KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

COLLEGE OF HEALTH SCIENCES

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

METHOD DEVELOPMENT FOR THE QUANTIFICATION OF PIPERAQUINE PHOSPHATE AND DIHYDROARTEMISININ IN COMBINATION TABLETS

BY

FREDA DANSOAH KISSI

August 2010

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmaceutical Chemistry, KNUST. This work has not been submitted for any other degree.

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Freda Dansoah Kissi

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Prof. J.S.K Ayim Reimmel Adosraku, PhD

(Supervisor) (Head of Department)

DEDICATION

This work is dedicated to my parents, Samuel Kissi and Esther Kissi; my sister, Felice Kissi and to my good friends both at home and in school.

ACKNOWLEDGEMENTS

I will praise God's name in song and glorify Him with thanksgiving. (Psalm 69: 30)

My sincere gratitude goes to my supervisor Professor J.S.K Ayim for his help and advice.

Appreciation goes to all my colleagues, the teaching and non-teaching staff of the Department of Pharmaceutical Chemistry at the Kwame Nkrumah University of Science and Technology (KNUST).

I am also grateful to my parents and siblings who made it possible for me to be here.

ABSTRACT

This research developed a spectrometric method of analyzing Piperaquine phosphate and Dihydroartemisinin in combination tablets.

Piperaquine phosphate in the tablets was analyzed by extracting with 0.001M HCl solution and taking its absorbance at 349nm. The LOD was 5.56×10^{-5} %w/v and the LOQ was 1.68×10^{-4} %w/v.

HPLC conditions were ODS column with a mobile phase of acetonitrile and water with 0.1%v/v Trifluoroacetic acid (TFA), (40:60) at a flow rate of 1.0ml/min at a wavelength of 349nm and an injection volume of 20µl. The retention time was 3.2 minutes. The LOD was 4.9×10^{-5} %w/v and the LOQ was 1.48×10^{-4} %w/v.

Dihydroartemisinin in the tablets was analysed by sonicating the tablet powder in diethyl ether for some time, filtering and evaporating the diethyl ether off. The residue was dissolved in methanol and reacted with conc HCl for 30 minutes at 30^{O}C and the absorbance taken at 254nm. The LOD was 1.09×10^{-4} %w/v and the LOQ was 3.31×10^{-3} %w/v.

HPLC conditions were ODS column with a mobile phase of acetonitrile and water with 0.1%v/v TFA (90:10) at a flow rate of 1.0ml/min at a wavelength of 220nm and an injection volume of 20µl.The retention time was 3.8 minutes. The LOD was 0.013 %w/v and the LOQ was 0.040 %w/v.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 MALARIA

In Ghana, malaria is the number one cause of morbidity accounting for 40-60% of outpatients. It is also the leading cause of mortality in children under five years, a significant cause of adult morbidity, and the leading cause of workdays lost due to illness (**Asante, Asenso-Okyere et al., 2003**).

Malaria is caused by parasitic protozoa of the genus Plasmodium, and it is transmitted to humans by the female Anopheles mosquitoes, which are present in almost all tropical and subtropical countries. There are approximately 380 anopheline species, but only about 60 transmit malaria. There are more than 120 species of Plasmodium protozoa but only four infect humans (**Ursos, Roepe et al., 2002**).

The four species include

- *P. vivax*; causes benign tertian malaria and produces mild clinical attacks
- *P. ovale*; causes tertian fever and produces mild clinical attacks.
- *P. malariae*; causes quartian malaria
- *P. falciparum*; causes malignant tertian malaria and can be fatal if untreated

Most deaths are caused by the *P. falciparum* **(Olaniyi, 2005).**

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**).

1.1.1 THE LIFECYCLE OF THE *P. FALCIPARUM* IN HUMANS

After the bite of an infected female Anopheles mosquito, sporozoites migrate to the liver and invade hepatocytes within 1h. After 5–7 days, the infected hepatocytes rupture, releasing thousands of merozoites, which will invade the erythrocytes. The parasite will develop and replicate within the erythrocytes. After 24–26h in the cycle, the trophozoite will adhere to the endothelium of small blood vessels. This adherence, named sequestration, is thought to be responsible for the pathophysiology of falciparum malaria. In this period (26–48h) these older stages cannot normally be seen in the blood smear. They will grow, become schizonts and rupture after 48h, releasing their progeny (16–32 merozoites per schizont) in the blood; with schizont rupture it is thought that a still unidentified malaria toxin is released causing a cytokine response which leads to the typical malaria symptoms of high fever, chills and malaise (**Agmael, Eggete et al., 1999**).

1.2 ANTIMALARIALS

Antimalarials are drugs used for the treatment of malaria in individuals with suspected or confirmed infection. They are also used for the prevention of infection in individuals visiting a malaria-endemic region who have no immunity (prophylaxis), and they are used for Routine Intermittent treatment of certain groups in endemic regions.

Antimalarials should rapidly relieve symptoms of the disease, be harmless to the patient and have no unpleasant side effects, preferably destroy all stages of development of the malaria parasites including gametocytes. It should also be economical and easy to administer. There are different groups of antimalarials which attack different stages of the life cycle of the malarial parasites as well as the different species.

- Causal prophylactic drugs: These would be expected to act on sporozoites well before the infection develops i.e. the exoerythrocytic or the liver stage. No such drugs exist but the terms are used for compounds acting on the pre-erythrocytic infection (primary tissue phase) stage of the malaria parasite and it is to prevent erythrocytic infection. Examples are primaquine, proguanil, pyrimethamine and sulphonamides.
- Schizontocidal drugs: Suppressive drugs which act on asexual erythrocytic forms of all species of malarial parasites. They include the secondary tissue schizontocides (anti

relapse drugs); primaquine and the blood schizontocides; quinine, mepacrine, Chloroquine, mefloquine, artemisinin.

- Gametocidal drugs: These kill gametes of *P. vivax* and *P. malariae* but no direct action on gametocytes of *P. falciparum*. Examples are primaquine, quinine, mepacrine. Complete eradication of the parasites from the body (radical cure) is possible with drugs that destroy the exo-erythrocytic forms as well as the erythrocytic forms. These are the secondary tissue schizontocides.
- Sporontocides: These prevent sporogony and multiplication of the parasites in the mosquito when ingested with the blood of the human host. Examples are proguanil, pyrimethamine, primaquine **(Olaniyi, 2005).**

1.2.1 CLASSES OF ANTIMALARIAL DRUGS WITH THEIR CHEMICAL GROUPS

1. Naturally occurring antimalarials; Quinine, Artemisinin
2. 8-aminoquinolines; Primaquine
3. 4-aminoquinolines; Chloroquine, Mefloquine
4. 9-aminoacridines; Mepacrine, Pyronaridine
5. Naphthoquinones; Atovaquone
6. The dihydrofolate reductase inhibitors; The Biguanides (Proguanil, Chlorproguanil), Diaminopyrimidine Derivatives (Pyrimethamine), Trimethoprim
7. Phenanthrene methanols; Halofantrine
8. Sulphonamides; Sulphadoxine
9. Other antibiotics; Desoxytetracyline **(Olaniyi, 2005).**

1.3 ARTEMISININ COMBINATION THERAPIES (ACT)

Sixty years ago, hopes of malaria eradication were raised by the discovery and implementation of chloroquine (CQ). The use of this highly effective, fast-acting and inexpensive 4-aminoquinoline, along with the potent insecticide dichlorodiphenyltrichloroethane (DDT), quickly proved successful in substantially reducing the incidence and severity of malaria primarily in regions with temperate climates and seasonal malaria transmission **(Sinclair et al., 2009**).

Subsequently, resistance to Chloroquine emerged in the most lethal human malarial pathogen, *Plasmodium falciparum* in 1959 (**Ursos, Roepe et al., 2002**).
In some areas, the switch to either mefloquine (MFQ) or quinine resulted in the appearance of multidrug-resistant parasites, particularly in Southeast Asia (**Sinclair et al., 2009**).

The new strains of Plasmodium falciparum, have developed resistance to most first- and second-line antimalarial drugs (Ioset and Kaur, 2009). Artemisinin (ART) is not used directly anymore because of its poor oral availability but modified into so-called derivatives like dihydroartemisinin, artesunate and artemether (**Buzzi, Presser et al., 2007**).

Artemisinin derivatives (artesunate, dihydroartemisinin, artemether, arteether) derived from the herb, qinghaosu, sweet wormwood or Artemisia annua L (Asteraceae), are the most effective antimalarial drugs available providing rapid cures (**Ioset and Kaur, 2009**).

The poor pharmacokinetic properties of ART and its derivatives, including the short half-lives of this chemical class, translate into substantial treatment failure rates when used as monotherapy. Combining a member of this class with a longer-acting partner drug assures sustained antimalarial pressure after the plasma concentrations of the ART derivatives have fallen below therapeutic levels. This increases the antimalarial treatment efficacy and reduces the selective pressure for resistance (**Eastman and Fidock, 2009**).

The key partner drugs which are currently in use are;

- Mefloquine, (a fluorinated 4-quinoline) used in combination with artemisinin.
- Lumefantrine (also called benflumetol is structurally related to the hydrophobic arylaminoalcohol antimalarials, including Mefloquine, quinine and halofantrine) is used in combination with artemether.
- Amodiaquine (a potent 4-aminoquinoline antimalarial) is in combination with artesunate.
- Piperaquine (a bisquinoline and is also structurally related to Chloroquine) is used in combination with Dihydroartemisinin (**Eastman and Fidock, 2009**).

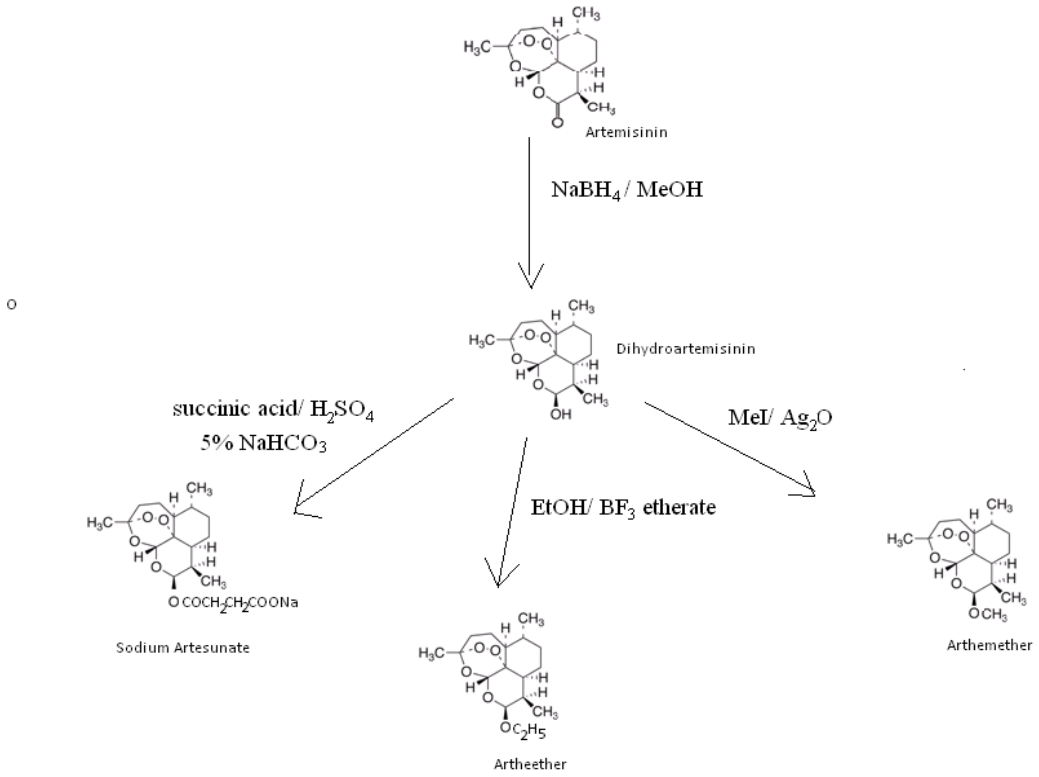


Figure 1.1 Artemisinin and its derivatives

1.3.1 MODE OF ACTION

These drugs have high affinity for hemozoin, a stage form of hemin, which is retained by the parasite after digestion of heamoglobin, leading to a highly selective accumulation of the drug by the parasite. The drug then comes in the presence of iron probably from the hemozoin and releases free radicals. This results in changes in membrane integrity and depression of protein synthesis resulting ultimately in cytotoxicity, phargocytosis and clearance by most leucocytes (**Meshnick et al., 1991, 1993**).

1.4 AIMS AND OBJECTIVES FOR THIS WORK

Since these combination drugs (ACT) are new in the system, there has not been an easy way to analyze them especially in the analysis of artemisinin and its derivatives. These compounds lack fluorescent chromophores and do not possess functional groups with the potential for

derivatization (**Amponsaa-Karikari, Kishikawa et al., 2006**), even though a few methods have been given;

The aim of this work is to develop and validate a quantification method of analyzing Piperaquine phosphate and Dihydroartemisinin in combination Tablets.

The objectives of this work are to develop and validate:

- UV quantification method to analyze Piperaquine phosphate,
- HPLC conditions for analyzing Piperaquine phosphate,
- a simpler way of derivatising Dihydroartemisinin for UV spectrophotometry to analyse Dihydroartemisinin,
- HPLC conditions for analyzing Dihydroartemisinin.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 PIPERAQUINE

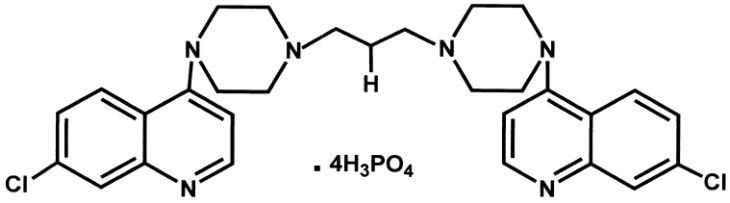


Figure 2.1: Structure of Piperaquine phosphate

Piperaquine is a bisquinoline antimalarial drug which was synthesized by the Shangai Research Institute of Pharmaceutical Industry in 1966 (**Moore, Batty et al., 2007**). It has a structure identical to one which was earlier synthesized by Rhone Poulence, France (**Tarning, 2007; Hung, Daus et al., 2003**). It was highly effective against Chloroquine resistant *P. falciparum* and *P. vivax* malaria in many areas of China and it replaced Chloroquine as the first line treatment in 1978. It is estimated that approximately 200 metric tons were dispensed between 1978 and 1992 for mass prophylaxis until it developed a very high resistance and monotherapeutic use was largely abandoned. Recently it has been used in a combination drug with artemisinin derivatives (**Moore, Batty et al., 2007**).

Piperaquine or 1,3-bis-[4-(7-chloroquinolyl-4)-piperazinyl-1]-propane is a base which has a molecular weight of 535.5g/mol. It is a pale white to yellow powder with a melting point of 212 –213^{o}C and UV absorption peaks at 225nm, 239nm and 340nm.. It belongs to the 4-aminoquinolines group (the molecule is based on the 7-chloro-4-aminoquinoline structure of Chloroquine) and it is highly lipophilic and it is poorly soluble in water even when fully ionized but soluble in acids (**Tarning, 2007**).

Piperaquine phosphate is its salt form. It has a molecular weight of 927.5g/mol with a melting point of 248–252^{o}C. It is soluble in water and also acidic water solution (**Tarning, Lindegardh et al., 2008**).

2.2 DIHYDROARTEMISININ

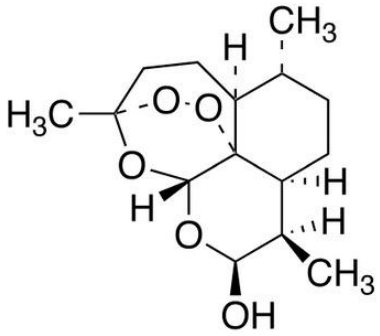


Figure 2.2: Structure of Dihydroartemisinin

Dihydroartemisinin (DHA), (also known as artenimolum or artenimol) is a derivative of artemisinin, a naturally occurring antimalarial. Artemisinin (also known as qinghaosu) comes from the Chinese wormwood Artemisia annua L (**Cabri, Ciogli et al., 2008**). This sesquiterpene lactone endoperoxide is extremely potent against chloroquine and sulphadoxine–pyrimethamine resistant P. falciparum in vitro and in vivo and can produce faster parasite clearance and fever resolution times than any other licensed antimalarial, including quinine (**Eastman and Fidock., 2009**).

Two methods have been used to convert artemisinin to dihydroartemisinin. The first method proposes to convert artemisinin to dihydroartemisinin by reduction with sodium borohydride in methanol or ethanol at about 0° to 5°C. The second method shows the reduction with DIBAL-H in dichloromethane at -78°C. The disadvantages of DIBAL-H are the smaller yield and the higher prices of both the solvent and the means of reduction (**Buzzi, Presser et al., 2007**).

