving it in methanol. The solution was made up to the100 ml mark in a 100ml volumetric flask. The solution was sonicated and filtered. Serial dilutions were done with methanol to obtain concentration such as **0.3, 0.2, 0.15, 0.1** and **0.05 %** ^{w}/v. The solutions were filtered injected and results recorded.

Determination of content of Dihydroartemisinin in Artesunate tablet

An equivalent of 20mg of Artesunate was weighed and diluted with sufficient methanol to make up to 10ml. The solution was shaken thoroughly and filtered. The solution was filtered injected and results recorded.

Validation of analytical method

To demonstrate to linearity, 400mg of Dihydroartemisinin pure powder was accurately weighed and dissolved in methanol to give a 100ml solution. Concentrations of approximately **0.05, 0.1, 0.15, 0.2, 0.3** and **0.4%** ^{w}/v were prepared by serial dilution from the stock solution. The solutions were filtered and injected and results recorded. A graph of absorbance against concentration was plotted with the readings and analyzed.

Precision was determined by weighing 20mg of Dihydroartemisinin pure powder and dissolved in sufficient methanol to obtain 10ml. The solution was filtered and injected and results recorded. The procedure was repeated with the same concentration for six determinations. For reproducibility, three different concentrations were weighed three times and injected and results recorded.

For robustness, different parameters were varied each at a time whilst keeping all other chromatograph parameters constant. The parameters varied were: **mobile phase composition** and **flow rate**.

The LOD and LOQ were also calculated from the calibration graph.

CHAPTER 4- RESULTS

4.1 IDENTIFICATION TEST

4.1.1 IDENTIFICATION TESTS FOR ARTESUNATE (AS)

Table 3 Identification of Artesunate (AS) pure powder by colour reactions

Test	Observation	Expected observation from IP
About 100mg of AS + 40 ml of dehydrated ethanol. AS residue + 5ml dehydrated ethanol + 1ml Vanillin sulphuric acid reagent	A reddish -brown colour was produced.	Reddish-brown colouration

4.1.2 MELTING POINT DETERMINATION OF AS

Table 4 Melting Point Determination of AS

Sample	Melting point (°C)		IP Reference Range (°C)
	1st Determination	2nd Determination	
Artesunate	132-134°C	132-135°C	132 - 135°C

4.1.3 SOLUBILITY TEST FOR ARTESUNATE

Table 5 Solubility test for Artesunate

Sample + solvent	Observation	Expected observation from IP
50mg of AS +10ml water	Sparingly soluble	Slightly soluble
50mg of AS + 5ml	Very soluble	Very soluble

dichloromethane		
50mg of AS + 5ml ethanol	Freely soluble	Freely soluble
50mg of AS + 5ml acetone	Freely soluble	Freely soluble

4.1.4 IDENTIFICATION OF DHA BY COLOUR REACTIONS

Table 6 Identification of DHA pure powder by colour reactions

Test	Observation	Expected observation from IP
5 mg + about 0.5 ml of dehydrated ethanol + 1.0 ml of potassium iodide + 2.5 ml of sulfuric acid + 4 drops of starch	A violet colour produced immediately	Violet colouration

4.1.5 MELTING POINT DETERMINATION OF DIHYDROARTEMISININ

Table 7 Melting Point Determination of DHA

Sample	Melting point (˚C)		Reference Range (˚C)
	1st Determination	2nd Determination	
DHA	157-159°C	158-160°C	158-160°C

4.1.6 SOLUBILITY TEST FOR DIHYDROARTEMISININ

Table 8 Solubility test for DHA

Sample + solvent	Observation	Expected observation from IP
50mg of DHA +5ml water	Insoluble	Insoluble

50mg of DHA + 5ml dichloromethane	Slightly soluble	Slightly soluble
50mg of DHA+ 5ml ethanol	Slightly soluble	Slightly soluble
50mg of DHA + 5ml acetonitrile	Slightly soluble	Slightly soluble

4.2 TLC OF ARTESUNATE AND DIHYDROARTEMISININ

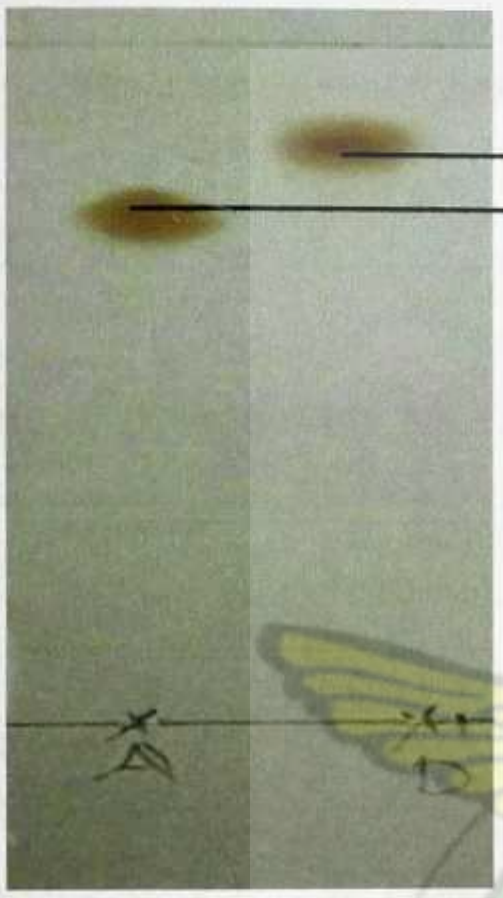


Fig. 6 TLC chromatogram of AS and DHA Pure Samples

Fig. 7 TLC chromatogram of AS, DHA and AS tablet

4.2.1 INTERPRETATION OF TLC CHROMATOGRAM

Table 9 Interpretation of TLC chromatogram

Spot	Interpretation
Fig. 5 Spot A	Spot of Artesunate pure sample
Fig. 5 Spot D	Spot of DHA pure sample
Fig. 6 Spot A	Spot of Artesunate pure sample
Fig. 6 Spot D	Spot of DHA pure sample
Fig 6 Spot T_1	Spot of Artesunate pure sample

4.2.2 CALCULATION OF R_F VALUES

R_f = Distance moved by solute
Distance moved by solvent front

For Artesunate pure sample

- Distance sample moved from origin = 3.0cm
- Distance solvent travelled from origin = 4.5cm

Rf = $\frac{3.0}{4.5}$ = **0.67**

Table 10 R_f values for AS Tablets

Sample	R_f value of AS reference sample	R_f value of DHA reference sample	R_f value (s) of AS tablet
T_1 (2 spots obtained for tablet)	0.67	0.78	0.67 0.78
T_2	0.68	0.80	0.67
T_3	0.72	0.83	0.70
T_4	0.69	0.79	0.69
T_5 (2 spots obtained for tablet)	0.68	0.82	0.68 0.82
T_6	0.70	0.80	.....

4.3 PHARMACOPEIAL TESTS

4.3.1 UNIFORMITY OF WEIGHT

Table 11 Results of Uniformity of Weight Test of Tablets

Brand of tablet	Results of uniformity of weight
T_1	PASSED
T_2	PASSED
T_3	PASSED
T_4	PASSED
T_5	PASSED

Table 12 Results for disintegration of tablets

Brand of tablet	Average disintegration time (min)	Tablet Disintegration test
T_1	8.0	PASSED
T_2	0.14	PASSED
T_3	2.1	PASSED
T_4	6.57	PASSED
T_5	13.6	PASSED
T_6	1.35	PASSED

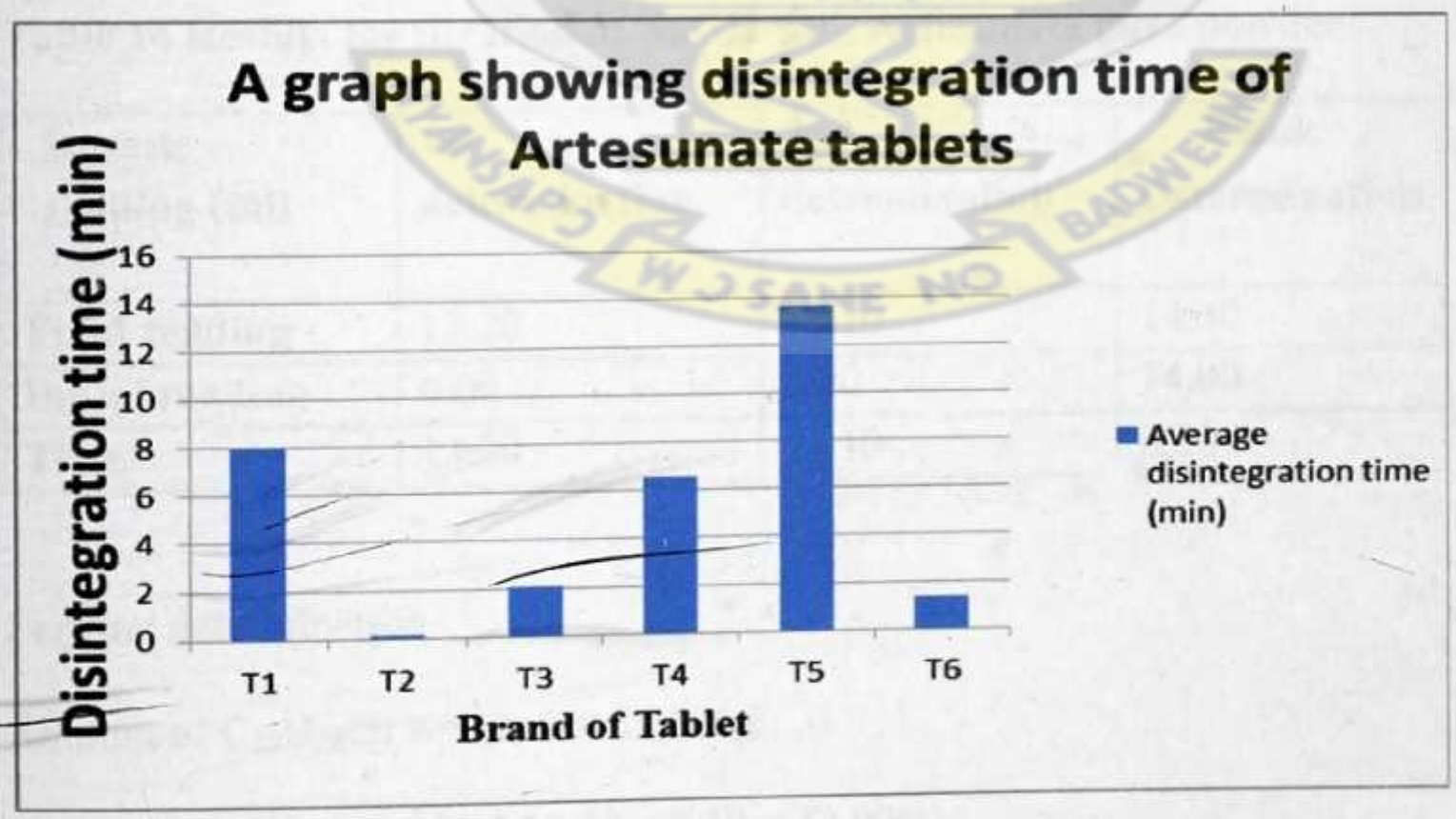


Fig. 8 Disintegration time of Artesunate Tablets

4.4 ASSAY OF AS PURE POWDER BY TITRATION

4.4.1 STANDARDIZATION OF NAOH USING SULPHAMIC ACID

Table 13 Results for titration of NaOH with Sulphamic acid

Burette reading (ml)	1st determination	2nd determination	3rd Determination
Final reading	24.80	49.70	24.70
Initial reading	0.00	25.00	0.00
Titre	24.80	24.70	24.70

97.07g of NH_2SO_3H in 1000ml ≡1M NaOH

0.0048535g of NH_2SO_3H in 1000ml ≡0.05M NaOH

Factor of $C_{19}H_{28}O_8$ $= \frac{0.4907}{0.4904} = 1.0006$

Factor of NaOH $= \frac{1.0006 \times 25.00}{24.73} = 1.0115$

4.4.2 DETERMINATION OF PERCENTAGE PURITY OF AS PURE POWDER

Table 14 Results for titration of NaOH with Artesunate pure powder

Burette reading (ml)	1st determination	2nd determination	Blank Determination
Final reading	13.20	13.10	14.30
Initial reading	0.00	0.00	14.00
Titre	13.20	13.10	0.30

For first determination

Amount of $C_{19}H_{28}O_8$ weighed = 0.2504g

Volume of 0.04M NaOH = 13.20 – 0.30 = 12.90ml

Actual volume of NaOH = 12.90 x 1.0115 = 13.05ml

1ml of 0.05MNaOH is equivalent to 19.22mg of $C_{19}H_{28}O_8$

Amount of $C_{19}H_{28}O_8$ equivalent to 13.05ml of 0.05M NaOH = 13.05 × 0.01922 = 0.2508g

Percentage Purity of Artesunate = 0.2508/0.25 × 100

= **100.32%^{w}/w**

For second determination

Amount of $C_{19}H_{28}O_8$ weighed = 0.2503g

Volume of 0.04M NaOH = 13.10 – 0.30

= 12.80ml

Actual volume of NaOH = 12.80 x 1.0115 = 12.95ml

1ml of 0.05MNaOH is equivalent to 19.22mg of $C_{19}H_{28}O_8$

Amount of $C_{19}H_{28}O_8$ equivalent to 12.95ml of 0.05M NaOH = 12.95 × 0.01922 = 0.2488g

Percentage Purity of Artesunate = 0.2488/0.25 × 100

= **99.54%^{w}/w**

Table 15 Percentage purity of AS pure powder

Percentage Purity (%w/w)	Reference range (%^{w}/w) (IP)
100.32%^{w}/w	99-101
99.54%^{w}/w	

4.4.3 ASSAY OF ARTESUNATE TABLETS BY TITRATION

Table 16 Percentage content of AS in tablets

Tablet	Percentage content (%^{w}/w)	Reference range (%^{w}/w) (IP)
T_1	125.215 ± 0.54472	90.0 - 110.0%
T_2	97.64 ± 0.39	
T_3	103.31 ± 1.36	
T_4	102.63 ± 0.68	
T_5	99.69 ± 4.40	
T_6	.....	

4.5 UV SPECTROPHOTOMETRIC METHOD DEVELOPMENT AND VALIDATION

4.5.1 UV SPECTRUM OF ARTESUNATE

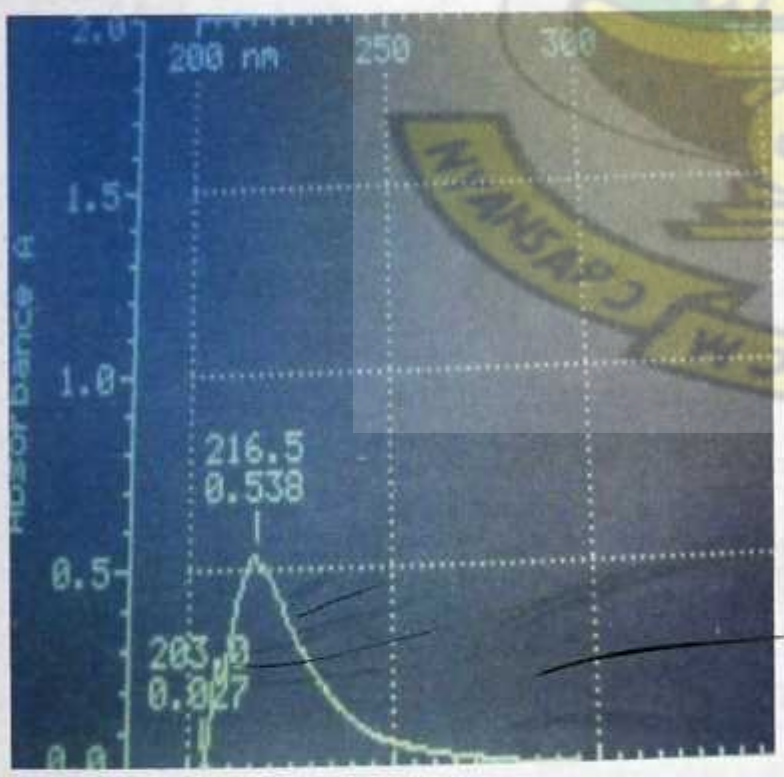


Fig. 9 UV Spectrum of AS in Methanol

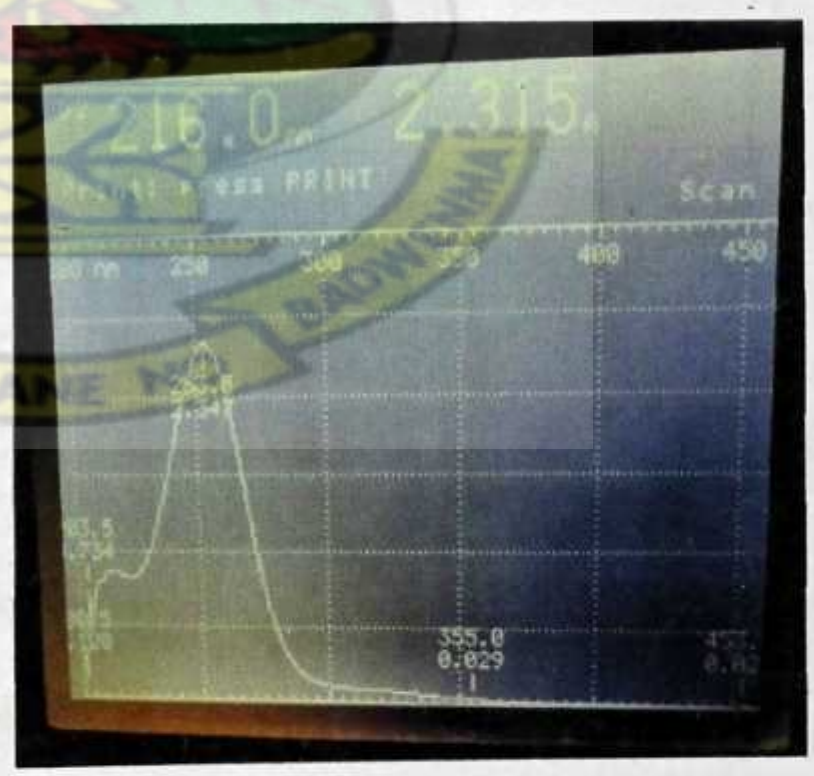


Fig. 10 UV spectrum of AS HCl decomposition product

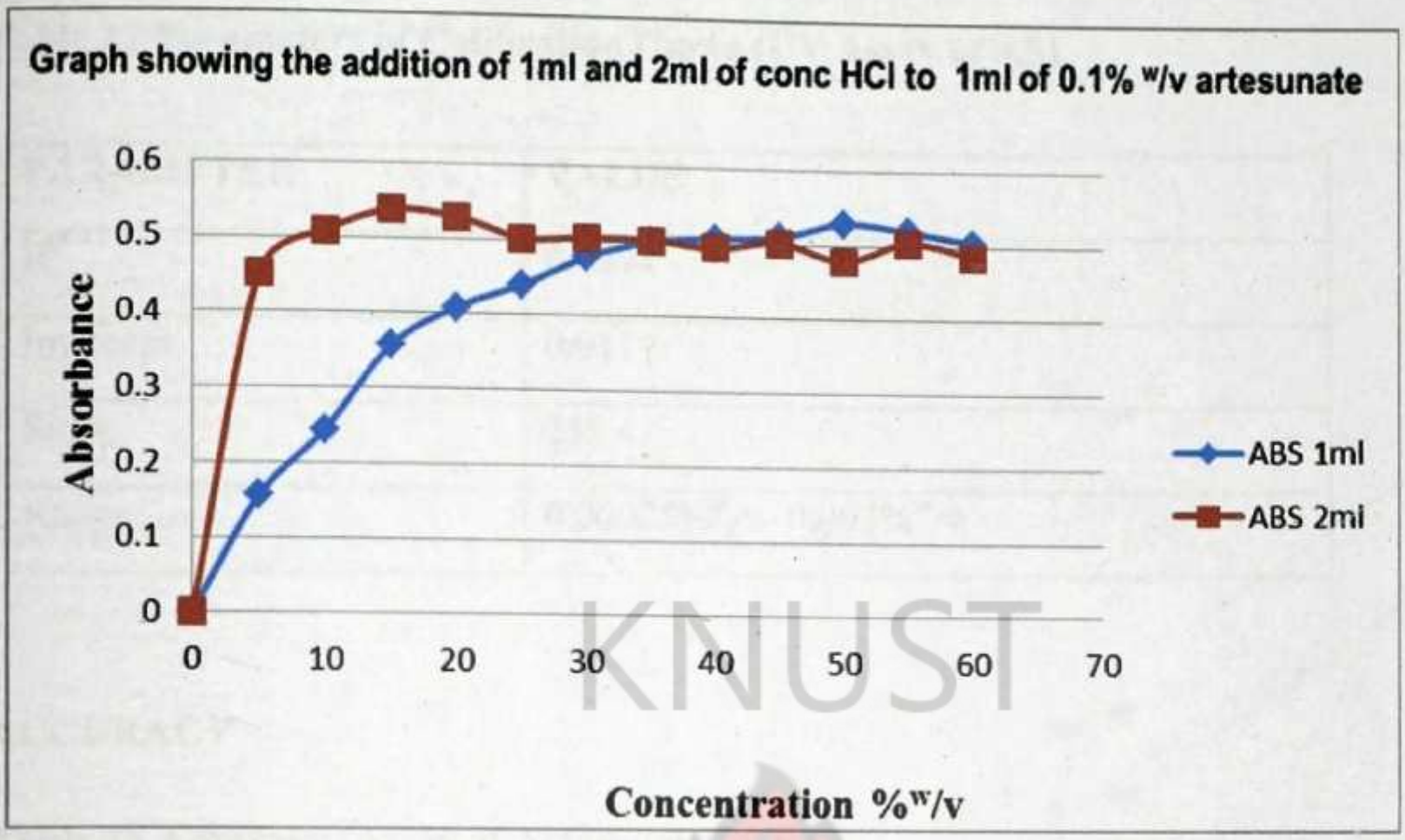


Fig. 11 Addition of 1ml and 2ml of conc. HCl to 0.1%^{w}/v AS

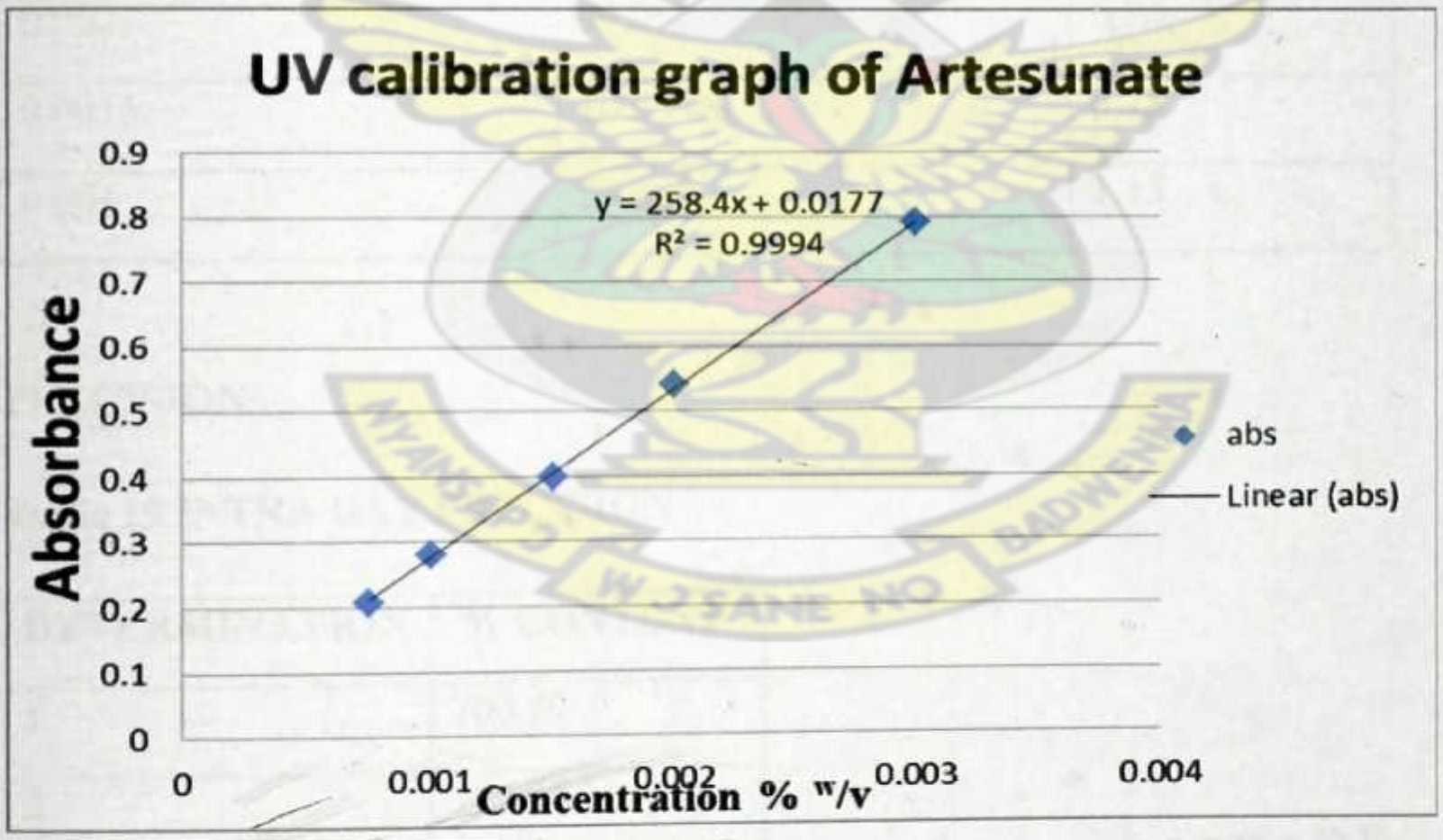


Fig. 12 Calibration Curve for AS (UV Method)