The conversion of the lactone carbonyl group at C-10 of artemisinin into the hydroxyl (hemiacetal) group in DHA yields a new sterically labile centre in the molecule, which, in turn, provides two lactol hemiacetal epimers, namely, α and β. The α-epimer bears the hydroxyl group in the equatorial position (absolute stereochemistry at C-10: *R*), whereas the β-epimer possesses an axial hydroxyl group. Although DHA has a chair-like pyranose ring, such nomenclature is

the reverse of that normally used for designating the stereochemistry of sugars and glycosides, in which, for example, α-D-glucopyranose possesses an axial hydroxyl group (**Cabri, Ciogli et al., 2008**).

It has a molecular mass of 284.4g/mol. It is a colourless needlike or white to almost white crystalline powder with a melting point of 158–160^{O}C. It is insoluble in water, slightly soluble in acetonitrile and soluble in methanol, ethanol, acetone and chloroform.

2.3 WORKS DONE ON PIPERAQUINE PHOSPHATE AND DIHYDROARTEMISININ

2.3.1 PIPERAQUINE

- A bioanalytical method for the determination of Piperaquine in 100 microlitre blood applied onto sampling paper, by solid-phase extraction and liquid chromatography, has been developed and validated (**Malm et al., 2004**).
- Four impurities in Piperaquine phosphate bulk drug substance were detected by a newly developed gradient reverse phase high performance liquid chromatographic (HPLC) method. These impurities were identified by LC/MS/MS (**Dongre et al., 2007)**.
- A significant contaminant of the antimalarial drug Piperaquine (1,3-bis-[4-(7-chloroquinolyl-4)-piperazinyl-1]propane) has been identified using liquid chromatography-mass spectrometry (LC-MS) and 2D NMR spectroscopy (1H-1H COSY, 1H-13C HSQC, 1H-13C HMBC). The impurity was identified as the positional isomer which is the 1-[(5-chloroquinolin-4)-piperazinyl]-3-[(7-chloroquinolin-4) piperazinyl]propan (**Lindegardh et al., 2006**).
- Investigation of the pharmacokinetic properties of Piperaquine after repeated oral administration of the antimalarial combination CV8 in healthy subjects (**Roshammar et al., 2006**).

2.3.2 DIHYDROARTEMISININ

- A development and validation of a simple, sensitive, and specific liquid chromatography-mass spectrometry (LC-MS) analytical method used for the co-quantification of artesunate and its active metabolite, dihydroartemisinin, in human plasma, using artemisinin as an internal standard (**Thuy le et al., 2008**).

- A new dihydroartemisinin Tablet formulation has been developed by the Thai Government Pharmaceutical Organization (GPO). In this report, its in vitro dissolution and in vivo pharmacokinetics as well as its safety in healthy volunteers were evaluated, using the DHA Tablet made by Dafra Pharma NV as a reference (**Kongpatanakul et al., 2007)**.
- The two fixed-dose combinations of dihydroartemisinin and Piperaquine (Artekin and Arterakine) were found to be bioinequivalent in healthy Vietnamese subjects. However, because the peak plasma concentrations and areas under the concentration-time curves of dihydroartemisinin and Piperaquine were only marginally different between the two formulations, similar therapeutic efficacies are expected in the treatment of malaria infections (**Chinh et al., 2009**).
- As none of the pharmacopoeial dissolution methods are suitable to evaluate the release rate of artemether and dihydroartemisinin from Tablets, a 'two-phase partition-dissolution' method, based on the one of [J. Pharm. Sci. 85 (1996) 1060] was developed (**Gabriels and Plaizier-Vercammen, 2004**).

2.4 THEORY AND INSTRUMENTATION OF ANALYTICAL METHODS

2.4.1 IDENTIFICATION TESTS

When a sample is received, the most important preliminary test is the identification test. This is done before any other experimental work is done. This is done to confirm the identity of the sample and its purity.

Identification tests used were:

- Melting point
- Thin layer chromatography
- Colour reactions

Melting point

This is characteristic of every solid compound. Melting point determines the purity of a sample. The range of temperature over which a pure compound melts is very small. Impurities in the sample both lower the melting point and also widen the melting point range.

Colour reactions

Many substances give distinct colours when brought into contact with certain chemical reagents. In some cases, the colour produced with a particular reagent may be specific for the compound under investigation but more often the colour reaction is not confined to a single compound but it is produced by a number of the compounds in a given class and sometimes by a substance not in that class (**Clarke, 1986**).

2.4.2 CHROMATOGRAPHY

This is the process by which a mobile phase passes over a stationary phase. When a sample compound is placed in the mobile phase, it will distribute between the mobile phase and the stationary phase usually in a fixed column or on a solid surface. Mobile phases may be gas, liquid or supercritical fluid. Stationary phase may be solid or liquid. The speed with which the sample moves over the stationary phase depends on the distribution ratio between the two phases i.e.

- Adsorption: This is based on surface phenomena using a solid stationary phase and a liquid or gas mobile phase.(column methods like GC or HPLC)
- Partition: This is based on relative solubilities of the solute in the two surfaces, the stationary phase forming a thin film on the surface of a solid(planar methods like TLC)
- Ion exchange: This is based on the exchange of ions between the stationary ion exchange resin and the components of the mixture.
- Molecular exclusion: Gel filtration or gel permeation is based on separation of molecules by molecular size (**Olaniyi, 2000**).

2.4.3 THIN LAYER CHROMATOGRAPHY (TLC)

The mobile phase moves by capillary action across a uniform thin layer of finely divided stationary phase (an adsorbent like silica gel, alumina) normally mixed with a binding agent – 13% w/v $CaSO_4$ and 1.5% of a fluorescent indicator having a maximum intensity of 254nm and bonded to a plate made of glass or aluminium foil or plastic. When a mixture of organic substances is applied to the plate and developed with the mobile phase, the substances move across the plate at different rates depending on their solubilities, pKa values and capability of H-bonding and so become separated.

Technique of TLC

The plate is prepared. The sample is dissolved in a suitable solvent and spotted on the plate with a melting point tube about 1.5cm from the end of the plate. The plate is placed in a developing chamber with the mobile phase of depth 0.5-1.0cm. 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 in UV light (254 and 366nm) or sprayed with suitable chromogenic agents which will react with the substances to give characteristic spot colouration.

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

Rf varies from 0-1. TLC is usually run with a reference standard or pure sample (**Olaniyi, 2000**).

2.4.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

This uses the concept of column chromatography in which the resolving agent is packed into a narrow-diameter; long length, high resolution column and high inlet pressure (up to 6000psi) to accelerate the separation process through the generation of more theoretical plates per unit length of column.

Instrumentation

HPLC consists of:

- Mobile phase reservoirs: They are made of glass or stainless steel equipped with degassers and filters to remove dissolved gases (O_2, N_2) and dust or particulate matter from the solvents.
- Pumping systems: They must be able to provide pressure up to 6000psi, pulse free output, flow rates ranging from 0.1-10ml/min flow control. There are three kinds of pumps viz reciprocating pump, displacement pump and constant pressure pump.
- Sample injection systems: Constant volume loop permits introduction of samples at pressures up to 7000psi and sample size is from 5-500µl. overloading columns must be avoided to prevent band broadening. Auto injectors or syringes may be used.
- Column: They are made of stainless steel of 2-4mm internal diameter for analytical separation and larger ones for preparative separations and lengths of 10-30cm. particle sizes

are 3, 5 and 10 in diameter and composed of silica gel. Bonded phase packing with coating is non polar (reverse phase) and polar functional groups such as cyano, diol, amino, diamino and dimethylamino are the normal phase.

- Detectors: They should be sensitive and of good stability
  a) Bulk property detector: It detects differences in bulk properties between a pure mobile phase and a mobile phase containing dissolved solute. They are valuable for any compound that does not show any UV absorption e.g: refractive index detector.
  b) Solute property detector: This is based on a unique chemical property of solutes like UV-vis absorption, fluorescence or redox e.g.: UV-vis light absorption detector
  c) Desolvation detector: They utilize some properties of the analyte after the mobile phase has been removed e.g.: mass spectrometer detectors
- Recorder: This gives a hard copy of the separation profile.

  (**Olaniyi, 2000**).

Column efficiency

This is dependent on the degree of band broadening relative to the time taken to elute. An efficient column must maintain sharp narrow peaks on a function of retention time. A quantitative measurement of column efficiency is the 'number of theoretical plates', N.

N= 16(Rt/W)2 or N= 5.54 ($Rt/W_{1/2}$)2 plates/m

Where Rt – retention time

W – Idealized peak width at base

$W_{1/2}$ – peak width at half height

The larger N is, the more efficient the column is.

Another measurement is the 'height equivalent to theoretical plate', HETP or the H value i.e. the length of the column required to generate one plate.

H = L/N

Where L – length of column

N – number of theoretical plates

A column with a small value of H is better **(Olaniyi, 2000).**

Column care

Proper care of the column is extremely important for its lifetime and the quality of analysis.

- **pH stability**: In general silica based columns are stable between a pH range of 2 to 8. The measurement should be done in the aqueous media before mixing it with the organic solvents to give a more accurate and consistent results. New bonding chemistry allows for operating as low as pH 1 with some stationary phases. Stationary phases based on ultra pure silica gel can also be used at a pH as high as 11 depending on the chemical nature of the modifier used in the mobile phase.
- **Mechanical stability:** Stationary phases based on silica are mechanically very stable and well packed columns can be used for more than 6000psi without any problem. However pressure shocks to the column must be avoided. They lead to channeling in the bed column which may result in peak splitting.
- **Mobile phases (eluents):** HPLC grade solvents must be used. Also prepared buffers must be filtered through a 0.45µm filter before using. The use of non pure solvents causes irreversible adsorption of impurities on the column head. They block adsorption sites, change the selectivity of the column and eventually lead to peak splitting. The use of a pre column increases the life time of the column drastically. It filters the particulate material coming from the pump seals or injection rotors. An alternative to a pre column is an in-line filter. These filters are placed between the column and the injector.
- **Storage:** For short-term storage i.e. overnight, columns can be stored in eluents. For middle-term storage i.e. about 2 days or over the weekend, columns should be flushed with pure water to prevent algal growth. For long-term storage, silica based columns should be stored in an aprotic solvent. The water content should not be greater than 50%. The best solvent for storage is acetonitrile. Buffers should be washed out of the column before exchanging aqueous mobile phases by organic solvents.

2.4.5 UV SPECTROPHOTOMETRY

The technique of ultraviolet-visible spectrophotometry is one of the most frequently employed in pharmaceutical analysis. It involves the measurement of the amount of ultraviolet (190-380nm) or visible (300-800nm) radiation absorbed by a substance in solution.

When a molecule absorbs energy of a specific wavelength from an electromagnetic radiation, an electron involved in a bond (with energy E_1) is promoted from the bonding orbital to the antibonding orbital (higher energy E_2). Hence there is a change in energy i.e. $\Delta E = E_2 - E_1$.
This energy is supplied by the UV-vis region of the electromagnetic spectrum and any region of the spectrum can be described in terms of wavelength, λ; wavenumber, v; and frequency, v.

Groups such as >C=O, >C=C , -N = N absorb UV-vis radiation at characteristic wavelengths. They are called chromophores. Groups like -OH, $-NH_2$ and $-OCH_3$ do not absorb specific UV or visible radiation but when conjugated with a chromophore, increases the wavelength of absorption of the chromophore. These are auxochromes. Bathochromic or red shift is the movement to a higher wavelength and hypsochromic or blue shift is the vice versa. Hyperchromic effect is an effect leading to an increased absorption intensity and hypochromic shift is the vice versa.

Laws of light absorption

When an organic molecule is exposed to light of intensity I_0, part of it is absorbed and the other part is not (I). This results in a decrease in intensity. Strength of absorption of electromagnetic radiation by a sample is known as absorbance.

Absorbance (A) = $\log_{10} (I_0/I)$

Transmittance (T) = (I/I_0)

% Transmittance (T) = (I/I_0) x 100%

Where I_0 – incident light

I – transmitted light

Lamberts law: $A = \log_{10} (I_0/I) = kl$

Where l = path length

K = proportionality constant for a particular material at a particular wavelength of light.

Beers law: $\log_{10} (I_0/I) = kc$

Where c =constant

Beer-lamberts law: Absorbance of a solution of a substance

$\log_{10} (I_0/I) = \varepsilon cl$

Absorptivity (ε) = A/cl

Where ε – constant for a particular solvent at a particular wavelength of the incident light used.

Absorptivity depends on

- When c is expressed in molarity (i.e. gram moles/ litre) and l in cm, the constant is called molar absorptivity.
- When c is in % w/v (i.e. grams/100ml) and l in cm, the constant is known as specific absorbance with the symbol $A_{(1\%,1cm)}$

(**Olaniyi, 2000**)

Instrumentation

UV spectrophotometers consist of:

- **Radiation source:** Tungsten filament or xenon are lamps for wavelength 350-800nm and deuterium discharge lamp or a low pressure hydrogen discharge tube for use in the UV range (185-375nm)
- **Monochromator (wavelength selector):** This enables the instrument to separate the different wavelengths of the UV or visible radiation using a prism or a diffraction grating
- **Sample and reference cells (cUVettes**): These are made of fused quartz or silica. Glass is not transparent in the UV region and absorbs strongly at wavelengths less than 350nm. Silicate glasses can be employed in the region between 350-2000nm. Cells of path length 1cm are used.
- **The sample compartment:** This is the part of the instrument where the resolved radiation (monochromatic light) interacts with the sample. In a double beam machine, there is a light-chopping device or a beam-switching assembly attached to a beam-splitter which allows the beam coming from the monochromator exit slit to split into two equal beams; one passing through the reference and the other, through the sample.
- **The photo detector:** This is usually a photomultiplier which consists of a cathode coated with a photoemissive compound. The ratio of the reference beam to sample beam intensities (I_0/I) as detected if fed into a pen recorder. The recorder trace is usually absorbance against wavelength **(Olaniyi, 2000).**

Solvents

The choice of solvent is governed by the solubility of the absorbing substance and by the absorption of the solvent at the analytical wavelength. The solubility in polar and non-polar solvents can often be predicted from a consideration of its chemical structure. Example of substances that are essentially hydrocarbon in nature and devoid of polar functional groups are lipophilic and are usually soluble in non-polar solvents such as Cyclohexane. Substances with several functional groups that confer polarity are normally hydrophilic and soluble in polar solvents such as water. Suitable solvents must be transparent within the range being examined i.e. their cut off points. The cutoff point is that wavelength where a solvent alone gives an absorbance of about 1unit when measured with water in the reference cell.

Table 2.1 Cut off points of some solvents (nm)

Acetonitrile	190
Acetone	330
Chlorobenzene	287
Chloroform	245
Cyclohexane	200
Ethanol	210
Etyl ether	215
Methanol	205
Trifluoroacetic acid	210
Water	190

Source: **(Olaniyi, 2000).**

Calibration of UV spectrophotometer

- **Control of wavelengths:** The wavelength scale is verified using the absorption maxima of holmium perchlorate solution, the line of a hydrogen or deuterium discharge lamp or the lines of a mercury vapour shown below. The permitted tolerance is ± 1 nm for the ultraviolet range and ± 3 nm for the visible range. Suitable certified reference materials may also be used.

Table 2.2 Absorption maxima for control of wavelength scale

241.15nm (Ho)	404.66nm (Hg)
253.7nm (Hg)	435.83nm (Hg)
287.15nm (Ho)	486.0nm (Dβ)
302.25nm (Hg)	486.1nm (Hβ)
313.16nm (Hg)	536.3nm (Ho)
334.15nm (Hg)	546.07nm(Hg)
361.5nm (Ho)	576.96nm (Hg)
365.48nm (Hg)	579.07nm (Hg)

Source: (**British Pharmacopoeia, 2005)**

- **Control of wavelengths:** The absorbance is checked using suitable filters or a solution of potassium dichromate at the wavelengths indicated below which gives for each wavelength the exact values and the permitted limits of the specific absorbance. The tolerance for the absorbance is ± 0.01.
- For the control of absorbance, solutions of potassium dichromate which has previously been dried to a constant mass at 130°C are used. For the control of absorbance at 430 nm, 57.0-63.0 mg of potassium dichromate is dissolved in 0.005 M sulphuric acid and diluted to 100.ml with the same acid. Suitable certified reference materials may also be used.