Table 17 Parameters of Calibration Curve (UV Assay of AS)

PARAMETER	VALUE
R^2	0.9994
Intercept	0.0177
Slope	258.4
Range	0.00075%^{w}/v -0.003%^{w}/v

ACCURACY

Table 18 Accuracy (Assay of Artesunate by UV)

CONCENTRATION %^{W}/V	MEAN %RECOVERY	RSD
0.002	99.0%	0.74
0.0015	100.34%	0.75
0.001	99.56%	1.15

PRECISION

Table 19 INTRA DAY PRECISION

DETERMINATION	% CONTENT
1	100.29
2	102.23
3	100.68
4	100.48
5	99.90
6	100.29

Mean= **100.65%**

RSD = **0.81**

INTER DAY PRECISION

Table 20 Inter Day precision

DETERMINATION	**% CONTENT**
Day 1	
1	100.29
2	102.23
3	100.68
4	100.48
5	99.9
6	100.29
Day 2	
7	101.64
8	101.84
9	100.68
10	100.29
11	101.84
12	100.48
Day 3	
13	99.90
14	100.29
15	101.64
16	101.84
17	100.48
18	101.61
Mean	**100.91**
RSD	**0.77**

Limit of Detection (LOD)

LOD = (3.3σ)/ S

Limit of Quantification (LOQ)

LOQ = (10 σ)/ S

Where σ = standard deviation of the response and

S= slope of the calibration curve

Limit of Detection (LOD) = **0.0000729%^{w}/v**

Limit of Quantitation (LOQ) = **0.000221%^{w}/v**

SPECIFICITY

There was no absorbance at 254nm when the tablets excipients alone were taken through the same procedure.

Table 21 Percentage content of AS in commercial samples (UV method)

Tablet	Percentage content (%^{w}/v)	Reference range (%^{w}/v) (IP)
T_1	108.81	90-110%
T_2	106.68	
T_3	106.10	
T_4	101.74	
T_5	105.71	

4.6 HPLC METHOD DEVELOPMENT AND VALIDATION

4.6.1 ARTESUNATE

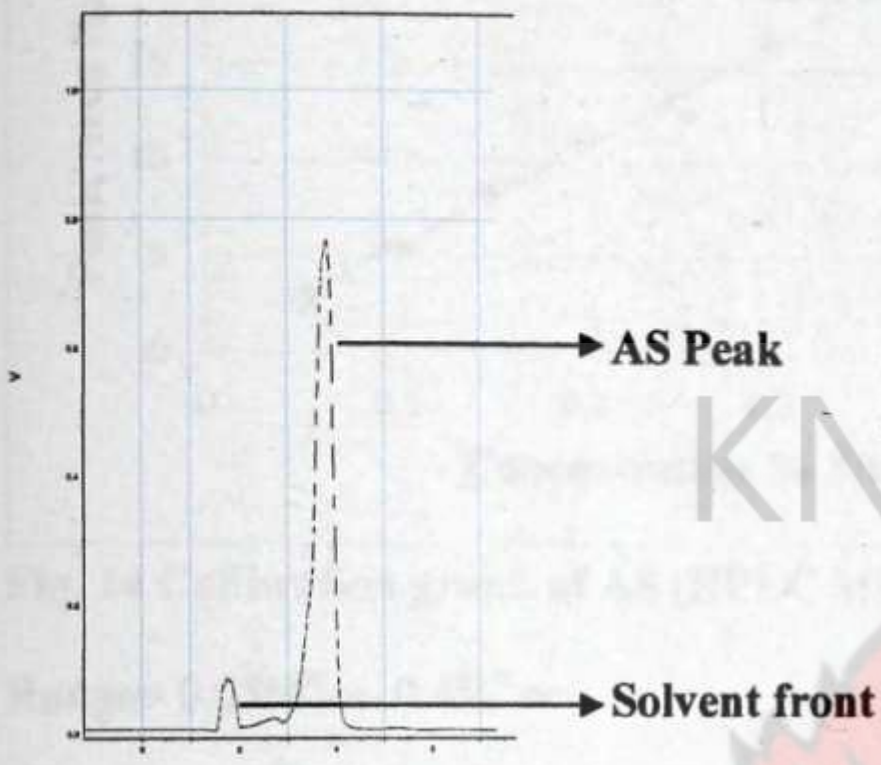


Fig. 13 HPLC Chromatogram of AS

Table 22 Retention time of AS

PARAMETER	VALUE (min)
Retention time	3.74 ± 0.06

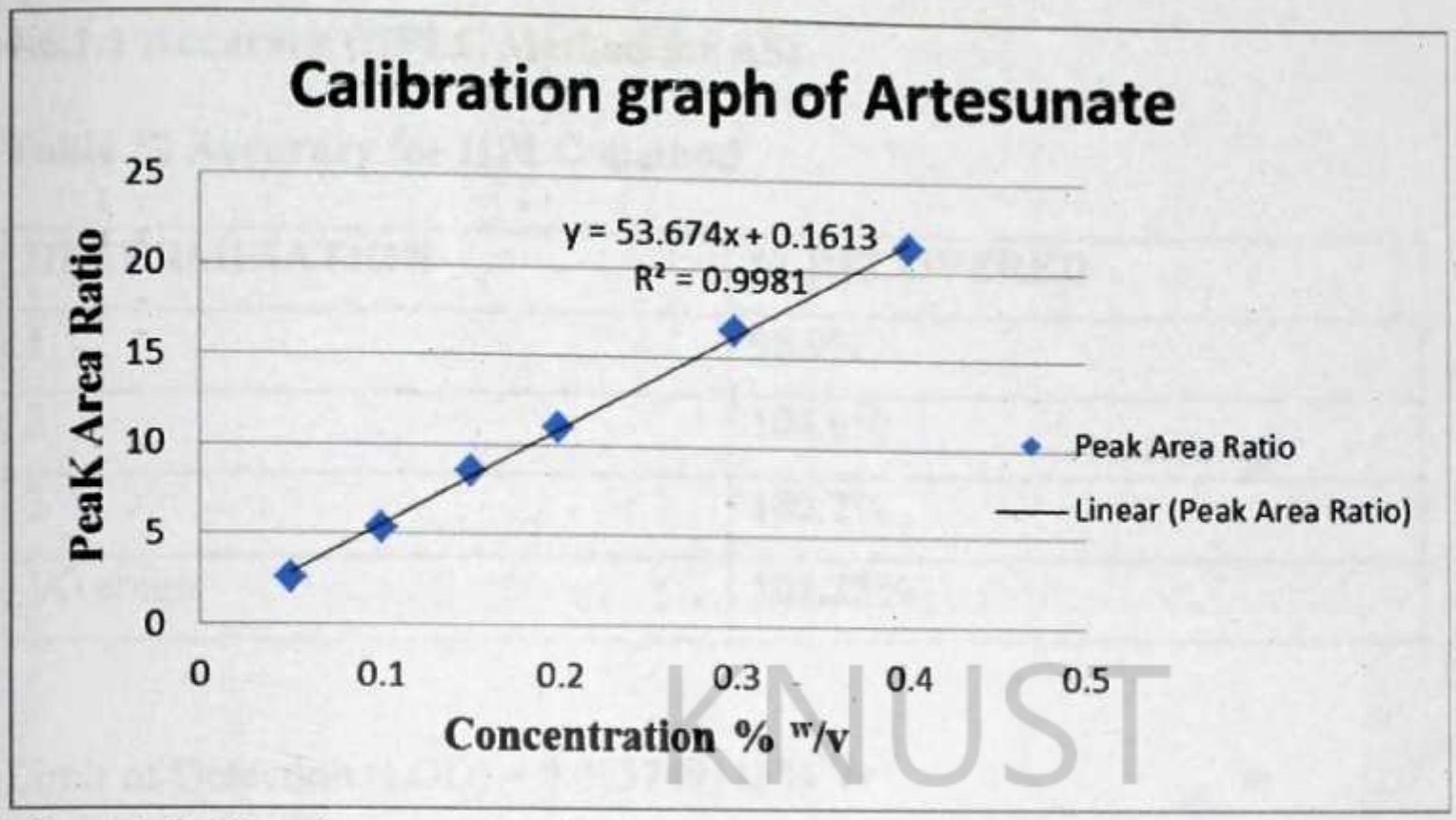


Fig. 14 Calibration graph of AS (HPLC METHOD)

Range= 0.05%^{w}/v- 0.4%^{w}/v

Sample Calculation

The equation of a line is y = mx + c,

Where;

y = Peak volume Ratio, m = Slope of Calibration Curve, x = Concentration,

c = y - intercept

From the graph,

y=53.674x + 0.1613

x= y- 0.1613 / 53.674

For y = 14.64, = 14.64- 0.1613/ 53.674

X =0.26975258

% Content = (0.266387227 / 0.269296797) × 100

= **100.17%^{w}/v**

4.6.1.1 Accuracy (HPLC Method for AS)

Table 23 Accuracy for HPLC method

DETERMINATION	% RECOVERED
1	98.9%
2	104.6%
3	100.2%
Average	**101.23%**

Limit of Detection (LOD) = **0.003749113%^{w}/v**

Limit of Quantification (LOQ) = **0.01136095%^{w}/v**

4.6.1.2 Precision

Repeatability (Intraday)

Table 24 Repeatability for HPLC method

Determination	% Recovery	
1	98.40	
2	100.96	
3	100.64	
4	99.20	RSD = 1.18
5	98.56	
6	100.8	

Table 25 Inter day precision for AS (HPLC method)

Concentration %w/v	% Recovery	RSD
1	100.96	1.34
2	98.56	
3	100.8	
4	100.96	0.94
5	100.64	
6	99.2	
7	100.8	1.23
8	100.18	
9	98.43	

RSD = 1.04

4.6.1.3 Robustness

Table 26 Robustness- Flow rate (HPLC Assay of AS)

Flow rate (ml/min)	%Recovery
1.20	96.08
1.40	101.9

4.6.2 DIHYDROARTEMISININ

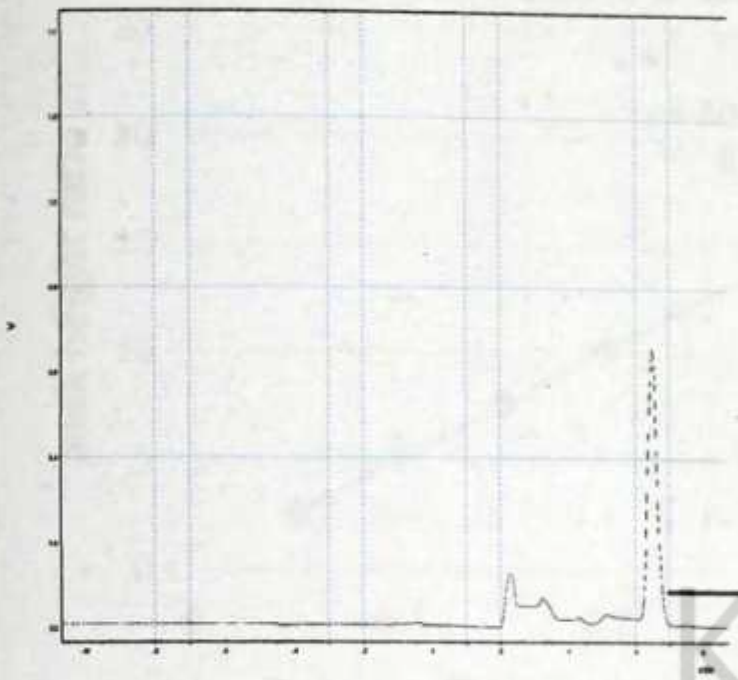


Fig. 15 Chromatogram of DHA (HPLC method)

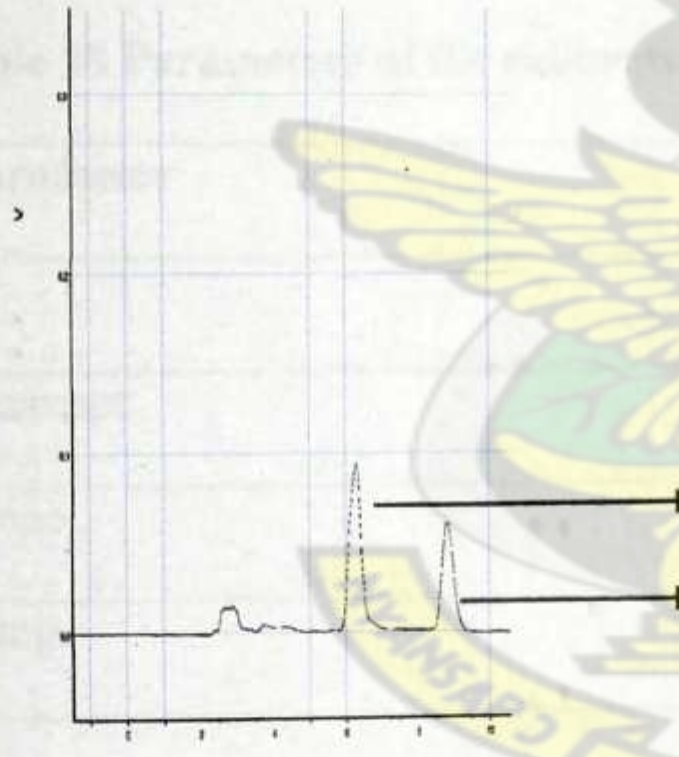


Fig. 16 Chromatogram of AS and DHA mixed together (HPLC method)

Table 27 Parameters of Chromatograms (HPLC Assay of DHA)

PARAMETER	VALUE
Retention time	5.13 ± 0.26

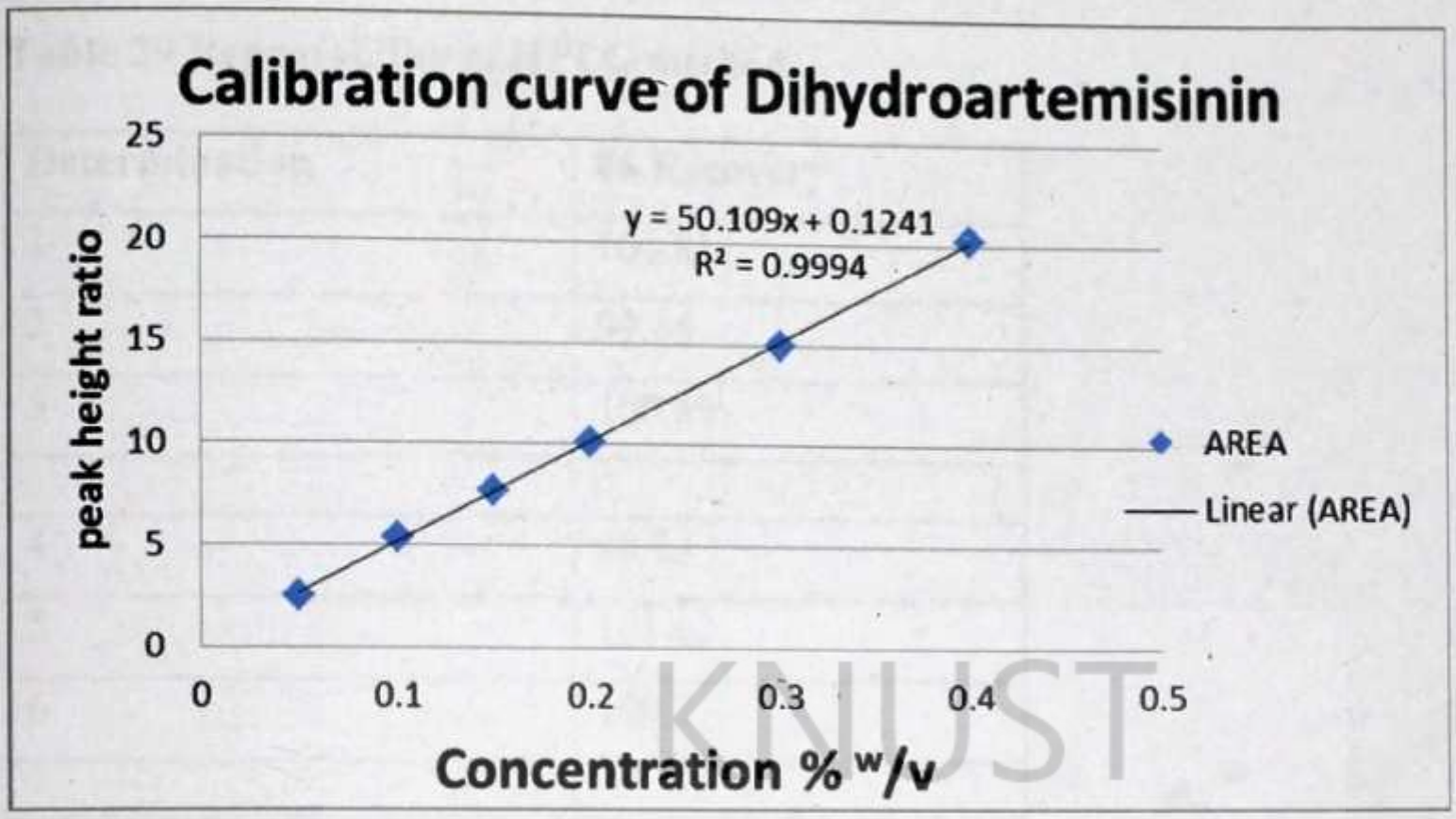


Fig. 17 Calibration graph of DHA (HPLC method)

Table 28 Parameters of the calibration curve

Parameter	Value
R^2	0.9994
Intercept	0.1241
Slope	50.109
Range	0.05%^{w}/v- 0.4%^{w}/v

Limit of Detection (LOD) = **0.004235491%^{w}/v**

Limit of Quantitation (LOQ) = **0.01283482%^{w}/v**

Table 29 Repeatability of HPLC method

Determination	% Recovery
1	101.81
2	99.86
3	100.69
4	98.73
5	101.62
6	100.2
7	98.59
8	101.64
9	99.84
RSD	**1.21**

4.6.3 ASSAY OF TABLETS

Table 30 Assay of Brands of AS Tablets

BRAND	AS (HPLC ANALYSIS)	DHA (HPLC ANALYSIS)	AS (HPLC STANDARD METHOD)
T_1	72.86 ± 6.76	21.35 ± 0.35	70.82 ± 0.19
T_2	97.95 ± 7.71	-	100.34 ± 1.74
T_3	102.26 ± 3.87	0.71± 0.35	96.23 ± 3.87
T_4	95.13 ± 4.71	0.06	97.50 ± 1.17
T_5	98.68± 3.49	5.40 ± 1.22	100.69 ± 2.56

Chapter 5

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

5.1.1 IDENTIFICATION TEST FOR REFERENCE SAMPLES

5.1.1.1 Artesunate

Identification tests are essential to verify the quality and identity of drug or product obtained. It is therefore essential to ascertain their authenticity, purity and quality before use. The melting point observed for Artesunate reference sample was 132-134 and 132-135°C for the 1st and 2nd determinations respectively. This agrees with the melting point range of 132-135°C stated in the international pharmacopoeia (IP). Identification by colour reactions also produced a reddish-brown colour upon addition of 1 drop of vanillin/Sulfuric acid solution which also indicates that the sample is Artesunate. TLC for the reference sample revealed only one principal spot giving an indication that the sample used was pure. Solubility tests done on Artesunate reference sample also agreed with the requirement of the international pharmacopoeia. The sample was very soluble in dichloromethane, freely soluble in acetone and ethanol and sparingly soluble in water.

5.1.1.2 Dihydroartemisinin

Identification of Dihydroartemisinin reference sample was done using the IP method which involves TLC and colour reactions. The presence of only one spot on the TLC plate and the violet colour produced instantly upon the addition of 1ml of KI, 2.5ml of

sulphuric acid and 4 drops of starch to DHA dissolved in dehydrated ethanol shows that the sample was DHA and that the sample was pure.

5.1.2 PHARMACOPOEIAL TESTS

5.1.2.1 Uniformity of weight

According to the international pharmacopoeia not more than 18 of the twenty tablets weighed should deviate by 5% and not more than two of the tablets should deviate by 10% for a tablet of mass greater than 250mg **(IP, 2006)**. Based on the stated conditions all the brands of tablets analysed passed the uniformity of weight test.

5.1.2.2 Tablet disintegration test

The requirement of disintegration is that all the six tablets disintegrate within 15 minutes at a temperature of 37 ^{0}C ± 2. If 1 or 2 dosage units fail to disintegrate, the test should be repeated on 12 additional dosage units **(BP, 2007)**. The average disintegration times for T_1, T_2, T_3, T_4, T_5, and T_6 were 8.0, 0.14, 2.1, 6.57, 13.6, and 1.35 minutes respectively. This implies that all the brands of Artesunate tablets used passed the tablet disintegration test. The difference in the disintegration time is probably due to the differences in the manufacturing conditions and the different excipients used.

5.1.3 ASSAY OF ARTESUNATE PURE SAMPLE BY TITRATION

The percentage purity obtained for Artesunate reference sample were 100.32%^{w}/w and 99.54%^{w}/w for two determinations. The mean percentage purity of the Artesunate reference sample is 99.93 ± 0.55. The international pharmacopoeia requirement for

Artesunate reference sample assayed by method of titration is 99-101 %^{w}/w **(IP, 2006)**. This implies that the reference sample used for the analysis met stated requirements.

5.1.4 ASSAY OF TABLETS BY TITRATION

The percentage content obtained for tablets labelled T_1, T_2, T_3, T_4, and T_5 was 125.22, 97.64, 103.31, 102.63, 99.69 %^{w}/w respectively. Tablets T_6 gave burette readings similar to that of the blank sample used during the titration. For Artesunate tablets to meet requirement, it should contain not less than 90.0% and not more than 110.0%. From the results obtained it could be inferred that tablets T_2, T_3, T_4, and T_5 passed. However, tablets T_1 and T_6 did not meet the requirements. Results obtained from tablet T_6 were similar to that obtained for the blank samples and this implies that the tablet may probably not contain Artesunate. For tablet T_1, it could be due to the breakdown of Artesunate to succinic acid which has two hydroxyl ends with the capability of reacting with NaOH hence increasing the percentage content. Artesunate has high chemical instability which causes breakdown to DHA and succinic acid. These breakdown products may be responsible for the high percentage content obtained.

5.1.5 THIN LAYER CHROMATOGRAPHY OF ARTESUNATE TABLETS

Thin layer chromatography was performed on the tablets in comparison with the Artesunate reference sample to ascertain whether the tablets purchased contained Artesunate. The results revealed the presence of a second spot in the tablet. An investigation of the second spot became necessary. The second spot was compared to other compounds such as Artemether, and DHA and it was realized that the second spot was comparable in appearance and R_f value to DHA. The mobile phase system used

comprised of ethyl acetate, acetone and glacial acetic acid (18: 4: 0.1). Tablets T_2, T_3, T_4, showed only one principal spot which was similar in appearance and R_f to the spot of Artesunate reference sample. Tablets T_1 and T_5 showed two spots each with the first spot being comparable to that of Artesunate and the second spot was similar to that of DHA. This may be due to the fact that the two tablets may have broken down and therefore contain DHA. Tablet T_6 showed no principal spot on the TLC plate, this may be due to the fact that it may not contain any Artesunate. The R_f values of T_1, are 0.67 and 0.78 and T_5 are 0.68 and 0.82 whilst the R_{fs} of tablets T_2, T_3, and T_4 are 0.67, 0.70, and 0.69 respectively.

5.1.6 UV SPECTROPHOTOMETRIC METHOD DEVELOPMENT

The Artemisinins generally lack chromophores which makes it difficult for UV spectrophotometric analysis due to its low wavelength of absorption with a lot of interferences. However, the international pharmacopoeia makes use of HCl in the assay of Artemether to form an HCl decomposition product which is described as an α β unsaturated decalone. This absorbs at a wavelength of 254nm and NaOH decomposition product in the assay of DHA. This concept served as the basis for the UV method development. From the results obtained, addition of 2ml of concentrated HCl to 1ml methanolic solution of Artesunate was chosen for the analysis. This is because addition of 2ml of concentrated HCl to 1ml methanolic solution of Artesunate at room temperature resulted in the formation of a stable HCl decomposition product after about 25 minutes and the complex formed was stable thereafter. In the addition of 1ml of concentrated HCl to the Artesunate solution, it was observed that although the complex

was formed after 25 minutes, the absorbance readings obtained were not constant even up to the 50th minute. Addition of 3ml and 4ml of concentrated HCl resulted in the formation of the complex at an earlier time and maximum absorption occurred after 15 minutes. However, the absorbance readings obtained were lower compared to that of the addition of 2ml of HCl. Addition of 2ml of the Artesunate solution to 1ml of concentrated HCl produced very low absorbance readings and reached a maximum after 50 minutes. Addition of 2ml and 3ml of concentrated HCl to 2ml of Artesunate solution resulted in the formation of the complex after 15 minutes, however, the absorbance readings increased even up to the 40th minute. This is because the reaction was still in progress. Addition of 2ml of concentrated HCl to 1ml of Artesunate solution was therefore used for calibration and analysis.