Table 2.3 Absorption maxima for control of absorbance scale

Wavelength (nm)	Specific absorbance $A_{(1\%, 1cm)}$	Maximum tolerance
235	124.5	122.9 – 126.2
257	144.5	142.8 – 146.2
313	48.6	47.0 – 50.3
350	107.3	105.6 – 109.0
430	15.9	15.7 – 16.1

Source: (**British Pharmacopoeia, 2005)**

- **Limit of stray light:** Stray light may be detected at a given wavelength with suitable filters or solutions: for example the absorbance of a 12 g/l solution of potassium chloride R in a 1 cm cell increases steeply between 220 nm and 200 nm and is greater than 2.0 at a wavelength between 198 nm and 202 nm when compared with water as compensation liquid. Suitable certified reference materials may also be used.
- **Resolution (for qualitative analysis):** When prescribed in a monograph, the resolution of the apparatus is measured as follows: the spectrum of a 0.02 per cent v/v solution of toluene R is recorded in hexane R. The minimum ratio of the absorbance at the maximum at 269 nm to that at the minimum at 266 nm is stated in the monograph. Suitable certified reference materials may also be used.
- **Spectral slit-width (for quantitative analysis):** To avoid errors due to spectral slit-width, when using an instrument on which the slit-width is variable at the selected wavelength, the slit-width must be small compared with the half-width of the absorption band but it must be as large as possible to obtain a high value of I_0. Therefore, a slit-width is chosen such that further reduction does not result in a change in absorbance reading.
- **Cells:** The tolerance on the path length of the cells used is ± 0.005 cm. When filled with the same solvent, the cells intended to contain the solution to be examined and the

compensation liquid must have the same transmittance. If this is not the case, an appropriate correction must be applied. The cells must be cleaned and handled with care. (**British Pharmacopoeia, 2005)**

2.4.6 CHEMICAL DERIVATISATION

Indirect spectrophotometric assays are based on the conversion of the analyte by a chemical reagent to a derivative that has different spectral properties. When an excess of the reagent is used, to ensure complete conversion, the absorbance of the derivative is usually, but not always, proportional to the concentration of the analyte. The majority of indirect spectrophotometric procedures involve the conversion of the analyte to a derivative that has a longer wavelength and or a higher Absorptivity. Chemical derivatisation procedures may be adopted for any of several reasons.

- If the analyte absorbs weakly in the ultraviolet region, a more sensitive method of assay is obtained by converting the substance to a derivative with a more intensely absorbing chromophore. E.g. Dihydroartemisinin which does not absorb above 220nm can be determined spectrophotometrically by heating with NaOH at $50^{O}C$ for 30minutes and measuring the absorbance of the derivative at 289nm.
- The interference from irrelevant adsorption may be avoided by converting the analyte to a derivative which absorbs in the visible region, where irrelevant absorption is negligible. E.g. the condensation of ketosteroids (e.g. methyltestosterone in methyltestosterone Tablets) with hydrazine reagents, or the oxidation of the α-ketol group by tetrazolium salts, produces derivatives that absorb in the visible reagion free of interference from irrelevant absorptions.
- Indirect spectrophotometric procedures are also used to improve the selectivity of the assay of ultraviolet-absorbing components. e.g. the assay of the low concentrations of adrenaline (20µg/ml) in procaine and adrenaline injection by a direct measurement of absorbance at the absorption maximum of adrenaline around 279nm is subject to gross inference from the bactericide. Only adrenaline forms the purple derivative in the presence of iron(II) and is measured colorimetrically free of interference from the other ultraviolet-absorbing components.

- The adoption of a visible spectrophotometric procedure, instead of an ultraviolet procedure, may be based on cost considerations. In general, single beam manually adjusted visible spectrophotometers (sometimes called colorimeters) are much cheaper than ultraviolet visible spectrophotometers.
  (**Beckett and Stenlake, 1988**)

2.4.7 EXTRACTION

Extraction is the withdrawing of an active ingredient or waste substance from a solid or liquid mixture with a liquid solvent. The solvent is not or only partially miscible with the solid or liquid. By intensive contact, the active ingredient transfers from the solid or liquid mixture (raffinate) into the solvent (extract). After mixing, the two phases are separated by either gravity or centrifugal force. For the recovery of the solvent and to get the active agent in pure form, a further separation process is necessary (rectification or re-extraction)

Types of extraction include;

- Solid-liquid extraction: This allows soluble components to be removed from solids using a solvent. A sequence of solvents of varying polarity or pH can be used to separate complex mixtures into groups. Applications include obtaining oil from oil seeds.
- Liquid-liquid extraction: This involves using a liquid solvent to remove a liquid component from a liquid mixture. The component dissolves preferably in the solvent. Applications of this process include removal of vitaminutes from aqueous solutions and aromatic compounds from crude oil fractions.
- Gas-liquid extraction: This is known as absorption.

The solvent for extraction should have:

- Selectivity: Only the active agent has to be extracted and no further substances, i.e. high selectivity is required.
- Capacity: The capacity of the solvent has to be high.
- Miscibility: To achieve simple regeneration of the solvent, the miscibility of the solvent and the primary solvent has to be low.

- Difference in density: After extraction, the two phases have to be separated and high difference in density is required.
- Recovery: The solvent has to be separated from the extract phase easily to produce the free active agents.

  (**Gamse, 2002).**

2.4.8 STANDARDS

The term standard in chromatography is employed in two ways. It can be used to describe a reference substance, the retention time of which is compared with the retention time of an unknown substance for identification purposes. Alternatively, it can be used to provide a reference peak height or peak area which can be compared with the peak heights or areas of the substances of interest to provide quantitative information. A standard employed in either application can be used in two ways, either as an internal standard or as an external standard. An internal standard is added as a known quantity to the sample itself, but must be chosen so that it is adequately resolved from its neighbors so that accurate measurements can be made. The external standard is used when a suitable internal standard that can be separated from the components of the mixture cannot be selected. In this case the external standard is run as a separate chromatogram under exactly the same conditions.

2.4.9 TABLETS

Uniformity of weight test:

20 Tablets are taken at random or, for single-dose preparations presented in individual containers, the contents of 20units. Capsules are weighed intact and then the shells are also weighed. The mass of the contents is the difference between the weights. This is done for 19 other capsules. The average mass is determined. Not more than 2 of the individual masses should deviate from the average mass by more than the percentage deviation shown in the Table below and none should deviate by more than twice that percentage.

Table 2.4 Percentage deviation for the average mass of some pharmaceutical forms

Pharmaceutical form	Average mass	Percentage deviation
Tablets (uncoated and film coated)	80mg or less More than 80mg and less than 250mg 250mg or more	10 7.5 5
Capsules, granules (uncoated, single-dose) and powders (single dose)	Less than 300mg 300mg or more	10 7.5

Source: British Pharmacopoeia

*when the average mass is equal to or below 40mg, the preparation is not submitted to the test for uniformity of mass but to the test of uniformity of content of single dose preparations.

Disintegration test:

To be absorbed, a drug substance must be in solution and the disintegration test is a measure only of the time required under a given set of conditions for a group of Tablets to disintegrate into particles. For compressed uncoated Tablets, the testing fluid is usually water at 37^{O}C. If one or two Tablets fail to disintegrate, the test is to be repeated using 12 Tablets. Of the 18 Tablets then tested, 16 must have disintegrated within a given period of time. For most uncoated Tablets, the period is normally 30minutes although some may vary. For coated Tablets, up to 120minutes may be required while for sublingual Tablets such as CT isoproterenol hydrochloride, the disintegration time is 3minutes (**Gennaro, 1990**)

2.5 METHOD VALIDATION

Typical validation characteristics

- Accuracy
- Precision (repeatability and reproducibility)
- Specificity
- Limit of detection (LOD)
- Limit of quantification (LOQ)
- Linearity
- Range

- Robustness
- Stability

According to ICH Harmonised Tripartite Guidelines on Validation of Analytical procedures: methodology, ICH Topic Q2B (1996); the validation of analytical methods must meet the following parameters:

System suitability	%RSD should not be more than 2%
Linearity	R^2 should be between 0.995 and 1
Precision	RSD should not be more than 2%
Specificity	no interference with the placebo
Accuracy	% recovery should be between 98 and 102%
Tailing factor	Less than 2

LOD and LOQ are based on the SD of the response and slope:

LOD = $(3.3\sigma)/ S$ and LOQ = $(10\ \sigma)/ S$

Where σ = SD of response equal to the residual standard deviation.

Residual standard deviation = $\sqrt{\sum(y - y_{est})^2 / n - 1}$

y = observed values

y_{est} = values of y calculated from the equations of the regression line

S = slope

CHAPTER THREE

3.0 METHOD

3.1 EQUIPMENT

- Adam analytical balance
- Cecil CE 2041 2000 series UV Spectrophotometer
- Shimadzu SPD-20A prominence UV/Vis Detector
- Shimadzu DGU-20A3 prominence degasser
- Shimadzu LC-20AB prominence liquid chromatograph
- Phenomenex Hypersil 5micron C18 250 × 4.60 mm column
- Water bath
- Stuart Melting point apparatus SMP10

3.2 CHEMICALS, REAGENTS AND DRUG SAMPLES

- Hydrochloric acid, HCl (BDH)
- Sodium hydroxide, NaOH (BDH)
- Acetonitrile (BDH)
- Methanol, MeOH (BDH)
- Potassium dihydrogen orthophosphate (BDH)
- Phosphoric acid (BDH)
- Singly distilled water
- Water with 0.1(v/v) TFA (Merck)
- Silver nitrate (BDH)
- Potassium iodide (BDH)
- Starch (Maize products)
- Chloroform (Laboratory Rasayan)
- Ethanol (BDH)
- Ammonia, NH_3 (BDH)
- Sulphuric acid, H_2SO_4 (BDH)
- Ethyl acetate (BDH)

- Petroleum Ether (BDH)
- Diethyl ether (BDH)
- Glacial Acetic acid (BDH)

Table 3.1: Profile of Drug Samples

Drug sample	Batch number	Manufacturing date	Expiry date
Piperaquine phosphate pure powder	8001PUSJ	January 2008	December 2011
Dihydroartemisinin pure powder	7006DKRI	September 2007	August 2011
P-Alaxin Tablets (Tablet A)	GB – 07	January 2008	January 2011
Alaxin Tablets (Tablet B)	AX – 27	Macrh 2008	February 2011

3.3 GLASSWARE

- Volumetric flasks (250ml,100ml, 50ml, 25ml) (Supertek)
- Beakers (Pyrex)
- Measuring cylinders (Fisherbrand)
- Pipettes (HBG)
- Melting point tubes
- Chromatank

3.4 Physical Characteristics of Tablets

Uniformity of weight: Twenty Tablets each of **Tablet A** and **B** were weighed and their mean, deviation and percentage deviation were calculated.

Disintegration: One **Tablet A** was introduced into each of the six tubes. The assembly was suspended in the beaker containing water at 37^{O}C and operated for 30minutes. The same thing was done for **Tablet B**.

PIPERAQUINE PHOSPHATE:

3.4.1 IDENTIFICATION TESTS

0.1g of the Piperaquine phosphate pure sample was dissolved in 10ml of water and 2ml of 0.05M NaOH solution was added. It was shaken with 2 quantities of chloroform and the aqueous layer acidified with nitric acid. 5ml of 5%w/v silver nitrate solution was added to 5 ml of the aqueous layer and observed for a while, boiled and ammonia added **(British Pharmacopoeia).**

3.4.2 SOLUBILITY TESTS

0.005g of the Piperaquine phosphate pure sample was dissolved in 25ml of each of the following solvents; water, methanol, petroleum ether and diethyl ether. Absorption spectrums between 200.0nm and 400.0nm were determined.

3.4.3 MELTING POINT DETERMINATION

Melting point tubes were each filled to about 1mm with the Piperaquine phosphate pure sample and the melting point was determined using the melting point apparatus

3.4.4 THIN LAYER CHROMATOGRAPHY

Mobile phase consisted of *Chloroform: Methanol (9:1).* The Piperaquine phosphate pure sample was dissolved in water and spotted on an aluminuim foil coated with silica gel. The foil was viewed under a UV light of 254 to determine the spots.

DEVEOPMENT OF UV METHOD FOR PIPERAQUINE PHOSPHATE.

3.4.5 Determination of UV spectrum of Piperaquine phosphate in acid, base and water.

0.132g of Piperaquine Phosphate was dissolved in 100ml water (stock solution).

5ml of the stock solution was pipetted into a 50ml volumetric flask and topped up to the mark with distilled water and the absorption spectrum determined between 200.0nm and 400.0nm.

5ml of the stock solution was pipetted into a 50ml volumetric flask and topped up to the mark with 0.05M NaOH solution. The absorption spectrum was determined between 200.0nm and 400.0nm.5ml of the stock solution was pipetted into a 50ml volumetric flask and topped up to the mark with 0.001M HCl solution. The absorption spectrum was determined between 200.0nm and 400.0nm.

3.4.6 Calibration curves for the UV absorption peaks for Piperaquine phosphate.

0.1008g of Piperaquine Phosphate was dissolved in 50ml of water. 40ml of the resulting solution was topped up to 200ml with distilled water (stock solution). 10ml, 20ml, 25ml, 30ml and 40ml each were pipetted from the stock solution and topped up to 50ml with distilled water. 5ml of each of the resulting solutions, including the stock solution was pipetted, topped up to 50ml with 0.001M methanolic HCl and their absorbances taken at 225.0nm, 239.0nm and 349.0nm.

3.4.7 Stability studies for Piperaquine phosphate in solution

0.0826g of Piperaquine Phosphate was dissolved in 100ml of water. 10ml of the resulting solution was topped up to 200ml with 0.001M HCl solution (stock solution). 2.5ml, 5ml, 10ml, 20ml, 25ml, and 40ml were pipetted from the stock solution and each topped up to 50ml with 0.001M HCl solution and their absorbances taken at 349.0nm. The rest of the stock solution was left for 24 hours and the absorbances were taken for the same serial dilutions.

3.4.8 Calibration curve for Piperaquine phosphate (Linearity)

0.0808g of Piperaquine Phosphate was dissolved in 100ml of 0.001M HCl solution. 10ml of the resulting solution was topped up to 200ml with 0.001M HCl solution (stock solution). 5ml, 10ml, 20ml, 25ml, 30ml and 40ml were pipetted from the stock solution and each topped up to 50ml with 0.001M HCl solution and their absorbances and the absorbance of the stock solution were taken at 349.0nm.

3.4.9 Assay of Piperaquine Phosphate in the presence of other excipients

0.0404g of starch, **0.1583g** of methyl cellulose, **0.0084g** of talc and **0.3226g** of Piperaquine Phosphate were powdered together in a mortar with a pestle. **0.1420g** of the mixture was dissolved in 100ml of 0.001M HCl solution and filtered. About 20ml of the first filtrate was discarded and 5ml was pipetted from the rest and topped up to 100ml with 0.001M HCl solution (stock solution). 10ml, 25ml and 40ml were pipetted from the stock solution and topped up to 50ml with 0.001M HCl solution and their absorbances taken at 349.0nm. The percentage recovery was calculated for the various concentrations.

3.4.10 Assay of Piperaquine Phosphate containing Tablets

An amount of powdered **Tablet A** containing **0.0394g** of Piperaquine phosphate was dissolved in 100ml of 0.001M HCl solution and filtered. About 20ml of the first filtrate was discarded and 5ml was pipetted from the rest and topped up to 50ml with 0.001M HCl solution (stock solution). Three concentrations of **$7.98x10^{-4}$%, $3.20x10^{-3}$%** and **$1.99x10^{-3}$%** were prepared by taking 2ml, 5ml and 8ml of stock solution and topping up to 10ml with 0.001M HCl. Their absorbances were taken and the percentage content of Piperaquine phosphate was calculated. This was done for three days.

3.4.11 DEVELOPMENT OF HPLC METHOD FOR PIPERAQUINE PHOSPHATE

The ODS column was chosen because Piperaquine phosphate has some level of polarity. When C8 is compared to C18, C18 is more hydrophobic, retentive and has a more stable bonded phase. Acetonitrile was chosen because from previous works done, acetonitrile was one of the solvents used. Different ratios of acetonitrile to water with 0.1%v/v TFA were used and the best selected. 349nm was used as the wavelength because Piperaquine phosphate absorbs at that wavelength without any interference from any other compound.

Conditions used with the following mobile phases were a flow rate of 1.0ml/min at 349nm with an injection volume of 20µl.

Table 3.2 Ratios of Mobile phase used in HPLC

Acetonitrile: Water with 0.1%v/v TFA (60:40)
Acetonitrile: Water with 0.1%v/v TFA (50:50)
Acetonitrile: Water with 0.1%v/v TFA (40:60)
Acetonitrile: Water with 0.1%v/v TFA (20:80)

3.4.12 Calibration curve for Piperaquine phosphate (Linearity)

The mobile phase was Acetonitrile : Water with 0.1%v/v TFA (40 : 60). **0.017g** of Piperaquine phosphate was weighed and dissolved in water and topped up to 100ml (stock solution). Concentrations of **$1.70x10^{-3}$%, $1.36x10^{-3}$%, $1.02x10^{-3}$%, $6.80x10^{-4}$%** and **$3.40x10^{-4}$% (w/v)** were prepared from the stock solution. 20µl of each of the solutions was injected into the HPLC

at a wavelength of 349nm with a flow rate of 1.0ml/min. Calibration curve was drawn and the LOD and LOQ determined from the graph.

3.4.13 Assay of Piperaquine phosphate in Tablets

An amount of powdered **Tablet A** containing **0.025g** Piperaquine phosphate was dissolved in 20ml of water sonicated for 5minutes and then topped up to 100ml with water. It was filtered and 10ml was pipetted and diluted to 50ml. 20µl of the solution was injected into the HPLC at a wavelength of 349nm with a flow rate of 1.0ml/min. The injection was done six times for 3 days. Their mean percentage contents, standard deviations and relative standard deviations were calculated.