5.1.7 VALIDATION OF UV METHOD

The R^2 obtained from the calibration curve was 0.9994 within the range of 0.00075%^{w}/v -0.003%^{w}/v. The ICH guidelines on validation of analytical method recommend that R^2 should be at least 0.995 for a method to be considered linear. The results obtained reflect that a linear relationship exists between concentrations of Artesunate prepared and the absorbance readings recorded within the stated range at 254nm.

The percentage recovery obtained from a mixture of excipients and Artesunate were 99.0%, 100.34% and 99.56% for concentration of 0.002%^{w}/v, 0.0015%^{w}/v and 0.001%^{w}/v respectively. The relative standard deviations (RSD) obtained were 0.74, 0.75 and 1.15 respectively. The method may be considered to be accurate since the RSD was less than 2.

The RSD for the intraday precision was 0.81 whereas that of inter day precision was 0.77. The method designed could be said to have a good precision because the RSD obtained was less than 2.

The LOD and the LOQ were 0.0000729%^{w}/v, and0.000221%^{w}/v respectively.

The method also could be said to have a good specificity. This is because when the method was applied to the tablet excipients no absorbance reading was observed at 254nm whereas the excipients initially showed absorbance at 216nm, the wavelength of absorbance of Artesunate.

5.1.8 HPLC METHOD DEVELOPMENT

The mobile phase systems investigated were numerous, but it was observed that Acetonitrile and 0.1 % TFA (80: 20) and Acetonitrile: 0.05 glacial acetic acid (60:40) could elute Artesunate at 220nm using a UV detector. However, methanol was chosen as a suitable alternative in the method development because it is cheaper and hence makes the method cost effective.

The focus of the method development was to elute Artesunate and its major breakdown product DHA simultaneously. This was essential to make the assay of Artesunate and its major breakdown product easier and faster since the mobile phase systems and other chromatographic conditions are the same and therefore a single injection of an Artesunate tablet solution could give an indication and the extent of breakdown. This was made possible since Artesunate and DHA have the same wavelength of absorption and was both soluble in methanol.

5.1.8.1 Chromatographic conditions

The column chosen was C-18 and the wavelength of absorption was 220nm using a UV detector. Several mobile phase additives were tried including phosphate buffer (pH 3.0), different concentrations of glacial acetic acid and Triflouroacetic acid (TFA) for both Artesunate and DHA. The phosphate buffer and glacial acetic acid produced well resolved peaks for Artesunate but the peaks of Dihydroartemisinin were tailing and not well resolved. Trifluoroacetic acid was selected because of its excellent solvating and ion pairing characteristics. It produced sharper peaks and resolution was increased.

Several concentrations of TFA were analysed starting from 0.1%^{w}/v within which Artesunate produced excellent peaks whereas DHA tailed. Reducing the concentration of TFA favoured DHA and suitable adjustments were made to obtain well resolved peaks for both. DHA initially produced two resolved peaks due to inter conversion to its α and β forms. Increasing the flow rate however, resulted in the generation of a single peak for DHA. This is based on the fact that the rate of inter conversion is dependent on the pH as well as the rate of the chromatographic process such that if the rate of inter conversion is slower than the chromatographic process then a single peak is obtained.

The mobile phase system suitable for the elution of Artesunate and DHA was found to be Methanol and 0.006% TFA in the ratios of (80:20) with a flow rate of 1.5ml/min.

5.1.8.2 HPLC analysis of Artesunate

The suitable HPLC conditions were employed in the analysis of Artesunate. The Artesunate pure sample was dissolved in methanol throughout the analysis. The retention time for Artesunate was found to be 3.74 ± 0.06 min.

Validation of analytical method

Linearity of the method was determined from the calibration graph. The R^2 value obtained from the calibration graph was 0.9981. This gives an indication of a good linearity within the range of 0.05%^{w}/v- 0.4%^{w}/v.

There was an average percentage recovery of 101.23% when Artesunate reference sample was mixed with tablet excipients and analyzed which conforms to the ICH requirement of 98- 102%.

The RSD of the intraday and inter day precision for Artesunate reference sample were 1.18 and1.04 respectively. Since the RSD in both instances is less than 2%, a good precision was therefore observed.

The method could be said to be specific to Artesunate analysis since tablet excipients although was observed in the chromatograms did not interfere with the analysis of Artesunate.

For robustness of the method, there were no significant differences in the results obtained when variations were made in the flow rate from 1.0 to 1.5ml/min in addition

the method is also robust for variations in mobile phase such as 10% decrease or increase in mobile phase percentage in the analysis of Artesunate.

The LOD and LOQ for Artesunate were found to be 0.003749113%^{w}/v and 0.01136095%^{w}/v respectively. The LOD and LOQ obtained from the calibration graph of Artesunate were high in value and this could be accounted for by the lack of a chromophoric system needed for absorption in the UV region and therefore higher concentrations were needed for injections thereby limiting the detection and quantification limits.

5.1.8.3 HPLC analysis of Dihydroartemisinin

In HPLC analysis of DHA, numerous considerations were made to arrive at the mobile phase system and other chromatographic conditions used in the HPLC analysis. Better resolution was observed with a decrease in the concentration of TFA during the method development. The mobile phase system that was chosen due to its suitability was Methanol and 0.006 TFA in the ratios of (80:20) %^{w}/w at a wavelength of 220nm and a flow rate of 1.5ml/min. The retention time of DHA was found to be 5.13 ± 0.26 minutes.

Validation of analytical method

The ICH guidelines on validation of analytical method recommend that R^2 should be at least 0.995 for a method to be considered linear. The R^2 obtained from the calibration curve of DHA was 0.9994 within the range of 0.05%^{w}/v- 0.4%^{w}/v. This conforms to the requirement of ICH guidelines and the method could be said to demonstrate a good linearity.

In the determination of repeatability of the analytical method for DHA an RSD of 1.21 was obtained for three different concentrations repeated three times. Since the RSD obtained is less than 2.0 the method could be said to be precise.

The method could be specific since the excipients of DHA did not interfere in the analysis of DHA. The elution of the excipients occurred at earlier time than DHA. The retention time of DHA was 5.13 ± 0.26 minutes and therefore there was no interference. In addition injection of the tablet excipients alone did not resolve in a peak at the retention time of DHA.

The LOD and LOQ of DHA were 0.004235491%^{w}/v and 0.01283482%^{w}/v respectively. The high values of LOD and LOQ values obtained are characteristic of the Artemisinins since they lack chromophores necessary for ultraviolet absorption.

5.1.8.4 HPLC analysis of Artesunate tablets

The method developed was employed in the analysis of Artesunate tablets obtained from the market and the percentage content of Artesunate for tablets labelled T_1, T_2, T_3, T_4 and T_5 were **72.86 ± 6.76, 97.95 ± 7.71, 102.26 ± 3.87, 95.13 ± 4.71** and **98.68± 3.49** respectively. From the results stated it could be inferred that tablet T_1 did not pass since it did fall within the stated range of the IP. The other tablets conformed to the stated range of the IP.

In the analysis of Artesunate tablets for the presence and content of DHA, it was observed that four of the commercial samples had DHA present in it whilst only one did not show the presence of DHA in the tablet. The percentage content of DHA found in

the tablets were **21.35 ± 0.35, 5.40 ± 1.22, 0.06, 0.71± 0.35** for tablets T_1, T_3, T_4 and T_5 respectively. Tablet T_2 however, did not show the presence of DHA. This implies that most of the tablets analysed showed the presence of DHA in the tablets as compared to the TLC method which showed the presence of DHA in two of the brands analysed. However, it must be noted Tablet T_1 was six months close to its expiry date at the time of preliminary analysis but expired three months before the HPLC analysis was performed. It was used as a case study to monitor the breakdown of Artesunate towards the expiry date and therefore was the reason for the high content of DHA in the tablet T_1.

Comparison of the results of the titrimetric method to the HPLC standard method reveals that the titrimetric method has limitations since it produced high percentage content in the event of breakdown.

5.1.9 COMPARISON OF HPLC METHOD DEVELOPED TO THE STANDARD METHOD (USP SALMOUS STANDARD)

Analysis of the results obtained for the assay of Artesunate statistically revealed that the calculated value of F was 1.15 and the critical value of F was 9.605. The calculated value is less than the expected, so there is no significant difference between the variances at the 5% level for the HPLC method developed and the Standard HPLC method which presupposes that the t test could be applied. The theoretical value for t for 8 degrees of freedom at 95% confidence interval is 2.306 and the calculated value of t was 0.702. Since the theoretical value of t is greater than the calculated value of t at 95% confidence interval, it implies that the HPLC method developed does not differ

significantly from the standard HPLC method in the USP SALMOUS standard for the analysis of Artesunate.

5.2 CONCLUSION

5.2.1 IDENTIFICATION TEST

Identification tests performed revealed that the pure samples used for the analysis were indeed Artesunate pure sample and DHA pure sample.

5.2.2 PHARMACOPOEIAL TEST

All the tablets passed the uniformity of weight test and the tablet disintegration test.

5.2.3 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography confirmed that five of the brands of Artesunate obtained contained Artesunate whilst one brand did not contain Artesunate. The TLC method gave an indication of the presence of DHA in two brands of Artesunate tablets purchased namely tablets T_1 and T_5 respectively.

5.2.4 ASSAY OF THE ARTESUNATE TABLETS BY TITRATION

The percentage content obtained for tablets labelled T_1, T_2, T_3, T_4, and T_5, **125.22, 97.64, 103.31, 102.63, 99.69 %^{w}/w** respectively. This implies that T_2, T_3, T_4, and T_5 passed the test based on the International pharmacopoeias requirement whilst tablets T_1 and T_6 failed the test.

5.2.5 UV METHOD DEVELOPMENT

The R^2 obtained from the calibration curve was 0.9994 within the range of **0.00075%^{w}/v -0.003%^{w}/v**. The LOD and the LOQ were **0.0000729%^{w}/v**, and **0.000221%^{w}/v** respectively.

5.2.6 HPLC METHOD DEVELOPMENT FOR ARTESUNATE AND DIHYDROARTEMISININ

The mobile phase system suitable for simultaneous elution of Artesunate and Dihydroartemisinin was Methanol and 0.006% ^{w}/v Triflouroacetic acid in the ratios of 80:20. The R^2 obtained from the calibration graph of Artesunate and Dihydroartemisin were 0.9981 and 0.9994 respectively. The LOD and LOQ for Artesunate were **0.003749113%^{w}/v** and **0.01136095%^{w}/v** whereas the LOD and LOQ for Dihydroartemisinin were **0.004235491%^{w}/v** and **0.01283482%^{w}/v**. The HPLC method proved to be suitable for the assay of Artesunate and Dihydroartemisinin simultaneously and therefore could be used to assay Artesunate commercial samples for the presence and extent of breakdown of Artesunate to Dihydroartemisinin.

5.2.6.1 Assay of Artesunate Tablets by HPLC method

The percentage content of Artesunate in tablet T_1, T_2, T_3, T_4 and T_5 were **72.86 ± 6.76, 97.95 ± 7.71, 102.26 ± 3.87, 95.13 ± 4.71**, and **98.68± 3.49** %^{w}/v respectively. The percentage content of Dihydroartemisin in tablet T_1, T_3, T_4 and T_5 were **21.35 ± 0.35, 4.95 ± 1.22, 0.06** and **0.71 ± 0.35%^{w}/v** respectively. Tablet T_2, however, gave no peak for Dihydroartemisinin. Four of the commercial brands of Artesunate analysed contained

the required amount of Artesunate stated in the International pharmacopoeia. One of the commercial samples contained a quantity that was lesser than the stated amount. Tablet T_6 however, was not analysed by the HPLC method developed since the TLC and the titrimetric method indicated the absence of Artesunate in the tablet. Four of the commercial samples showed the presence of DHA in the tablet at different concentrations. The results indicate that Artesunate tablet may breakdown to DHA during its shelf-life.

5.3 RECOMMENDATIONS

Further stability studies should be done on Artesunate Commercial samples to determine the stability profile of the drugs throughout their shelf-life.

The research should be extended to more of the brands of Artesunate on the local market to monitor their stability.

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APPENDIX

PHARMACOPEIAL TESTS

Table 31 Uniformity of weight for tablet T_1

Sample	Weight (g) (x)	Deviation (x-t)	% Deviation (x-t)/t *100
1	0.3148	0.0087	2.8422
2	0.3032	-0.0029	-0.9474
3	0.3188	0.0127	4.1490
4	0.3156	0.0095	3.1036
5	0.3194	0.0133	4.3450
6	0.2943	-0.0118	-3.8549
7	0.2989	-0.0072	-2.3522
8	0.3024	-0.0037	-1.2088
9	0.3208	0.0147	4.8024
10	0.3051	-0.001	-0.3267
11	0.3014	-0.0047	-1.5354
12	0.2959	-0.0102	-3.3322
13	0.3074	0.0013	0.4247
14	0.2960	-0.0101	-3.2996
15	0.3058	-0.0003	-0.0980
16	0.2931	-0.013	-4.2470
17	0.2976	-0.0085	-2.7769
18	0.3112	0.0051	1.6661
19	0.2973	-0.0088	-2.8749
20	0.3220	0.0159	5.1944

Weight of twenty tablets = 6.121g

Average weight of twenty tablet = 0.3061g

Table 32 Uniformity of Weight Test for Tablet T_2

Sample	Weight (g)	Deviation (x-t)	% Deviation
1	0.2656	-0.0041	-1.5202
2	0.2697	0	0
3	0.2716	0.0019	0.7045
4	0.2681	-0.0016	-0.5933
5	0.267	-0.0027	-1.0011
6	0.266	-0.0037	-1.3719
7	0.2675	-0.0022	-0.8157
8	0.2684	-0.0013	-0.4820
9	0.2701	0.0004	0.1483
10	0.2704	0.0007	0.2595
11	0.2678	-0.0019	-0.7045
12	0.2788	0.0091	3.3741
13	0.2696	-0.0001	-0.0371
14	0.2725	0.0028	1.0382
15	0.2747	0.005	1.8539
16	0.2691	-0.0006	-0.2225
17	0.2732	0.0035	1.2977
18	0.2673	-0.0024	-0.8899
19	0.2683	-0.0014	-0.5191
20	0.269	-0.0007	-0.2595

Weight of twenty tablets = **5.3947g**

Average weight of twenty tablet = **0.2697g**

Table 33 Uniformity of Weight for tablet T_3

SAMPLE	Weight (G)	Deviation	% Deviation
1	0.2923	-0.0002	-0.0685
2	0.2961	-0.004	-1.3694
3	0.2867	0.005	1.7117
4	0.2918	0.0003	0.1027
5	0.2907	0.0014	0.4792
6	0.2882	0.0039	1.3352
7	0.2949	-0.0028	-0.9585
8	0.2929	-0.0008	-0.2739
9	0.2909	0.0012	0.4108
10	0.2941	-0.002	-0.6847
11	0.2882	0.0039	1.3352
12	0.2918	0.0003	0.1027
13	0.2825	0.0096	3.2865
14	0.2870	0.0051	1.7459
15	0.2906	0.0015	0.5135
16	0.3043	-0.0122	-4.1767
17	0.2830	0.0091	3.1154
18	0.2995	-0.0074	-2.5333
19	0.2968	-0.0047	-1.6090
20	0.2975	-0.0054	-1.8487

Weight of twenty tablet = **5.8416g**

Average weight of twenty tablets = **0.2921g**

Table 34 Uniformity of weight for tablet T_4

Uniformity of weight

SAMPLE	WEIGHT (G) (X)	DEVIATION (x-t)	% DEVIATION
1	0.5824	0.001	0.1720
2	0.5914	0.010	1.720
3	0.5820	0.0006	0.1032
4	0.5821	0.0007	0.1204
5	0.6051	0.0237	4.0764
6	0.5716	-0.0098	-1.6856
7	0.5819	0.0005	0.0856
8	0.5828	0.0014	0.2408
9	0.5816	0.0002	0.0344
10	0.5795	-0.0019	-0.3268
11	0.5702	-0.0112	-1.9264
12	0.5768	-0.0046	-0.7912
13	0.5828	0.0014	0.2408
14	0.5826	0.0012	0.2064
15	0.5796	-0.0018	-0.3096
16	0.5787	-0.0027	-0.4644
17	0.5823	0.0009	0.1548
18	0.5715	-0.0099	-1.7028
19	0.5819	0.0005	0.0860
20	0.5810	-0.0004	-0.0688

Weight of twenty tablets = **11.6278g**

Average weight of twenty tablet = **0.5814g**

Table 35 Uniformity of weight for tablet T_5

SAMPLE	WEIGHT (G) (X)	DEVIATION (x-t)	DEVIATION	% DEVIATION
1	0.2077	-0.0007	-0.0034	-0.3359
2	0.2069	-0.0015	-0.0072	-0.7198
3	0.2089	0.0005	0.0024	0.2399
4	0.2062	-0.0022	-0.0106	-1.056
5	0.2112	0.0028	0.0134	1.3436
6	0.2071	-0.0013	-0.0062	-0.6238
7	0.2027	-0.0057	-0.0274	-2.7351
8	0.2076	-0.0008	-0.0038	-0.3839
9	0.2099	0.0015	0.0072	0.7198
10	0.2078	-0.0006	-0.0029	-0.2879
11	0.213	0.0046	0.0221	2.2073
12	0.2108	0.0024	0.0115	1.1516
13	0.2136	0.0052	0.0250	2.4952
14	0.2071	-0.0013	-0.0062	-0.6238
15	0.207	-0.0014	-0.0067	-0.6718
16	0.2082	-0.0002	-0.0010	-0.0960
17	0.212	0.0036	0.0172	1.7274
18	0.2033	-0.0051	-0.0245	-2.4472
19	0.209	0.0006	0.0029	0.2879
20	0.207	-0.0014	-0.0067	-0.6718