DIHYDROARTEMISININ

3.5 IDENTIFICATION TEST

0.0055g of Dihydroartemisinin was dissolved in 0.5ml ethanol. 1ml of potassium iodide (8%w/v), 2.5ml H_2SO_4 (10%v/v) and 4 drops of starch were added and observed **(International Pharmacopoeia, 2008).**

3.5.1 SOLUBILITY TEST

0.0055g of pure sample of Dihydroartemisinin was dissolved in 25ml of water, methanol, ethanol, ethyl acetate, acetonitrile, acetic acid, petroleum ether and diethyl ether.

3.5.2 MELTING POINT DETERMINATION

Melting point tubes were each filled to about 1mm with the pure sample and the melting point was determined using the melting point apparatus

3.5.3 THIN LAYER CHROMATOGRAPHY

Mobile phase consisted of *ethyl acetate: cyclohexane: glacial acetic acid (5:20:2.5).* The pure sample was dissolved in methanol and spotted on an aluminuim paper coated with silica gel. The spots were determined by spraying with 20% Sulphuric acid in methanol and heated at 1408 for about 10 minutes in an oven and examined under daylight.

UV METHOD DEVEOPMENT

3.5.4 Derivatisation of Dihydroartemisinin with NaOH

0.0501g of Dihydroartemisinin was dissolved in 100ml ethanol. 25ml of the solution was pipetted and topped up to 100 ml (stock solution). 10ml of the stock solution was pipetted and topped up to 100ml with 0.05M NaOH solution. 25ml of the solution was heated at 50^{o}C for 30minutes. It was cooled for 10minutes and its absorption spectrum determined between 200.0nm and 400.0nm using a 1cm cell. The same thing was done for the same solution which had not been heated (International Pharmacopoeia, 2008).

0.1g of Dihydroartemisinin was dissolved in 100ml ethanol. 5ml of the solution was pipetted and each topped up to 100 ml (stock solution). 5ml of the stock solution was pipetted and topped up to 50ml with 0.05M NaOH solution and heated at 50^{o}C for 30minutes. It was cooled for 10minutes and its absorption spectrum determined between 200.0nm and 400.0nm.

0.1g of Dihydroartemisinin was dissolved in 10ml methanol. 5ml of the solution was pipetted and topped up to 50ml with 0.05M NaOH solution and heated at 50^{o}C for 30minutes. It was cooled for 10minutes and its absorption spectrum determined between 200.0nm and 400.0nm.

3.5.5 Calibration curve of Derivatised Dihydroartemisinin with NaOH (Linearity)

0.2192g of Dihydroartemisinin was dissolved in 100ml methanol (stock solution). 5ml, 10ml, 12.5ml, and 25ml were pipetted from the stock solution and each topped up to 50ml with ethanol. 5ml of each of the solutions including the stock solution was topped up to 50ml with 0.05M NaOH solution and heated at 50^{o}C for 30minutes. They were cooled for 10minutes and their absorbances taken at 289 nm.

3.5.6 Variation of volumes of NaOH for derivatisation of Dihydroartemisinin

0.2092g of Dihydroartemisinin was dissolved in 200ml methanol (stock solution). 10ml, 15ml, 20ml, 25ml, 30ml, 35ml, 40ml and 45ml were pipetted from the stock solution and each topped up to 50ml with 0.05M NaOH solution and heated at 50^{o}C for 30minutes. They were cooled for 10minutes and their absorbances taken at 289.0nm.

3.5.7 Variation of heating times for Dihydroartemisinin in NaOH

0.1052g of Dihydroartemisinin was dissolved in 50ml methanol. 50ml of the solution was pipetted and topped up to 200ml with methanol (stock solution).

20ml of the stock solution was pipetted and topped up to 200ml with 0.05M NaOH solution. 20ml of the solution was pipetted six times and each put into 6 different glass stoppered test tubes and heated at 50^{o}C for 10, 20, 30, 40, 50 and 60 minutes respectively. They were cooled for 10 minutes after heating and their absorbances taken at 289nm.

10ml of the stock solution was pipetted and topped up to 200ml with 0.05M NaOH solution. 20ml of the solution was pipetted six times and each put into 6 different glass stoppered test tubes and heated at 50^{o}C for 10, 20, 30, 40, 50 and 60 minutes respectively. They were cooled for 10 minutes after heating and their absorbances taken at 289nm.

3.5.8 Variation of concentrations of NaOH for derivatisation of Dihydroartemisinin and investigating the stability of the derivative in solution

0.104g of Dihydroartemisinin was dissolved in 100ml of methanol. 50ml of the solution was pipetted and topped up to 100ml. 10ml of the solution was pipetted six times and put in six different stoppered test tubes. 10ml of 0.02M, 0.04M, 0.08M, 0.10M, 0.12M and 0.20M NaOH were added to the stoppered test tubes containing the stock solutions. They were heated at 50^{o}C for 30minutes. Their absorbances at 289.0nm were taken after they were cooled for 10minutes. Their absorbances at 289.0nm were taken again after 10, 20, 30, 40, 50 and 60 minutes after the first absorbances were taken.

3.5.9 Investigation of the addition of different concentrations of acetic acid to derivatised Dihydroartemisinin in NaOH

0.1006g of Dihydroartemisinin was dissolved in 100ml of methanol. 50ml of the solution was pipetted and topped up to 100ml with methanol. 10ml of the solution was pipetted four times and put in four different stoppered test tubes. 1M NaOH solution was also added to each of the solutions and heated at 50^{o}C for 30minutes. Their absorbances were taken after they were cooled for 10minutes. The absorbances of each of the solutions were taken again after 10minues and 20minutes after the first absorbances were taken. Then 5ml of 1M acetic acid solution, 1ml of 1M acetic acid solution, 5ml of 0.5M acetic acid solution and 5ml of acetic acid solution were

added respectively to the solutions and the absorbances taken again at 30, 40 and 50 minutes after the first absorbances were taken.

3.5.10 Derivatisation of Dihydroartemisinin with NH_3

0.0253g of Dihydroartemisinin was dissolved in 100ml of Methanol. (stock solution) 2ml of 0.7M NH_3 was added to 5ml of the stock solution and heated for 30minutes at 50^OC, then another was heated at 60^OC and the absorption spectrum of both solutions were determined between 200nm and 400nm. 2ml of 0.7M NH_3 was added to 5ml of the stock solution and heated at 60^OC for 30minutes. Two more were heated for 40 and 50 minutes and their absorbances taken at 235nm. 2ml of 4M NH_3 was added to 5ml of the stock solution and heated for 30 minutes at 60^OC and then another one at 70^OC and the absorption spectrum determined between 200 and 400nm.

3.5.11 Variation of Concentration of NH_3 and investigating the stability of the derivative

0.054g of Dihydroartemisinin was dissolved in 100ml of methanol and 5ml of the solution was diluted to 50ml with methanol. (stock solution). 2ml of 2M NH_3 was added to 5ml of the stock solution and heated at 70^OC for 30minutes. Absorbances were taken after 10, 20, 30, 40, 50 and 60minutes after heating at 236nm.

3.5.12 Derivatisation of Dihydroartemisinin with HCl

0.0102g of DHA was dissolved in 50ml of methanol (stock solution). 1ml of conc HCl was added to 2ml of the stock solution and left for 10 minutes at 30^OC. It was topped up to 25ml with methanol and the absorbance was taken at 254.0 nm. This was done for 20, 30, 40, 50 and 60 minutes and their absorbances taken. The same thing was done for 2ml, 3ml and 4ml of HCl added to 2ml of the stock solution.

3.5.13 Calibration curve for Dihydroartemisinin (Linearity)

0.0128g of DHA was dissolved in 50ml of methanol (stock solution). 3ml of conc HCl was added to 2ml of the sock solution and left for 30minutes at 30^OC. It was then topped up to 25ml with methanol and the absorbance taken at 254nm. The same thing was done for 2ml, 4ml, 6ml and 8ml of stock solution diluted to 10ml.

3.5.14 Assay of DHA in the presence of other excipients

0.0412g of starch, **0.130g** of methyl cellulose, **0.0084g** of talc was added to **0.0197g** of DHA, powdered together and dissolved in 40ml of diethyl ether, sonicated for 5minutes and filtered. The ether was evaporated off and the percentage recovery was calculated for the residue.

3.5.15 Assay of DHA containing Tablets

An amount of powdered tablet containing **0.02g** DHA was dissolved in 30ml of diethyl ether and sonicated for 5minutes, filtered and extracted again with two 20ml of diethyl ether and the diethyl ether evaporated off. The residue was dissolved in 100ml of methanol (stock solution) 3ml of conc HCl was added to 2ml of the stock solution and to 2ml of two solutions of concentrations; $7.24x10^{-4}$% and $9.66x10^{-4}$%, prepared from the stock solution. They were left to stand for 30minutes and topped up to 25ml with methanol and their absorbances taken at 254nm. Their absorbances were taken and the percentage content of DHA was calculated. This was done three times for each concentration and done for three days. This was done for Tablets A and B.

3.5.16 DEVELOPMENT OF HPLC METHOD FOR DIHYDROARTEMISININ

The ODS column was chosen because Dihydroartemisinin has some level of polarity. When C8 is compared to C18, C18 is more hydrophobic, retentive and has a more stable bonded phase. Acetonitrile was chosen because methanol gave an unstable baseline due to its cut off point and the wavelength at which Dihydroartemisinin absorbs. 220nm was used as the wavelength because Dihydroartemisinin absorbs at that wavelength and high concentrations were used because low concentrations were not detecte. An external standard was chosen because, the peaks of available standards could not be separated from the peak of Dihydroartemisinin. Benzoic acid was therefore chosen finally.

HPLC conditions used was 20µl injection volume, a mobile phase of Acetonitrile : Water with 0.1%v/v TFA (90 : 10) at 220nm with a flow rate of 1.0ml/min.

3.5.17 Calibration curve for Dihydroartemisinin (Linearity)

The mobile phase was Acetonitrile: Water with 0.1%v/v TFA (90 : 10). 0.16g of DHA was weighed and dissolved in the mobile phase and topped up to 50ml (stock solution). Concentrations of **0.064%, 0.128%, 0.192%** and **0.256%** (w/v) were prepared from the stock

solution. 20µl of each of the solutions was injected into the HPLC at a wavelength of 220nm with a flow rate of 1.0ml/min and benzoic acid as external standard. Calibration curve was drawn and the LOD and LOQ determined from the graph.

3.5.18 Assay of Dihydroartemisinin in Tablets

An amount of powdered tablet containing 0.2g DHA was dissolved in 20ml of diethyl ether and sonicated for 5minutes, filtered and extracted again with two 20ml of diethyl ether and the diethyl ether evaporated off. The residue was dissolved in 100ml of mobile phase (stock solution). 20µl of the solution was injected into the HPLC at a wavelength of 220nm with a flow rate of 1.0ml/min and benzoic acid as external standard. The injection was done six times for 3 days. Their mean percentage contents, standard deviations and relative standard deviations were calculated. This was done for Tablets A and B.

CHAPTER FOUR

4.0 RESULTS AND CALCULATIONS

4.1 Physical Characteristics of Tablets

Uniformity of weight: All Tablets of A and B passed the uniformity of weight test.

Disintegration time: Tablet A was 7.833minutes and Tablet B was 2.267minutes.

Tablet A:

Average weight of Tablets = 0.5575g

Content of Piperaquine Phosphate in a Tablet = 0.32g

Content of Dihydroartemisinin in a Tablet = 0.04g

Total active ingredients in a Tablet = 0.36g

Content of excepients in a Tablet = 0.5575g – 0.36g

= 0.1975g

% content of excepients in a Tablet:

Starch = 20, talc = 1% and methyl cellulose = 79%

Content of starch = 0.0395g

Content of talc = 0.002g

Content of methyl cellulose = 0.1558g

Tablet B:

Average weight of Tablets = 0.2094

Content of Dihydroartemisinin in a Tablet = 0.06g

Total active ingredients in a Tablet = 0.06g

Content of excepients in a Tablet = 0.2094g – 0.06g

= 0.1494g

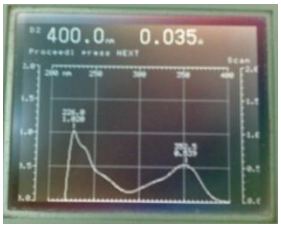
PIPERAQUINE PHOSPHATE

Table 4.1 Preliminary tests for Piperaquine phosphate

Test	Results
Identification test	A yellow precipitate was formed whose colour was not changed by boiling and which dissolved on addition of ammonia.
Solubility	Piperaquine phosphate showed absorbances for water, methanol but it did not show any absorbance for petroleum ether and diethyl ether
Melting point	248 – 250^{O}C
Thin layer chromatography	Rf value is 0.854

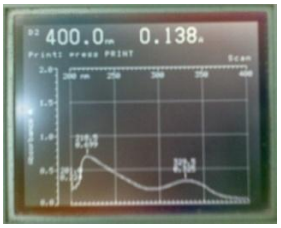
UV METHOD DEVELOPMENT

0.0132% (w/v) Piperaquine in water – stock solution



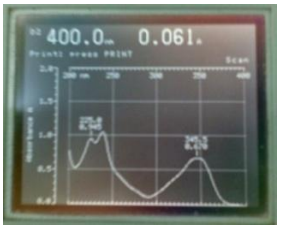
Wavelength (nm)	Absorbance
226.0	1.020
352.5	0.539

Figure 4.1: 5ml of stock solution diluted to 50ml with water.



Wavelength (nm)	Absorbance
218.0	0.699
329.5	0.325

Figure 4.2: 5ml of stock solution diluted to 50ml with 0.05M NaOH.



Wavelength (nm)	Absorbance
225.0	0.945
238.5	1.044
345.5	0.670

Figure 4.3: 5ml of stock solution diluted to 50ml with 0.1% (v/v) HCl.

4.2 CALIBRATION CURVES FOR UV ABSORPTION PEAKS OF PIPERAQUINE PHOSPHATE

Table 4.2: Absorbances of Piperaquine phosphate aqueous solution in 0.001M methanolic HCl at wavelengths of 225.0, 239.0 and 349.0nm

Concentration (%w/v)	Average Abs at wavelengths		
	225nm	239nm	349nm
0.0086	0.511 ± 0.0010	0.489 ± 0.0010	0.354 ± 0.0010
0.0101	0.679 ± 0.0010	0.647 ± 0.0012	0.466 ± 0.0010
0.0161	0.943 ± 0.0013	0.903 ± 0.0010	0.654 ± 0.0013
0.0202	1.334 ± 0.0015	1.284 ± 0.0010	0.932 ± 0.0012
0.0242	1.560 ± 0.0010	1.497 ± 0.0010	1.078 ± 0.0010
0.0403	2.332 ± 0.0010	2.265 ± 0.0015	1.653 ± 0.0010

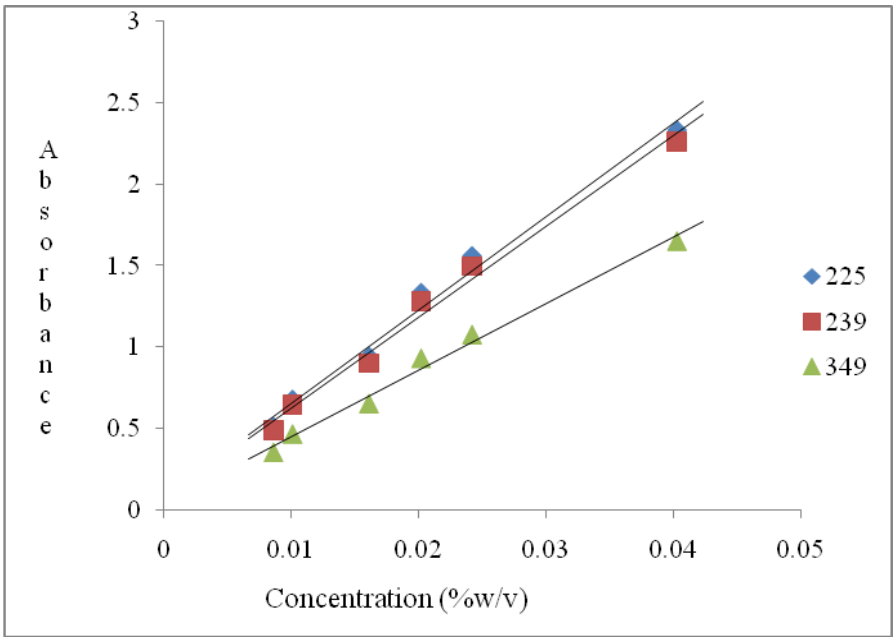


Figure 4.4: Graph of absorbance against concentration of Piperaquine phosphate aqueous solution in 0.001M methanolic HCl at wavelengths of 225.0, 239.0 and 349.0nm

4.2.1 STABILITY STUDIES FOR PIPERAQUINE PHOSPHATE IN SOLUTION

Table 4.3: Absorbances of Piperaquine phosphate solution in 0.001M HCl solution for a period of 2 days at a wavelength of 349.0nm