Weight of twenty tablets = **4.1670g**

Average weight of twenty tablet =**0.2084g**

UV method development

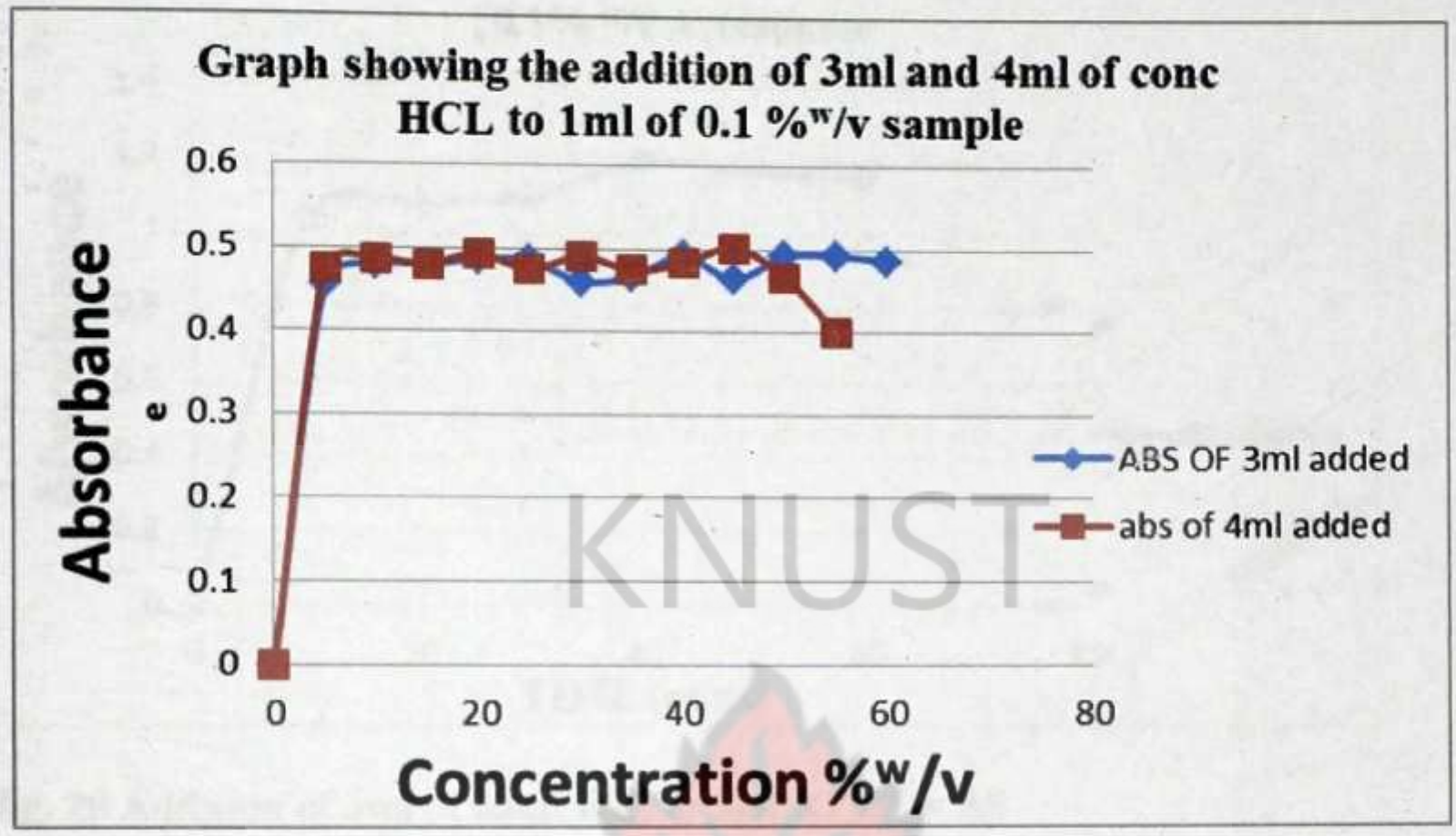

Fig. 18 Addition of 1ml and 2ml of conc. HCl to 0.1%^{w}/v AS

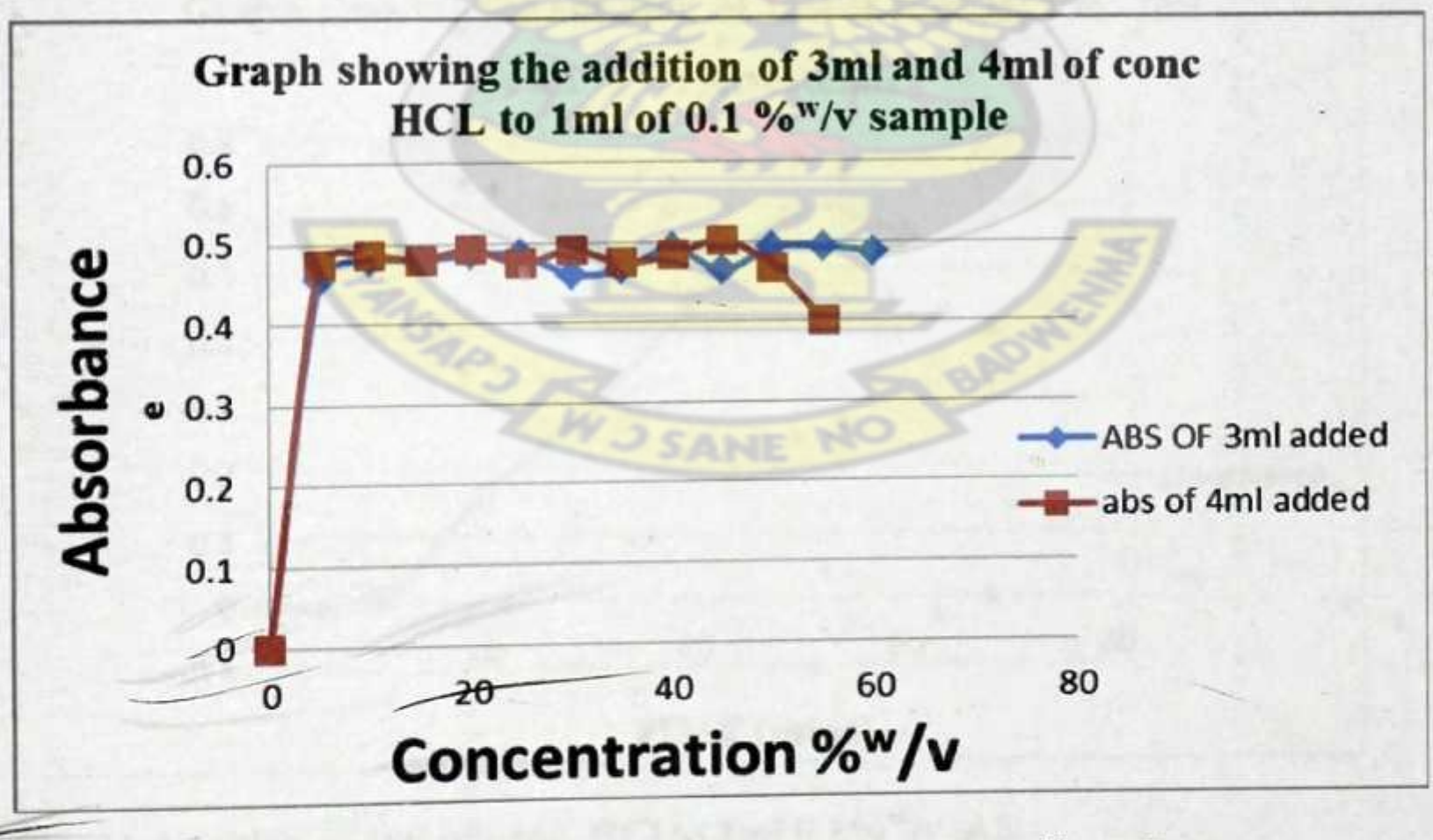

Fig. 19 Addition of 3ml and 4ml of conc. HCl to 1ml 0.1%^{w}/v AS

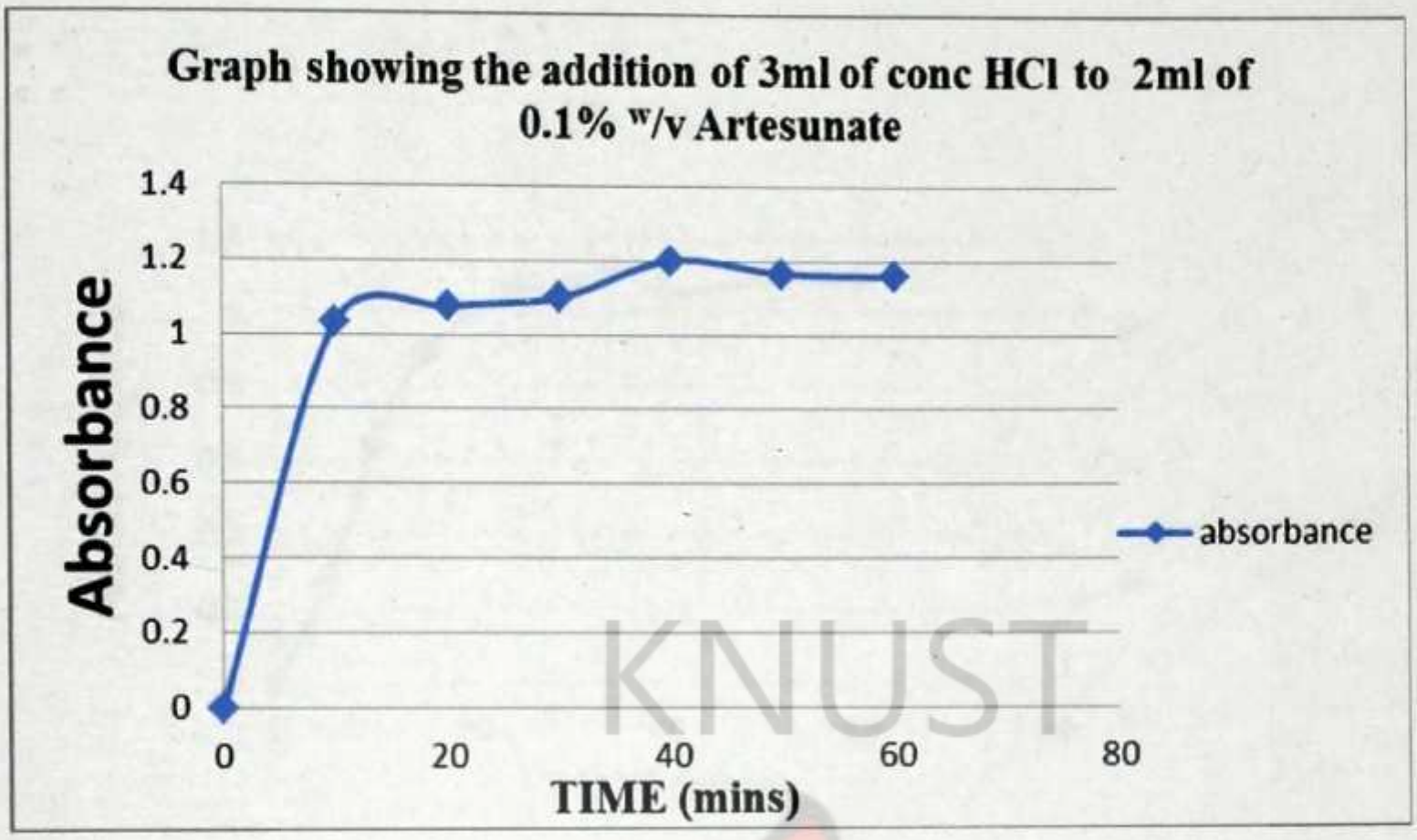

Fig. 20 Addition of 3ml of conc. HCl to 2ml 0.1%^{w}/v AS

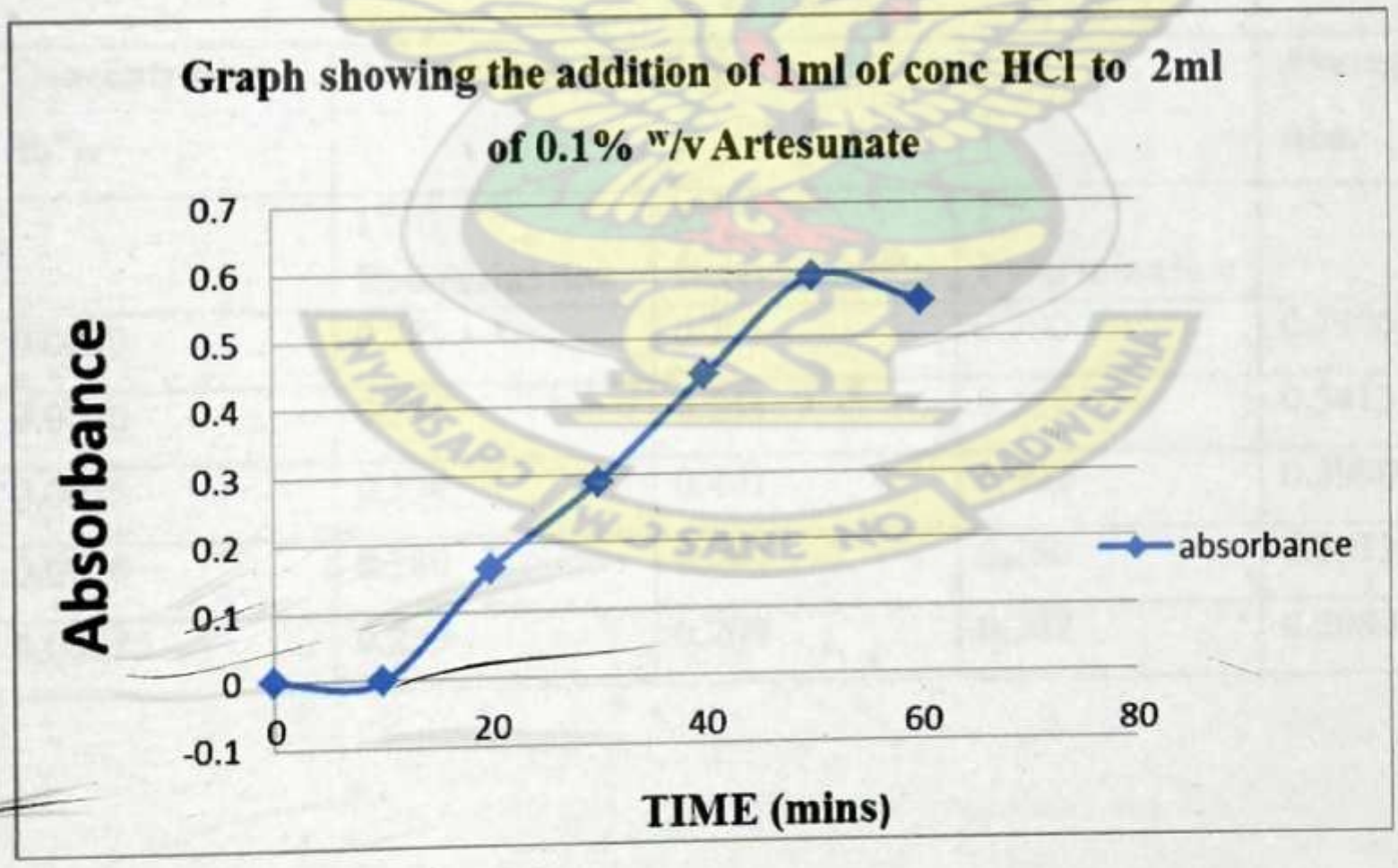

Fig. 21 Addition of 1ml of conc. HCl to 2ml 0.1%^{w}/v AS

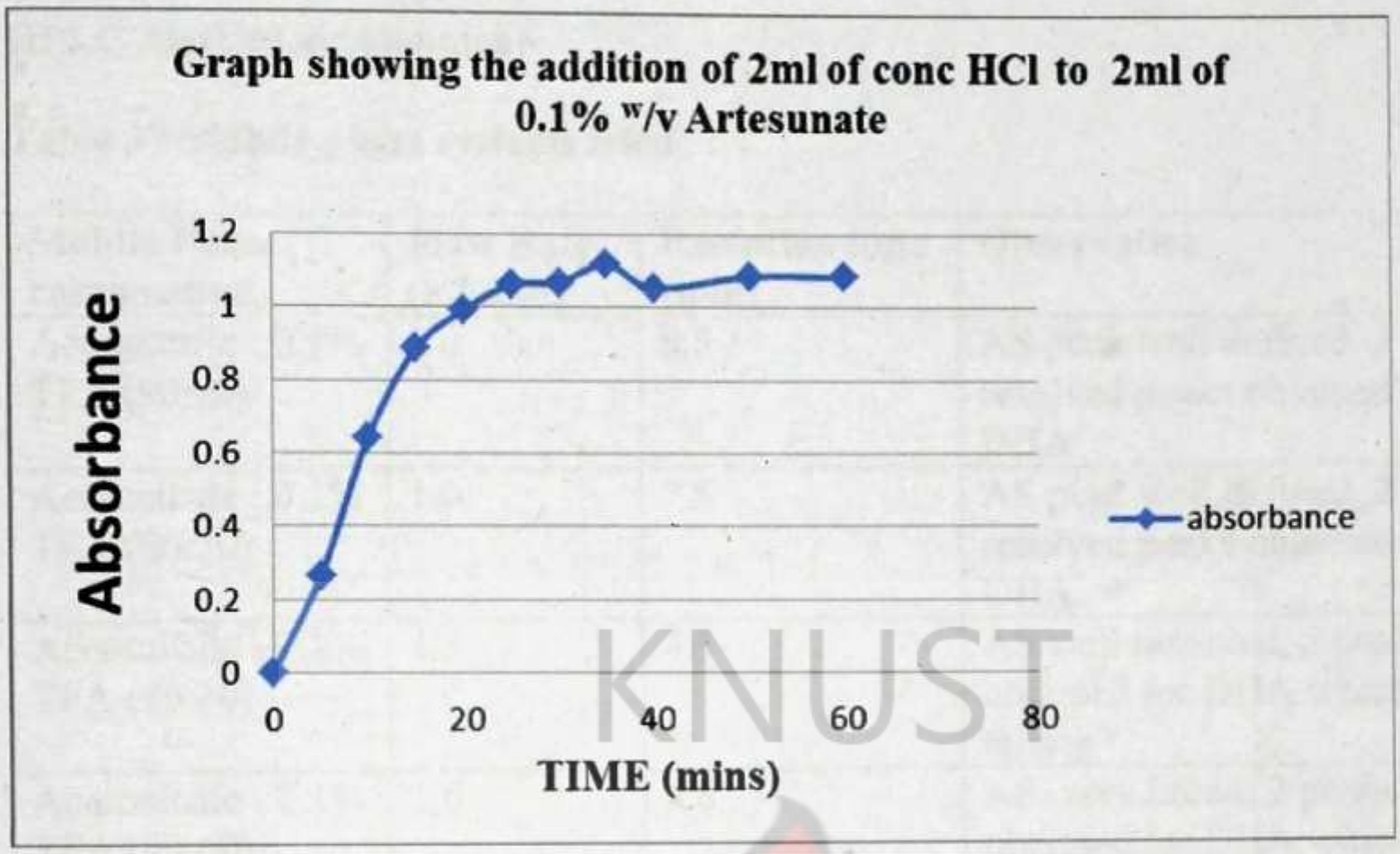

Fig. 22 Addition of 2ml of conc. HCl to 2ml 0.1%^{w}/v AS

Table 36 UV results for the calibration curve of Artesunate at 254nm

Concentration %^{w}/v	Absorbance Readings			Average Abs.
	1st Determination	2nd Determination	2nd Determination	
0.0030	0.789	0.793	0.790	0.7906
0.0020	0.546	0.541	0.537	0.5413
0.0015	0.394	0.401	0.402	0.3990
0.0010	0.280	0.284	0.280	0.2813
0.00075	0.209	0.208	0.207	0.2080

HPLC Method development

Table 37 Mobile phase systems tried

Mobile Phase composition	Flow Rate (ml/min)	Retention time (min)	Observation
Acetonitrile : 0.1% TFA (90:10)	1.0	6.5	AS peak well defined, 2 well resolved peaks obtained for DHA
Acetonitrile : 0.1% TFA (80:20)	1.0	7.5	AS peak well defined, 2 well resolved peaks obtained for DHA
Acetonitrile : 0.1% TFA (70:30)	1.5	4.5	AS well resolved, 2 peaks obtained for DHA which were tailing
Acetonitrile : 0.1% TFA (40:60)	1.0	8.8	AS very broad, 2 peaks obtained for DHA which were tailing
Acetonitrile : 0.1% glacial acetic acid (60: 40)	1.0	6.5	AS peak well defined, 2 un resolved peaks obtained for DHA
Methanol: : 0.1% TFA (60:40)	1.0	6.5	AS unresolved, 2 peaks obtained for DHA which were unresolved
Methanol: : 0.1% TFA (70:30)	1.0	5.5	AS peaks well resolved, DHA peaks unresolved
Methanol: : 0.1% TFA (80:20)	1.0	5.5	AS peaks well resolved, DHA peaks unresolved
Methanol: : 0.05% TFA (80:20)	1.0	5.5	AS peaks well resolved, 2 DHA peaks unresolved
Methanol: : 0.005% TFA (80:20)	1.5	4.2	AS peaks tailing, DHA peak (one peak) well resolved

HPLC calibration curves

Table 38 Results for the Calibration curve of Artesunate

CONC % $^{W}/V$	Average Peak Area Ratio
0.4	21.28
0.3	16.56
0.2	11.02

0.15	8.58
0.1	5.393
0.05	2.543

Table 39 Results for the Calibration curve of DHA

CONC % W/V	AVERAGE PEAK AREA RATIO
0.4	20.29
0.3	14.95
0.2	10.09
0.15	7.71
0.1	5.34
0.05	2.495

HPLC Chromatograms of Commercial samples

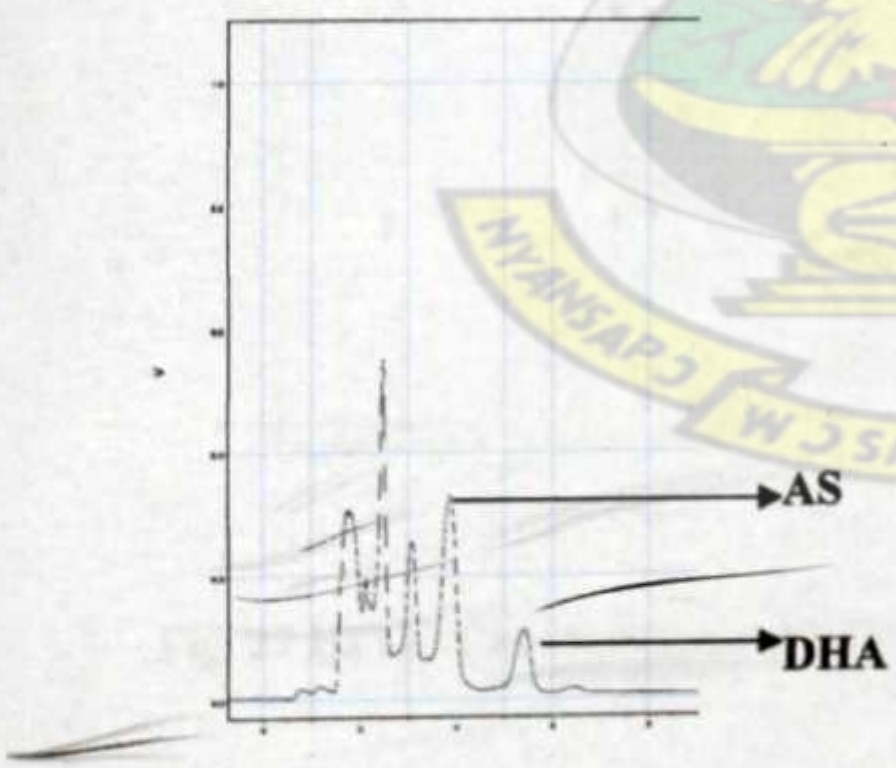

Fig. 23 HPLC Chromatogram of Tablet T_1

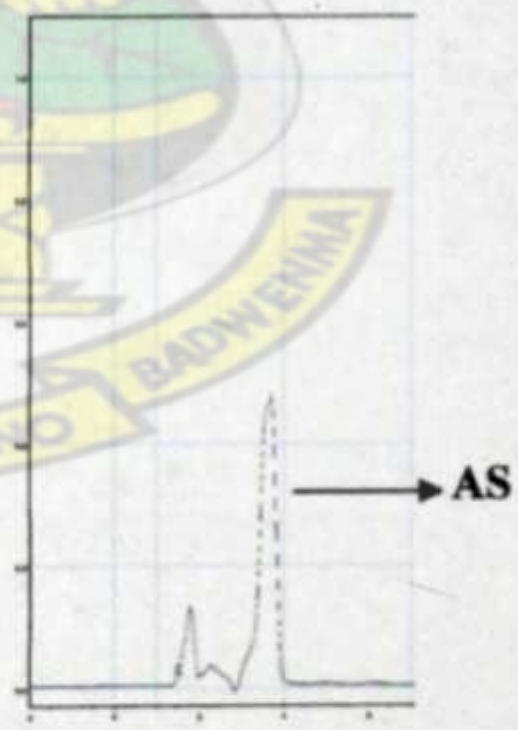

Fig. 24 HPLC Chromatogram of Tablet T_2

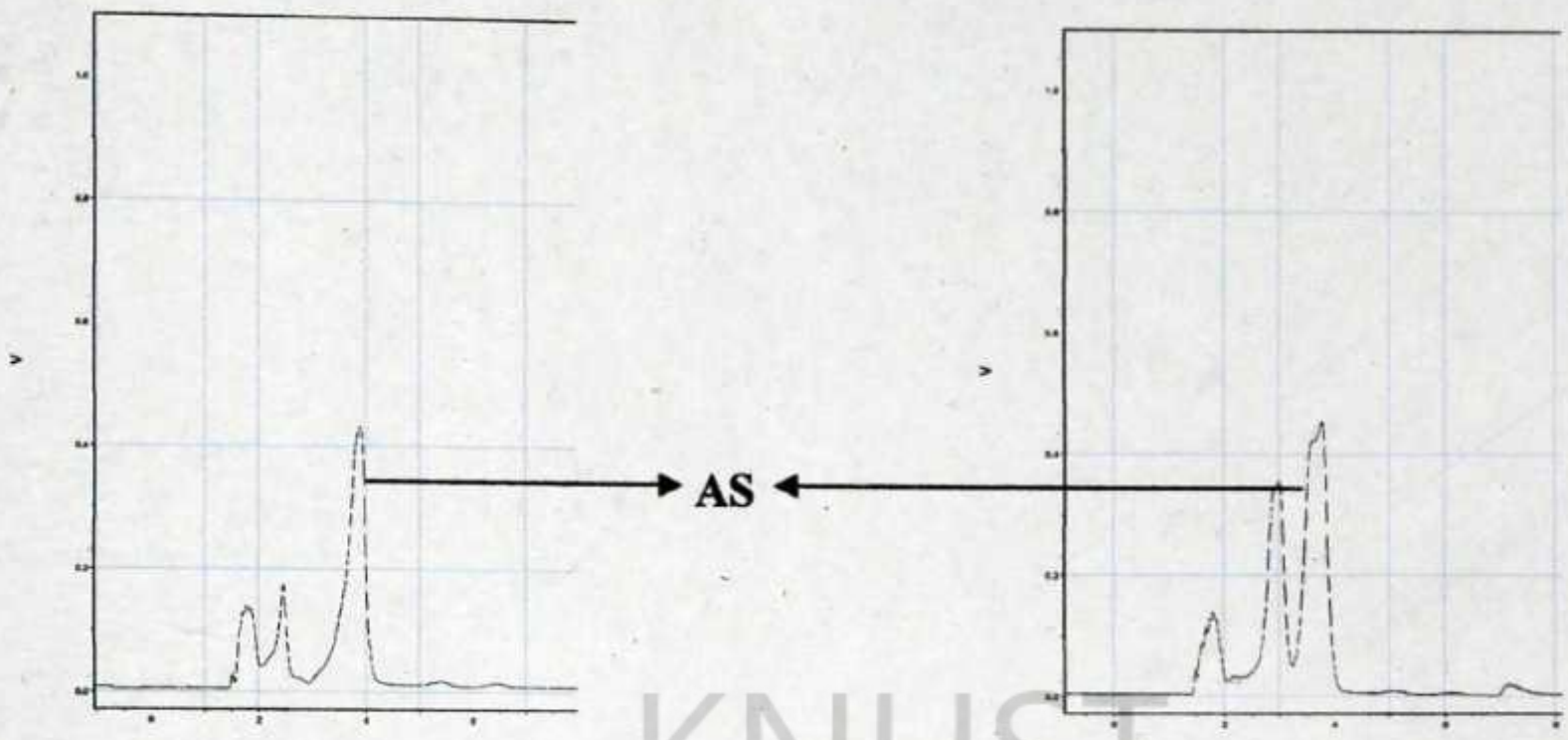

Fig. 24 HPLC Chromatogram of tablet T_3

Fig. 26 HPLC Chromatogram of Tablet T_4

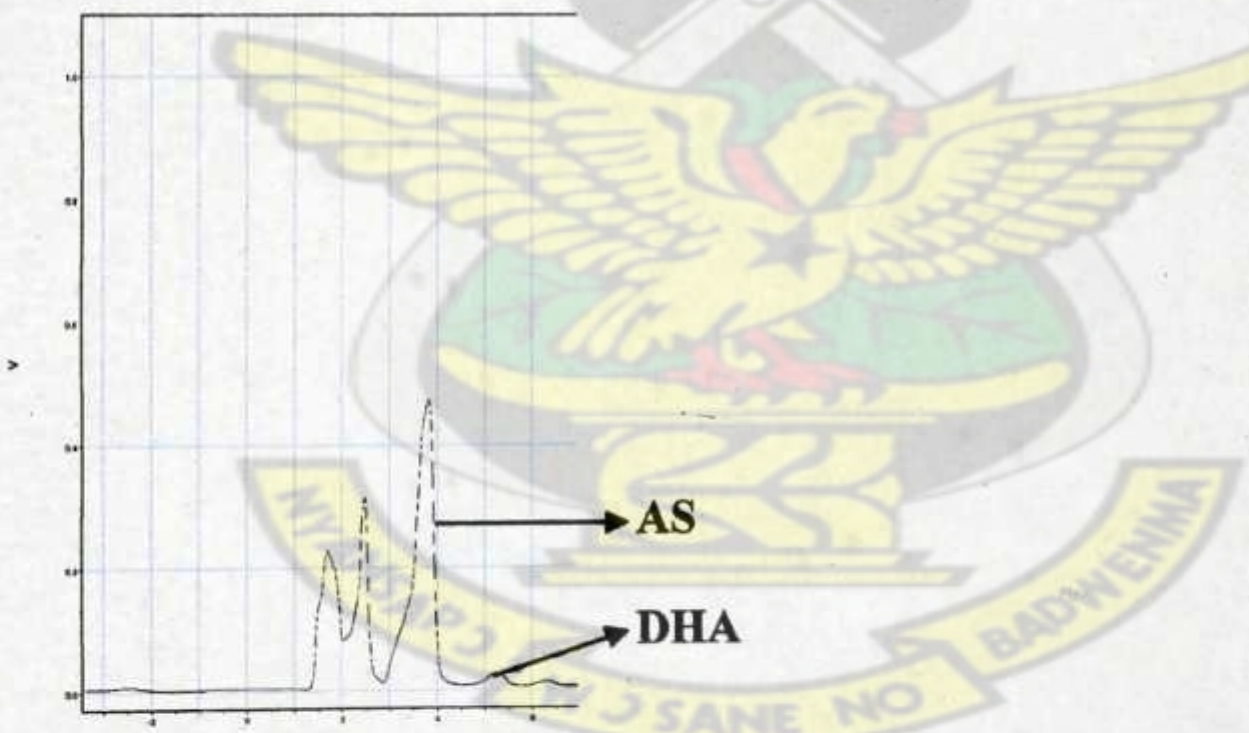

Fig. 27 HPLC Chromatogram of Tablet T_5