Concentration (%w/v)	Average absorbance Day1	Average absorbance Day 2
2.070×10^{-4}	0.118 ± 0.0010	0.081 ± 0.0012
4.130×10^{-4}	0.163 ± 0.0015	0.137 ± 0.0013
8.260×10^{-4}	0.292 ± 0.0010	0.244 ± 0.0010
1.652×10^{-3}	0.594 ± 0.0010	0.489 ± 0.0010
2.065×10^{-3}	0.753 ± 0.0015	0.622 ± 0.0010
3.304×10^{-3}	1.127 ± 0.0010	0.988 ± 0.0012

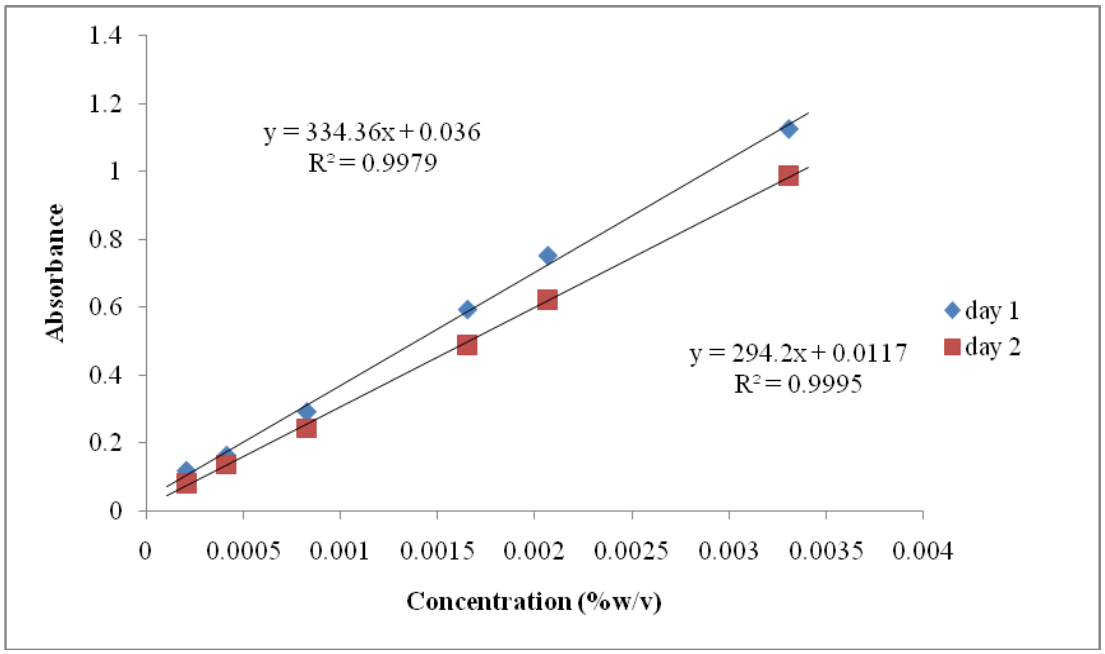


Figure 4.5: Graph of absorbances against concentration of Piperaquine phosphate in 0.001M HCl solution at 349.0nm for two days.

4.2.2 CALIBRATION CURVE FOR PIPERAQUINE PHOSPHATE(LINEARITY)

Table 4.4: Absorbance against concentration of Piperaquine Phosphate in 0.001M HCl solution at a wavelength of 349.0nm.

Concentration (%w/v)	Average Absorbance (n=3)
4.04×10^{-4}	0.122 ± 0.0010
8.08×10^{-4}	0.231 ± 0.0010
1.616×10^{-3}	0.456 ± 0.0012
2.424×10^{-3}	0.696 ± 0.0010
3.232×10^{-3}	0.932 ± 0.0015
4.04×10^{-3}	1.161 ± 0.0010

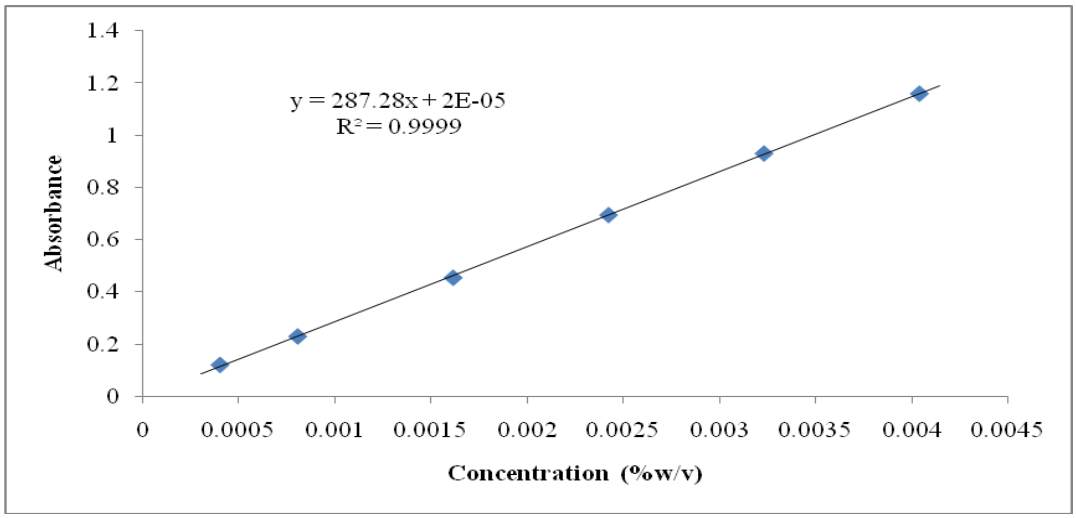


Figure 4.6: Graph of absorbance against concentration of Piperaquine Phosphate in 0.001M HCl solution at 349.0nm.

4.2.3 ASSAY OF PIPERAQUINE PHOSPHATE IN THE PRESENCE OF OTHER EXCEIPIENTS

Table 4.5: % content of Piperaquine phosphate in the presence of other excepients

Calculated concentration (% w/v)	Average absorbance	Mean % recovery	SD
8.16×10^{-4}	0.231	98.65	0.3570
2.04×10^{-3}	0.579	98.82	0.1706
3.28×10^{-3}	0.930	98.80	0.0888

4.2.4 ASSAY OF PIPERAQUINE PHOSPHATE IN TABLETS

Table 4.6: % content of Piperaquine Phosphate in Tablets Day 1

Calculated concentration (% w/v)	Average absorbance	Mean % content	SD	RSD
7.98×10^{-4}	0.259	93.79	0.3168	0.3378
1.99×10^{-3}	0.628	93.28	0.1071	0.1148
3.20×10^{-3}	1.001	93.66	0.2407	0.2578

Table 4.7: % content of Piperaquine Phosphate in Tablets Day 2

Calculated concentration (% w/v)	Average absorbance	Mean % content	SD	RSD
8.02×10^{-4}	0.261	93.28	0.2065	0.2214
2.01×10^{-3}	0.633	93.25	0.1504	0.1613
3.21×10^{-3}	1.004	93.43	0.1074	0.1150

Table 4.8 % content of Piperaquine phosphate in Tablets Day 3

Calculated concentration (% w/v)	Average absorbance	Mean % content	SD	RSD
8.20×10^{-4}	0.264	93.44	0.3292	0.3524
2.05×10^{-3}	0.645	93.48	0.1679	0.1796
3.28×10^{-3}	1.028	93.69	0.0770	0.0822

LOD = $(3.3\sigma)/ S$

$= (3.3 \times 0.00484) / 287.28$

$= 5.56 \times 10^{-5}$ %w/v

LOQ = $(10\ \sigma)/ S$

$= (10 \times 0.00484) / 287.28$

$= 1.68 \times 10^{-4}$ %w/v

4.3 HPLC METHOD DEVELOPMENT

Table 4.9: Retention times and peak tailing factor of the different mobile phases

Mobile Phase	Retention time (minutes)	Peak tailing factor
Acetonitrile: Water with 0.1%v/v TFA (50:50)	2.912	3.875
Acetonitrile: Water with 0.1%v/v TFA (40:60)	3.213	1.5
Acetonitrile: Water with 0.1%v/v TFA (60:40)	3.035	2.6
Acetonitrile: Water with 0.1%v/v TFA (20:80)	5.880	2.583

Table 4.10: Peak area ratio against concentration of Piperaquine phosphate

Concentration (%w/v)	Peak area of piperaquine phosphate	Peak area of standard	Peak area ratio
0.0017	4829249	3169922	1.52
0.00136	3859631	3104344	1.24
0.00102	3178342	3167446	1.00
0.00068	2382460	3176520	0.75
0.00034	1520035	3175572	0.48

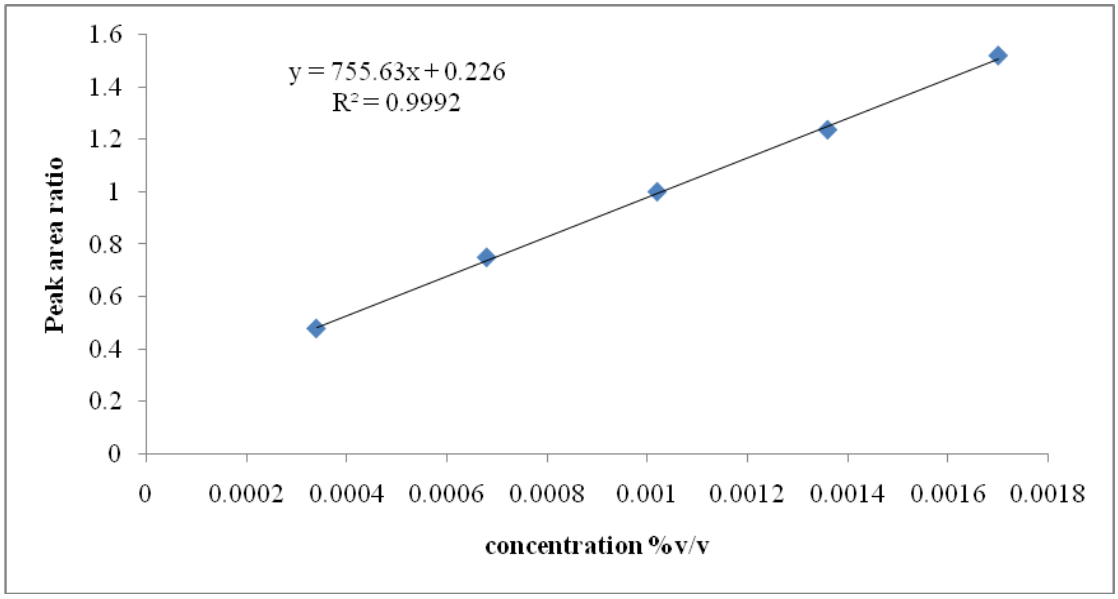


Figure 4.7: Graph of peak area ratio against concentration of Piperaquine phosphate

LOD = (3.3σ)/ S

= (3.3 x 0.011232) / 755.63

= 4.9 x 10^{-5} %w/v

LOQ = (10 σ)/ S

= (10 x 0.011232) / 755.63

= 1.48 x 10^{-4} %w/v

4.3.1 ASSAY OF PIPERAQUINE PHOSPHATE IN TABLETS

Table 4.11: % content of Piperaquine Phosphate in Tablets

Day	Average % content	SD	RSD
1	89.72	0.5340	0.5953
2	90.33	0.5926	0.6560
3	90.51	0.7035	0.7772

DIHYDROARTEMISININ

Table 4.12 Preliminary tests for Dihydroartemisinin

Test	Results
Identification test	A violet colour was immediately produced when the potassium iodide, sulphuric acid and starch were added to the Dihydroartemisinin powder dissolved in ethanol.
Solubility test	A clear solution was obtained when methanol, ethanol, ethyl acetate, acetonitrile, acetic acid, petroleum ether and diethyl ether was added to Dihydroartemisinin. A solution with visible particles was observed when water was used.
Melting point:	158 – 160^{O}C
Thin layer chromatography	Rf value: = 0.4906

UV METHOD DEVELOPMENT

4.4 Derivatisation of Dihydroartemisinin with NaOH

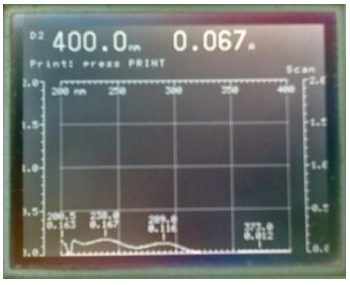
0.0125% (w/v) Dihydroartemisinin in ethanol – stock solution

Table 4.13: Absorbances and wavelengths of stock solution heated for 30minutes at 50^{o}C

Wavelength (nm)	Absorbance
201.5	0.168
205.5	0.130
209.0	0.179
214.5	0.305
240.5	0.286
289.5	0.706
376.0	0.034

Table 4.14: Absorbances and wavelengths of stock solution not heated

Wavelength (nm)	Absorbance
205.0	0.114
209.0	0.007



Wavelength (nm)	Absorbance
238.5	0.167
289.0	0.116

Figure 4.8: 5.00ml of 0.005% (w/v) Dihydroartemisinin in ethanol diluted to 50.00ml with 0.05M NaOH and heated for 30minutes at 50^{o}C

Wavelength (nm)	Absorbance
240.0	1.296
289.5	0.024

Figure 4.9: 5.00ml of 0.2%(w/v) dihydroartemisinin in methanol topped up to 50.00ml with 0.05M NaOH and heated at 50°C for 30minutes

Table 4.15: absorbances and concentrations of DHA in methanol and 0.05M NaOH heated at 50°C at a wavelength of 289.5nm.

Average Absorbance (n=3)	Concentration %w/v)
0.078 ± 0.0010	0.022
0.218 ± 0.0010	0.044
0.255 ± 0.0015	0.055
0.474 ± 0.0013	0.110
0.896 ± 0.0012	0.220

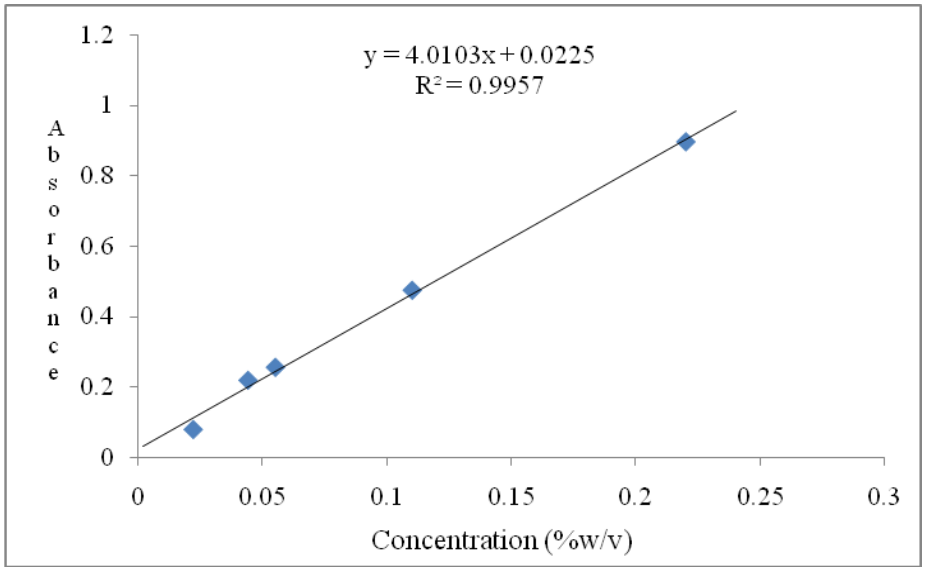


Figure 4.10: A graph of absorbance against concentration of DHA in methanol / 0.05M NaOH heated at 50°C at 289.5nm.

4.4.1 VARIATION OF VOLUMES OF NAOH

Table 4.16: Volumes and absorbances of 0.05MNaOH in a 50ml solution of 0.1046% w/v DHA in Methanol heated at 50^0C for 30minutes at a wavelength of 289.0nm

Volume (ml)	Average Absorbance (n=3)
10	0.665 ± 0.0010
15	0.893 ± 0.0010
20	1.004 ± 0.0015
25	1.477 ± 0.0010
30	1.203 ± 0.0010
35	1.128 ± 0.0013
40	0.837 ± 0.0010
45	0.444± 0.0010

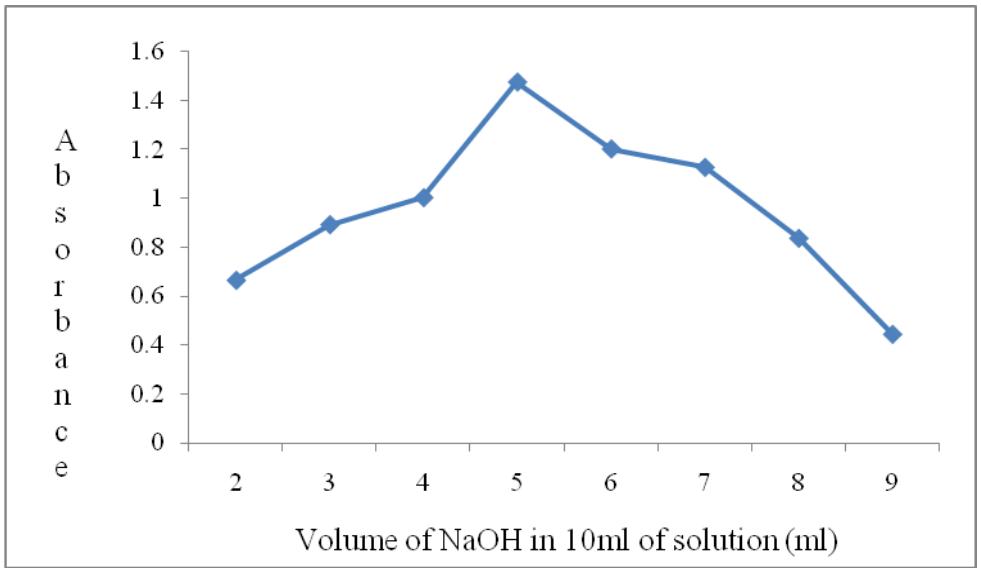


Figure 4.12: Effect of increasing volumes of 0.05MNaOH in a solution of 0.1046% w/v DHA in Methanol heated at 50^0C for 30minutes on absorbance at a wavelength of 289.0nm

Table 4.17: Absorbances of the two different ratios of DHA in methanol to NaOH solutions heated at 50^0C

Time	Average absorbance of 1ml DHA in methanol : 9ml NaOH (n=3)	Average absorbance of 5ml DHA in methanol : 5ml NaOH (n=3)
10	0.111± 0.0010	0.464± 0.0010
20	0.164± 0.0010	0.548± 0.0010
30	0.215± 0.0015	0.627± 0.0015
40	0.238± 0.0010	0.868± 0.0015
50	0.275± 0.0012	0.989± 0.0010
60	0.298± 0.0010	1.050± 0.0013

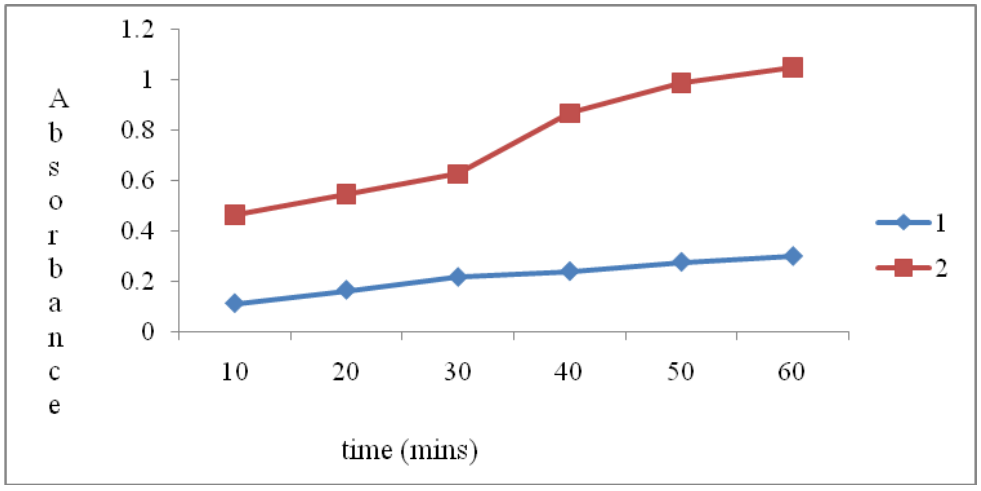


Figure 4.13: Effect of increasing heating time at 50^0C of a solution of 0.0526 %w/v DHA in methanol : 0.2%w/v NaOH on absorbance

Ratio of DHA in methanol to NaOH = 1(1:9) and 2(5:5)

4.4.2 VARIATION OF CONCENTRATIONS OF NaOHFOR DERIVATISATION OF DIHYDROARTEMISININ

Table 4.18: Absorbances of DHA in methanol to different concentrations of NaOH solutions heated at 50^0C for 30minutes.

Concentration (M)	Average Absorbance after standing for 60 minutes
0.02	0.294 ± 0.0015
0.04	0.474 ± 0.0012
0.08	0.813 ± 0.0010
0.10	0.924± 0.0010
0.12	0.961 ± 0.0013
0.20	0.984± 0.0010

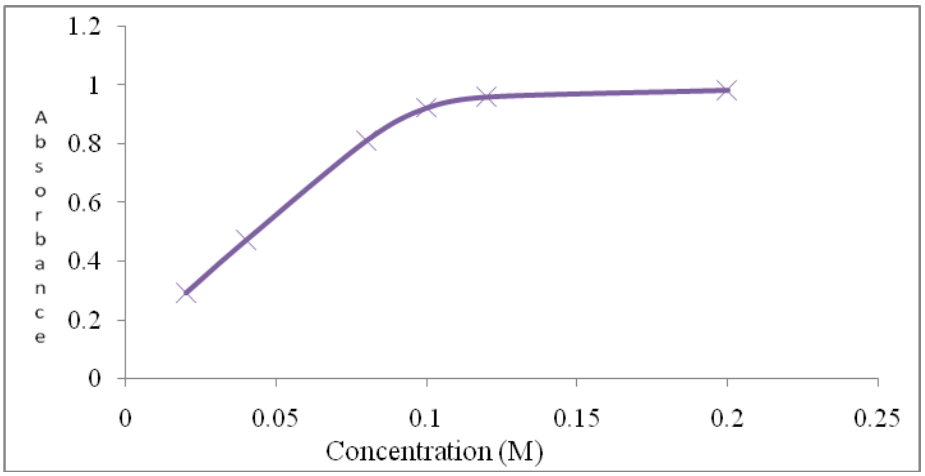


Figure 4.14: Graph of absorbance of DHA in methanol against different concentrations of NaOH solution heated for 30minutes and left standing for 60 minutes.

Table 4.19: Absorbances of DHA in methanol to different concentrations of NaOH solutions heated at 50^0C for 30minutes and left standing for 0-60minutes.

Concentration (M)	Average Absorbance 0 minutes	Average Absorbance 20 minutes	Average Absorbance 40 minutes	Average Absorbance 60 minutes
0.02	0.241± 0.001	0.263± 0.001	0.280± 0.001	0.294± 0.0012
0.04	0.407± 0.0013	0.429± 0.0010	0.450± 0.0010	0.474± 0.0017
0.08	0.716± 0.0010	0.751± 0.0010	0.782± 0.0013	0.813± 0.0010
0.10	0.789± 0.0010	0.840± 0.0015	0.888± 0.0012	0.924± 0.0010
0.12	0.853± 0.0012	0.895± 0.0010	0.926± 0.0010	0.961± 0.0010
0.20	0.904± 0.0015	0.922± 0.0013	0.944± 0.0010	0.984± 0.0010

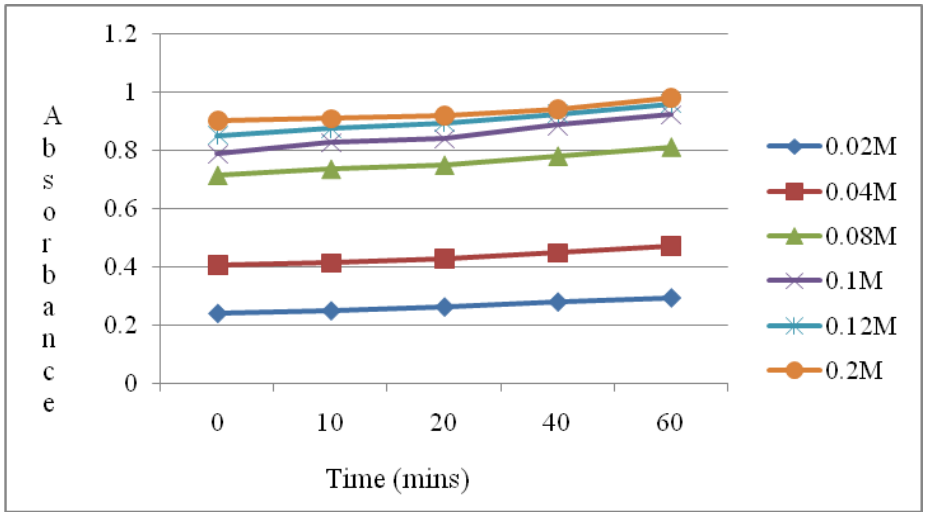


Figure 4.15: Graph of absorbance of DHA in methanol against different concentrations of NaOH solution heated for 30minutes and left standing for 60 minutes.

4.4.3 INVESTIGATION OF THE ADDITION OF DIFFERENT CONCENTRATIONS OF ACETIC ACID TO THE DERIVATIVE OF DIHYDROARTEMISININ AND NaOH

0.0503% (w/v) DHA in MeOH/ 0.05M NaOH heated at 50^0C for 30minutes.(stock solution) The absorbance was taken at 289.0nm after 10, 20 and 30minutes and then after30minutes, acetic acid was added to the solution and then the absorbance taken again.

Table 4.20: Absorbances and time of DHA in methanol and 0.05M NaOH heated at 50^oC at a wavelength of 289.0nm and 1M acetic acid added after 30minutes.

Time	Average Absorbance (n=3)
10	0.976 ± 0.0015
20	0.994 ± 0.0010
30	1.023 ± 0.0010
40	0.273 ± 0.0012

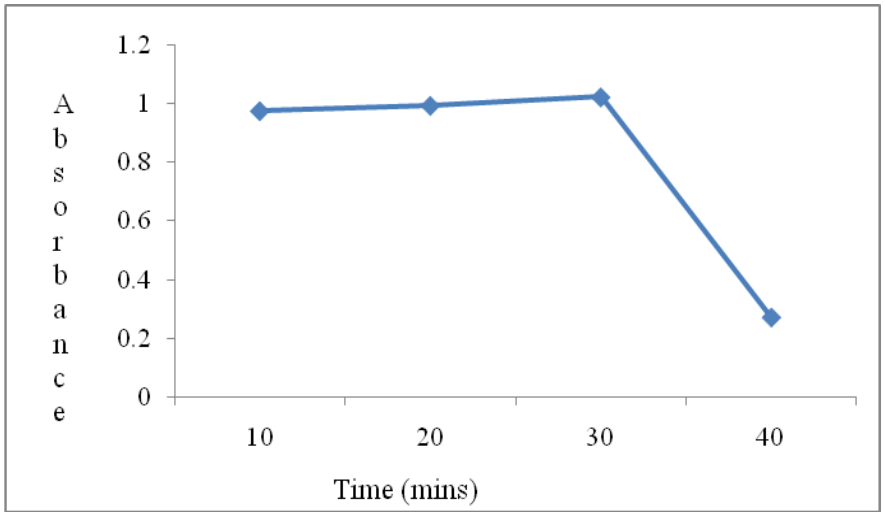


Figure 4.16: 5ml of 1M acetic added to stock solution

Table 4.21: absorbances and time of DHA in methanol and 0.05M NaOH heated at 50°C at a wavelength of 289.0nm and 1M acetic acid added after 30minutes.

Time	Average Absorbance (n=3)
10	0.766 ± 0.0010
20	0.797 ± 0.0013
30	0.839± 0.0010
40	0.760± 0.0017
50	0.772± 0.0013
60	0.779± 0.0015

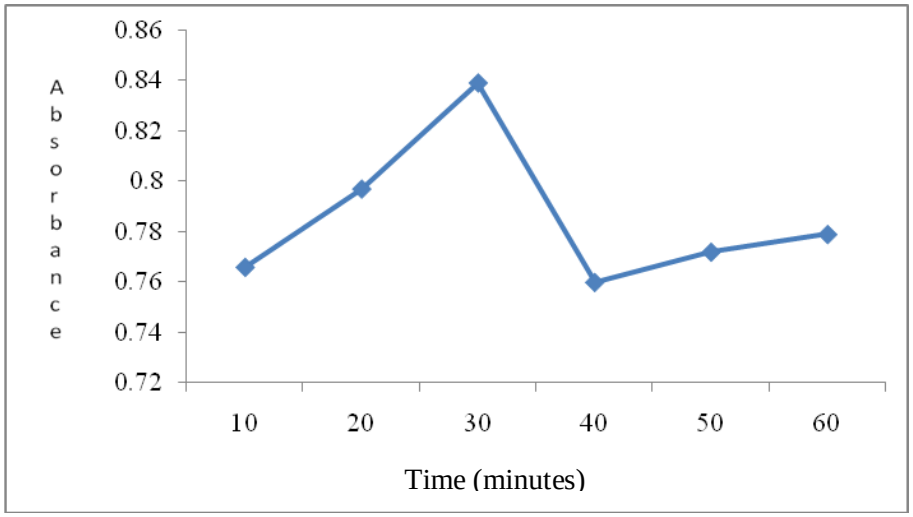


Figure 4.17: 1ml of 1M glacial acetic added to stock solution:

Table 4.22: Absorbances and time of DHA in methanol and 0.05M NaOH heated at 50°C at a wavelength of 289.0nm and 0.5M acetic acid added after 30minutes.

Time	Average Absorbance (n=3)
10	0.776 ± 0.0010
20	0.816 ± 0.0015
30	0.840 ± 0.0010
40	0.547 ± 0.0012
50	0.541 ± 0.0010
60	0.543 ± 0.0010

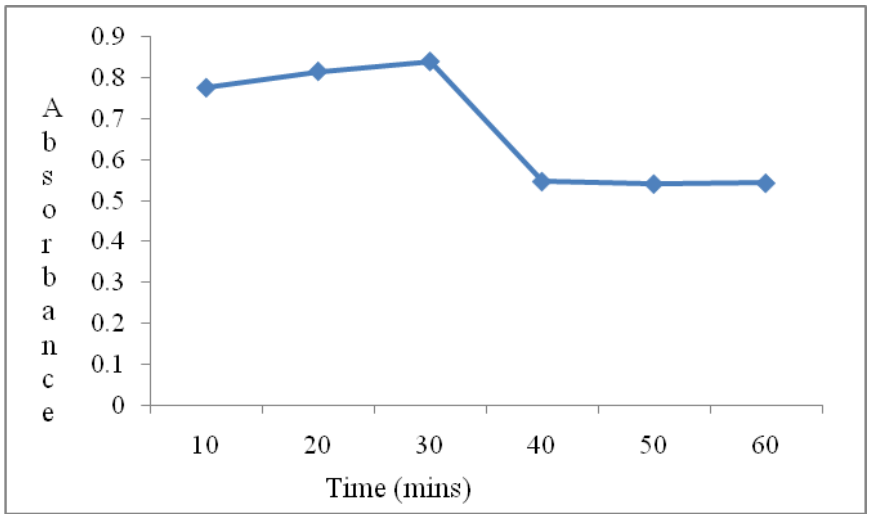


Figure 4.18: 5ml of 0.5M glacial acetic added to stock solution:

Table 4.23: absorbances and time of DHA in methanol and 0.05M NaOH heated at 50°C at a wavelength of 289.0nm and 0.1M acetic acid added after 30minutes.

Time	Average Absorbance (n=3)
10	0.826± 0.0010
20	0.847± 0.0015
30	0.862± 0.0010
40	0.554± 0.0012
50	0.560± 0.0010
60	0.562± 0.0010

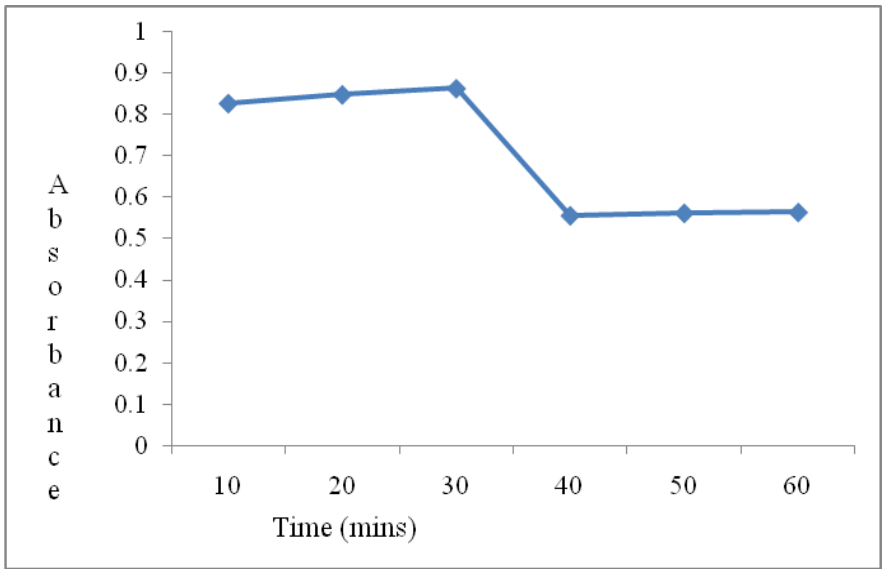
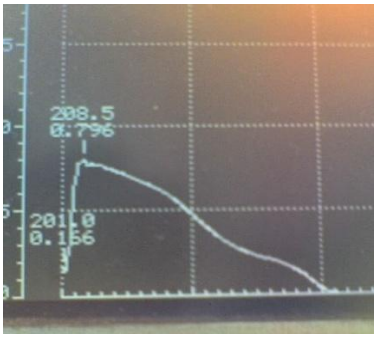


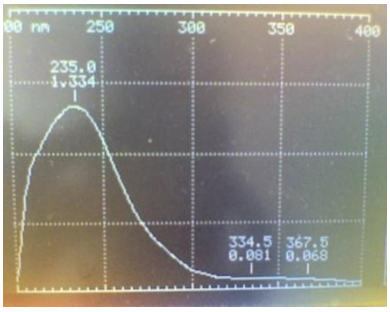
Figure 4.19: 5ml of 0.1M glacial acetic added to stock solution

4.4.4 DERIVATISATION OF DIHYDROARTEMISININ WITH AMMONIA (NH_3)



Wavelength (nm)	Absorbance
201.0	0.166
208.5	0.796

Figure 4.20: 5ml of 0.0253% DHA in MeOH and 2ml 0.7M NH_3 heated at 50^{O}C for 30minutes



Wavelength (nm)	Absorbance
235.0	1.334
334.5	0.081
367.5	0.068

Figure 4.21: 5ml of 0.0253% DHA in MeOH and 2ml 0.7M NH_3 heated at 60^{O}C for 30minutes

Table 4.24: 5ml of 0.0253% DHA in MeOH / 2ml of NH_3 (0.7M) and heated at 60^{0}C for :

Time (minutes)	Wavelength (nm)	Absorbance
30	235.0	1.334
40	235.0	1.432
50	235.0	1.561

Table 4.25: 5ml of 0.0253% DHA in MeOH / 2ml of NH_3 (4M) and heated for 30minutes at:

Temperature ^{0}C	Wavelength (nm)	Absorbance
60	239.5	2.053
70	232.5	2.266

4.4.5 STABILITY OF THE DERIVATIVE FORMED FROM DIHYDROARTEMISNIN AND HCl

Table 4.26: 5ml of 0.0054% DHA in MeOH / 2ml of NH_3 (4M) and heated at 70^0C.

Time (minutes)	Average Absorbance
10	0.542 ± 0.0010
20	0.689 ± 0.0010
30	0.702 ± 0.0012
50	0.733 ± 0.0015
60	0.798 ± 0.0010

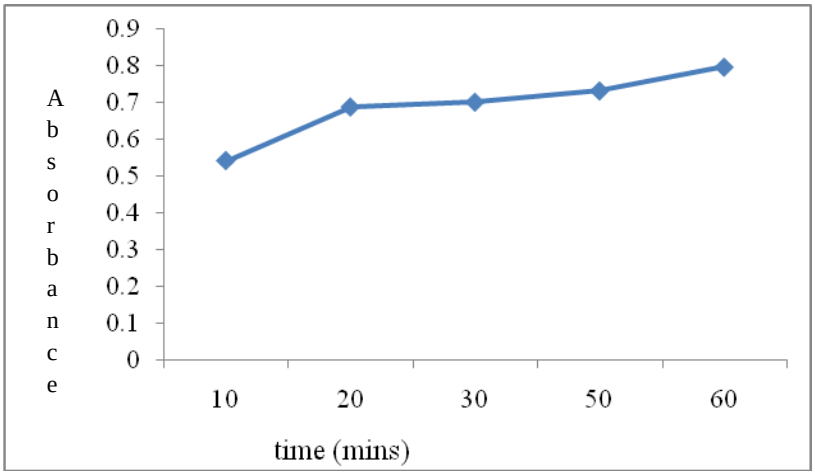
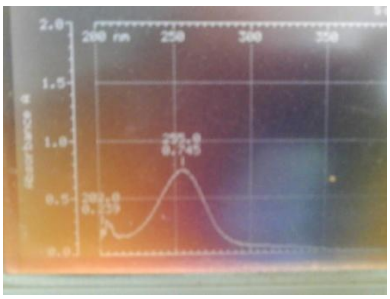
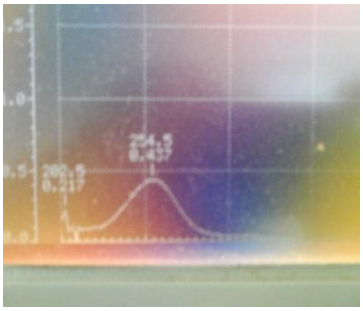


Figure 4.22: 5ml of 0.0054% DHA in MeOH / 2ml of 2M NH_3 heated at 70^0C for 30minutes and absorbance taken at 236nm when left to stand.



Wavelength nm	Absorbance
202	0.239
255	0.745

Figure 4.23: 2ml pure DHA in MeOH +3ml conc HCl left for 30minutes



Wavelength nm	Absorbance
202.5	0.217
254.5	0.437

Figure 4.24: 2ml of extracted DHA in MeOH +3ml conc HCl left for 30minutes

Table 4.27: Absorbances and time of 2ml DHA in MeOH and 1ml of HCl.

Time (minutes)	Average Absorbance
10	0.043 ± 0.0012
20	0.130 ± 0.0010
30	0.166 ± 0.0012
40	0.204 ± 0.0012
50	0.249 ± 0.0012
60	0.281 ± 0.0013

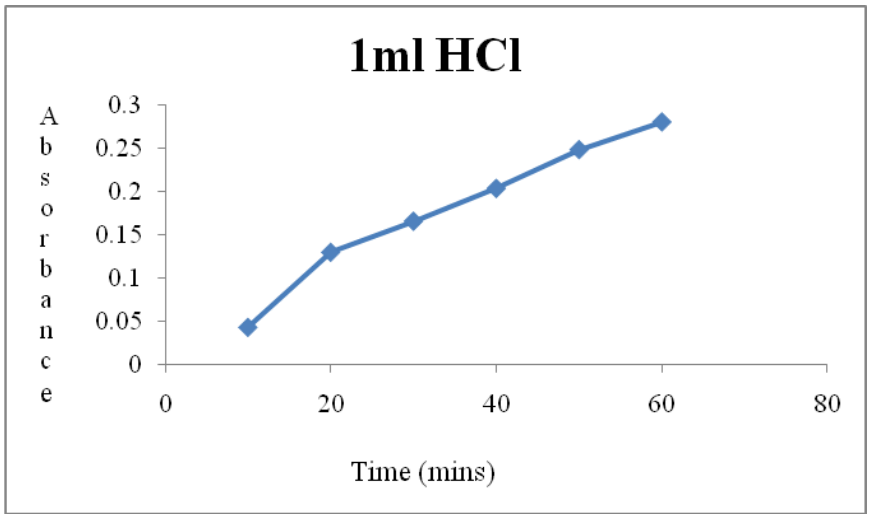


Figure 4.25: Graph of absorbance against time (minutes) of 2ml DHA in MeOH and1ml HCl

Table 4.28: Absorbances and time of 2ml DHA in MeOH and 2ml of HCl.

Time (minutes)	Average Absorbance
10	0.315 ± 0.0010
20	0.509 ± 0.0010
30	0.546 ± 0.0015
40	0.615 ± 0.0015
50	0.654 ± 0.0010
60	0.674 ± 0.0010

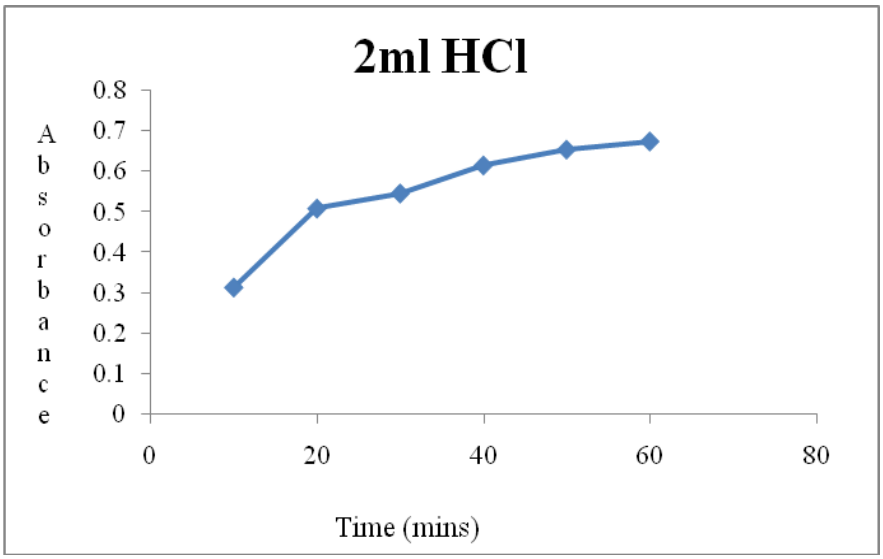


Figure 4.26: Graph of absorbance against time (minutes)of 2ml DHA in MeOH and 2ml HCl

Table 4.29: Absorbances and time of 2ml DHA in MeOH / 3ml of HCl.

Time (minutes)	Average Absorbance
10	0.619 ± 0.0012
20	0.725 ± 0.0012
30	0.730 ± 0.0010
40	0.731 ± 0.0012
50	0.731 ± 0.0015
60	0.732 ± 0.0012

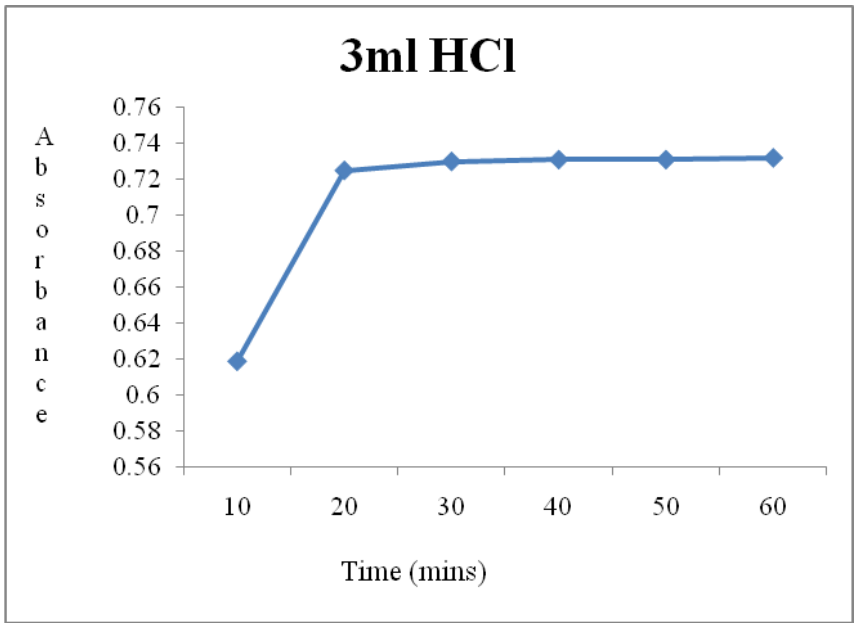


Figure 4.27: Graph of absorbance against time (minutes)of 2ml DHA in MeOH and 3ml HCl

Table 4.30: Absorbances and time of 2ml DHA in MeOH and 4ml of HCl.

Time (minutes)	Average Absorbance
10	0.588 ± 0.0012
20	0.646 ± 0.0010
30	0.709 ± 0.0010
40	0.709 ± 0.0010
50	0.710 ± 0.0012
60	0.710 ± 0.0015

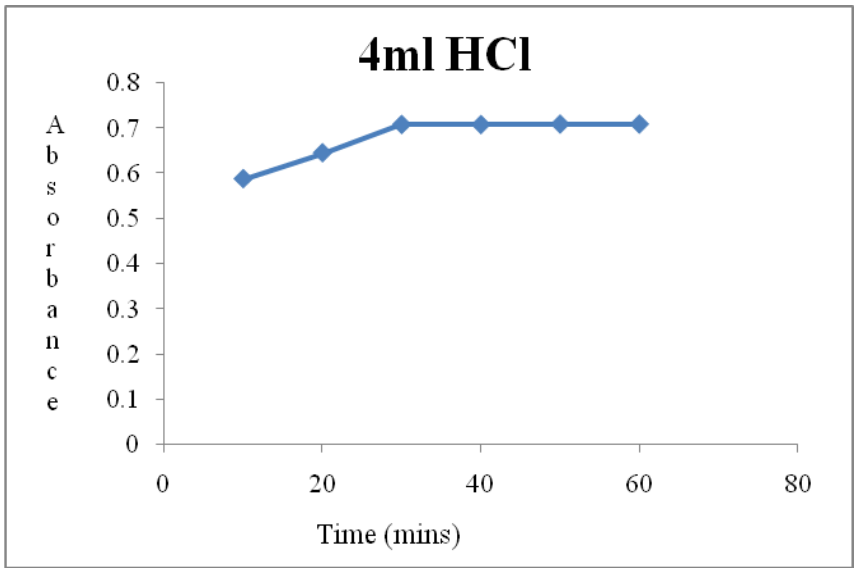


Figure 4.28: Graph of absorbance against time (minutes) of 2ml DHA in MeOH / 4ml HCl

Table 4.31: Table of absorbance against amount of HCl added to 2ml of DHA stock solution

Amount of HCl (ml)	Average Absorbance
1	0.130
2	0.545
3	0.736
4	0.883

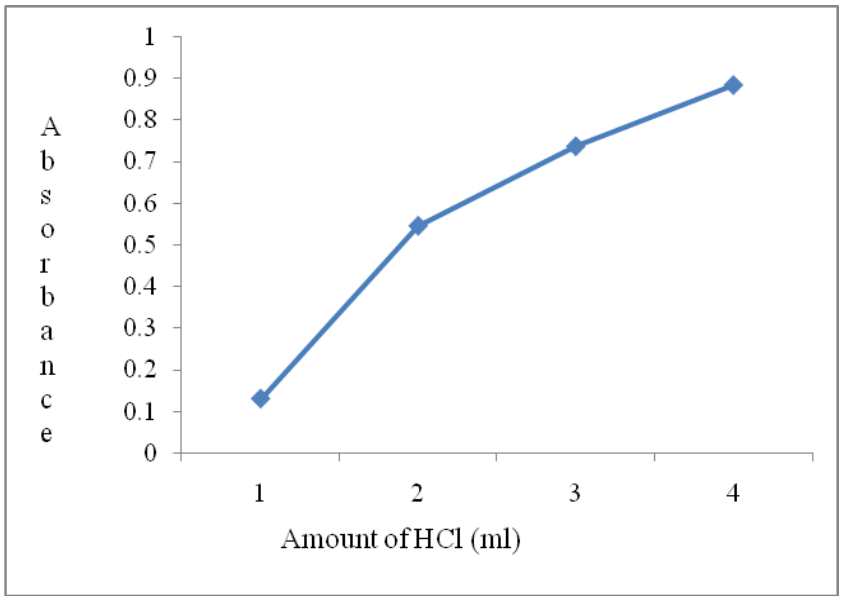


Figure 4.29: Graph of absorbance against amount of HCl added to 2ml of DHA stock solution

4.4.6 CALLIBRATION CURVE FOR DERIVATIVE OF DIHYDROARTEMISININ AND HCl (LINEARITY)

Table 4.32: Absorbance against concentration of DHA in MeOH/ HCl

Concentration (%w/v)	Average Absorbance
4.1×10^{-4}	0.169 ± 0.0012
8.2×10^{-4}	0.310 ± 0.0010
1.22×10^{-3}	0.443 ± 0.0010
1.64×10^{-3}	0.566 ± 0.0015
2.05×10^{-3}	0.677 ± 0.0010

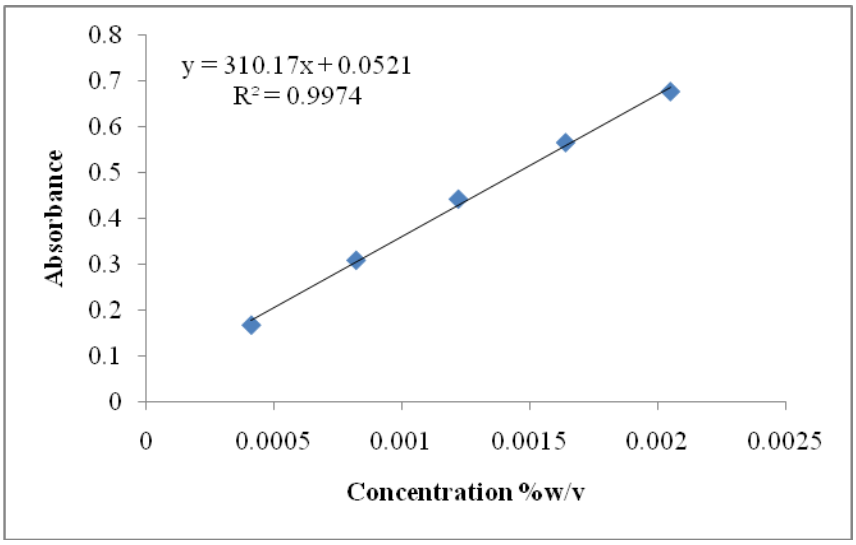


Figure 4.29: Graph of absorbance against concentration of DHA in MeOH/conc HCl solution

DHA in presence of other exciepients

Weight of pure sample DHA = 0.0197g

Weight of empty petri dish = 44.2531g

Weight of dish + residue = 44.2709g

Weight of residue = 0.0178g

% recovery = (0.0178 / 0.0197) x 100%

= 90.36%

Table 4.33: % content of DHA in Tablet A (Day 1)

Calculated concentration (% w/v)	Average absorbance	Mean % content	SD	RSD
7.24×10^{-4}	0.256 ± 0.0010	90.50	0.2598	0.2871
9.66×10^{-4}	0.323 ± 0.0010	90.79	0.5778	0.6359
1.45×10^{-3}	0.460 ± 0.0015	90.67	0.3361	0.3706

Table 4.34: % content of DHA in Tablet A (Day 2)

Calculated concentration (% w/v)	Average absorbance	Mean % content	SD	RSD
7.40×10^{-4}	0.295 ± 0.0010	91.27	0.2656	0.2910
9.84×10^{-4}	0.359 ± 0.0012	91.04	0.2021	0.2220
1.48×10^{-3}	0.490 ± 0.0012	90.54	0.1328	0.1467

Table 4.35: % content of DHA in Tablet A (Day 3)

Calculated concentration (% w/v)	Average absorbance	Mean % content	SD	RSD
7.12×10^{-4}	0.252 ± 0.0012	91.12	0.6928	0.7603
9.50×10^{-4}	0.320 ± 0.0012	90.85	0.4997	0.5501
1.42×10^{-3}	0.454 ± 0.0010	91.07	0.4732	0.5196

LOD = $(3.3\sigma)/ S$

= $(3.3 \times 0.0103) / 310.17$

= 1.09×10^{-4} %w/v

LOQ = $(10\ \sigma)/ S$

= $(10 \times 0.0103) / 310.17$

= 3.31×10^{-4} %w/v

Table 4.36: % content of DHA in Tablet B (Day 1)

Calculated concentration (% w/v)	Average absorbance	Mean % content	SD	RSD
4.99×10^{-4}	0.156 ± 0.0012	90.51	0.5802	0.6410
7.48×10^{-4}	0.239 ± 0.0015	92.37	0.5912	0.6400
1.50×10^{-3}	0.456 ± 0.0015	88.18	0.2956	0.3352

Table 4.37: % content of DHA in Tablet B (Day 2)

Calculated concentration (% w/v)	Average absorbance	Mean % content	SD	RSD
4.91×10^{-4}	0.154 ± 0.0015	90.41	0.6808	0.7531
7.36×10^{-4}	0.230 ± 0.0012	90.21	0.4542	0.5035
1.47×10^{-3}	0.451 ± 0.0015	88.76	0.1135	0.1279

Table 4.38: % content of DHA in Tablet B (Day 3)

Calculated concentration (% w/v)	Average absorbance	Mean % content	SD	RSD
4.77×10^{-4}	0.148 ± 0.0010	89.82	0.6069	0.6757
7.16×10^{-4}	0.221 ± 0.0010	89.36	0.8087	0.9050
1.43×10^{-3}	0.438 ± 0.0012	88.62	0.1167	0.1317

4.4.7 HPLC METHOD DEVELOPMENT

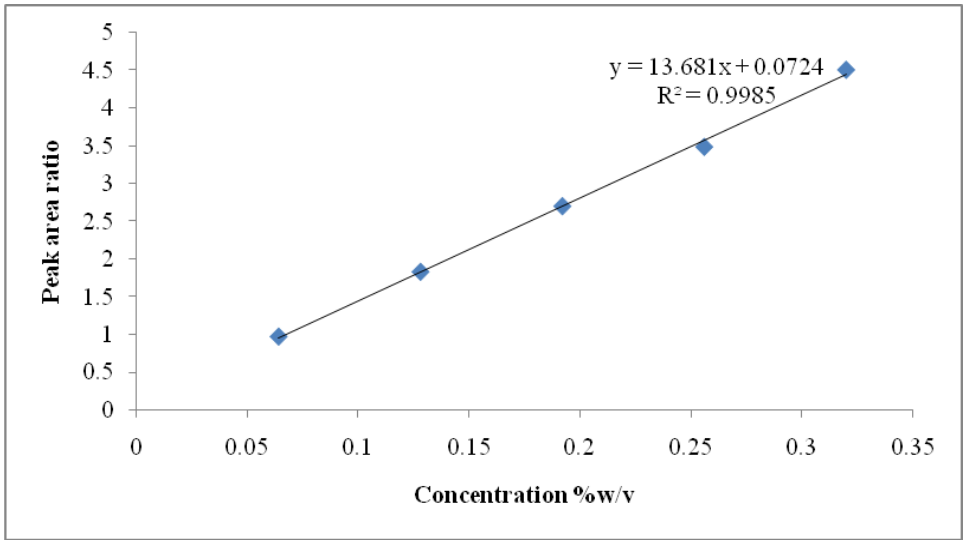
Retention time for mobile phase of Acetonitrile: water with 0.1%v/v TFA (90 : 10) at a flow rate of 1.0ml/min at 220nm was 3.853minutes.

4.4.8 CALIBRATION CURVE FOR DIHYDROARTEMISNIN (LINEARITY)

Table 4.39: Table of peak area against concentration of DHA

Concentration %w/v	Peak Area of DHA	Peak area of internal standard	Peak area ratio
0.32	2027828	449287	4.51
0.256	1568050	449488	3.49
0.192	1214413	449654	2.70
0.128	822344	450070	1.83
0.064	432375	447544	0.97

Figure 4.31: Graph of peak area ratio against concentration of DHA



LOD = (3.3σ)/ S

= (3.3 x 0.054221) / 13.681

= 0.013 %w/v

LOQ = (10 σ)/ S

= (10 x 0.054221) / 13.681

= 0.040%w/v

4.6.1 Assay of Dihydroartemisinin in Tablets

Table 4.40: % content of Dihydroartemisinin in Tablets A

Day	Average % content	SD	RSD
1	82.60	1.1879	1.4381
2	81.37	0.9764	1.1998
3	81.93	0.5188	0.6332

Table 4.41: % content of Dihydroartemisinin in Tablets B

Day	Average % content	SD	RSD
1	83.58	0.8507	1.0178
2	83.99	0.4758	0.5666
3	84.09	0.5829	0.6931

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 DISCUSSION

5.1.1 Physical characteristics of tablets

Uniformity of weight: Not more than 2 Tablets of A and B deviated from the average mass by 7.5% and none of the also deviated by twice that percentage. According to the standards of the British Pharmacopoeia 2005 (Page 28), Tablets A and B passed the uniformity of weight test.

Disintegration test: The disintegration time for most uncoated Tablets is 30minutes and for coated Tablets is normally up to 120 minutes (**Gennaro, 1990**). Tablet A is film coated and its disintegration time was 7.83minutes while Tablet B is uncoated and its disintegration time was found to be 2.27minutes. Both times were within the limits as stipulated by the BP 2005 and therefore both Tablets passed the test.

5.2 PIPERAQUINE PHOSPHATE

5.2.1 Preliminary tests for Piperaquine phosphate

Absorbing substances will only show absorbances when they are in solution. If they do not dissolve in a particular solution, there will be no absorbance. Piperaquine Phosphate shows absorbances in water, methanol but does not show any absorbance in petroleum ether, diethyl ether. Therefore it dissolves in water, methanol but not in petroleum ether or diethyl ether. In water, it dissolves completely leaving a clear solution but when it was dissolved in methanol, some of the particles settled below leaving a clear solution. Therefore water was chosen as a more suitable solvent in this case. A yellow precipitate was formed which dissolved on addition of ammonia when silver nitrate was added to the solution which showed the presence of a phosphate. It had a melting point of 248 – 250^{O}C which showed that it was a pure sample since the range was small and also confirmed its identity from literature which was 246 – 252^{O}C.

5.2.2 Determination of UV absorption spectrum of Piperaquine phosphate in acid, base and water.

Piperaquine Phosphate showed two peaks when it was dissolved in water (Figure 4.1) and 0.05M NaOH solution (Figure 4.2) and it showed 3 peaks when it was dissolved in 0.001M HCl solution (Figure 4.3). From literature (**Hung, Daus et al., 2003**), Piperaquine exhibits three peaks which are 225, 235 and 340nm and so the acidic medium was chosen.

5.2.3 Calibration curves for the UV absorption peaks for Piperaquine phosphate

A calibration curve was drawn for Piperaquine Phosphate using an aqueous solution of Methanolic HCl (Figure 4.4). Three different curves were drawn using the three different peaks at the wavelengths of 225.0nm, 239.0nm and 349.0nm. At 225.0nm, $y = 57.283x + 0.0856$ and $R^2 = 0.9871$, at 239.0nm, $y = 55.895x + 0.0676$ and $R^2 = 0.9885$ and at 349.0nm, $y = 40.865x + 0.0423$ and $R^2 = 0.9899$. Out of the three peaks, a wavelength of 349nm was chosen because at such a high wavelength very few compounds would absorb and therefore cause less interference.

5.2.4 Stability studies for Piperaquine phosphate in solution

The calibration curve was drawn using the wavelength of 349.0nm. The solution used was left to stand for 24hours and a calibration curve was drawn again (Figure 4.5). The second curve showed a drop in absorbance compared to the first but no change in the number of peaks. This shows that if the sample in solution is left to stand for a long time, its absorbance will change and repeatable results may not be obtained, so it is preferable to work with the solution when it is freshly prepared.

5.2.5 Calibration curve for Piperaquine phosphate (Linearity)

In method development, the simplest method is often preferred. The previous method required dissolving the powder in water before adding an amount of methanolic HCl. To make the method simpler, methanol was excluded. The calibration curve was drawn using the sample dissolved in a solution of HCl and similar results to the previous method were obtained. The simpler method was therefore chosen (Figure 4.6). From the graph, $y = 287.2x + 2E\ -5$ and $R^2 = 0.999$.

5.2.6 Assay for Piperaquine phosphate in the presence of excipients

Apart from the active ingredient in a Tablet, there are other substances included in its formulation and these are referred to as the excipients. They are added to improve the quality of its formulation. The methyl cellulose is acting as a binder. It is included in the Tablet to ensure structural integrity and provide free-flowing powders. It may interact with the active drug such that its release in a form suitable for absorption may be retarded. The talc is acting as a lubricant. This is used to prevent the tablets from sticking to punches and dies in the tablet machine. They are water repellent and may interfere with the wettable nature of the disintegrating tablet, thus with the rate at which the drug goes into solution. The starch is acting as a diluents or filler. It gives the powder bulk so that an acceptable size is produced (Olaniyi, 2000). An amount of each was weighed in addition to the Piperaquine phosphate according to their proportions and the absorbance taken. This was to determine if the excipients also showed absorbances which might interfere with the absorbances of the Piperaquine Phosphate. The percentage recovery obtained for the three different concentrations showed that the excipients do not interfere with the absorbances of the pure sample (Table 4.5).

The assay was done for three days (interday assay). Each assay was done for three different concentrations and for each concentration, the assay was repeated three times (intraday) and the average calculated. The mean percentage contents, standard deviations (SD), relative standard deviations (RSD) were calculated. From Tables 4.6, 4.7 and 4.8 the standard deviations and the relative standard deviations were all below 2%, the linearity and the percentage recovery were all within the limits of the guidelines of ICH (Page 29). The LOD was 5.56×10^{-5} %w/v and the LOQ was 1.68×10^{-4} %w/v.

5.2.7 HPLC METHOD DEVELOPMENT

Acetonitrile was chosen for this work based on the previous works done on Piperquine phosphate which used acetonitrile. At 349nm, there was no interference with the absorption of Piperaquine phosphate by other compounds so it was chosen. Piperaquine phosphate is based on the structure of chloroquine and it is a large molecule which makes it difficult to move through a stationary phase easily even in solution. The accuracy of quantitation decreases with increase in peak tailing. A tailing factor of less than or equal to 2 is desired (Table 4.9). The peaks for Piperaquine phosphate in Figures 6.5; 6.6 and 6.8 showed tailing factors of more than 2.

Therefore the mobile phase chosen for HPLC analysis of Piperaquine phosphate was Acetonitrile : Water with 0.1%v/v TFA (40 : 60), Figure 6.7, at a flow rate of 1.0ml/min at 349nm. It had a retention time of 3.241 ± 0.015minutes. The same concentration was injected several times to show that the method was reproducible (Figure 6.9). A calibration curve of peak area ratio against concentration was drawn for Piperaquine phosphate (Figure 4.8). y= 755.63x + 0.226 and R2= 0.9992. The LOD was 4.9 x 10^{-5} %w/v and the LOQ was 1.48 x 10^{-4} %w/v.

Tablets A were analysed and Figure 6.10 shows the peak of Piperaquine phosphate present in the Tablets without interference from any excepients. This shows that the method is specific to only Piperaquine phosphate. Table 4.31 shows the % content, standard deviation and relative standard deviations for 3 days which were all within the limits of the ICH guidelines.

5.2.8 Comparing the UV method to the HPLC method

The null hypothesis is an assumption that there is no significant difference between the values being compared. If the null hypothesis is rejected, then there is said to be a significant difference between the values.

The critical value for a two tailed f-test (f_{tab}) at p=0.05 is 39.30 and the calculated value for f_{cal} was 3.3897. This shows that there is no significant difference between the two variances and hence between the precisions of the two methods and the null hypothesis is accepted.

The critical value of $|t|$ (t_{tab}) for 7 degrees of freedom at p=0.05 is 2.36 and the calculated value for t_{cal} was 4.6276. This shows that there is a significant difference between the two means and hence between the means of the two methods and the null hypothesis is rejected. Therefore the HPLC method is preferred.

5.3 DIHYDROARTEMISININ

5.3.1 Preliminary tests on Dihydroartemisinin

The violet colour produced when the starch was added to a solution of dissolved Dihydroartemisinin in ethanol, sulphuric acid and potassium iodide shows the presence of Dihydroartemisinin in the solution.

When a sample is dissolved in a particular reagent, a clear solution shows that everything has dissolved and therefore it is soluble. This shows that Dihydroartemisinin is soluble in methanol,

ethanol, ethyl acetate, acetonitrile, acetic acid, petroleum ether and diethyl ether. Since there was not a clear solution when the water was added, it shows that Dihydroartemisinin does not dissolve in water. The melting range was 158^{o}C to 160^{o}C as stated in literature. The short range of the melting point shows its purity. The TLC was done compared with pure samples of Artemether mixed with Lumefantrine and also with Piperaquine phosphate. Figure 6.15 shows that the mobile phase worked for DHA, Arthemeter and Lumefantrine but did not work for Piperaquine phosphate. The Rf values of DHA, Artemether and Lumefantrine were 0.4906, 0.3491 and 0.7547 respectively.

5.3.2 Derivatisation of Dihydroartemisinin with NaOH

Due to the absence of appropriate chromophore for UV absorption at higher wavelength of the Dihydroartemisinin structure **(Hong *et al.*, 2008**), it absorbs light in the UV region between 210nm and 220nm with a poor extinction coefficient.

In Table 4.14, Dihydroartemisinin dissolved in ethanol absorbs at 205.0nm, in Table 4.13, there is an increase in wavelengths (bathochromic shift) and also higher absorbances (hyperchromic effect) in the spectrum; at 240.5nm there is an absorbance of 0.286 and at 289.5nm there is an absorbance of 0.706. This was observed after Dihydroartemisinin dissolved in ethanol was heated with 0.05M NaOH at 50^{o}C for 30minutes and left to cool at room temperature; Dihydroartemisinin was converted to a derivative with a longer wavelength and higher Absorptivity (**International Pharmacopoeia**).

The same thing was observed when Dihydroartemisinin was dissolved in methanol and heated with 0.05M NaOH at 50^{o}C for 30minutes and left to cool at room temperature (Figure 4.9).

The highest absorption was at 289.5nm, therefore it was chosen as the wavelength to be used.

A calibration curve of the pure sample of Dihydroartemisinin dissolved in methanol and heated with NaOH at 50^{o}C for 30minutes was drawn (Figure 4.10); $y = 4.0103x + 0.0225$ and $R^2 = 0.9957$.

5.3.3 Variation of volumes of NaOH for the derivatisation of Dihydroartemisnin

In the previous experiments the ratio of the volume of Dihydroartemisinin in methanol : volume of NaOH was 1:9. In Figure 4.12, the ratio of the volume of Dihydroartemisinin in methanol : the volume of NaOH was varied to obtain the best absorbance value at 289.0nm. From the graph,

it was observed that as the ratio of the volume of Dihydroartemisinin in methanol was becoming equal to the volume of NaOH, the absorbance also increased but as the amount of NaOH increased, the absorbance decresed. The highest absorption was obtained when there was equal volumes of Dihysroartemisinin in methanol and NaOH and the lowest one was when the amount of NaOH was more.

5.3.4 Variation of heating times of the solutions

The heating time was also varied for two different ratios of Dihydroartemisinin in methanol: NaOH that is, 1:1 and 1:9. Figure 4.13 shows that regardless of the amount of NaOH in the solution, increasing heating time results in higher absorbances.

5.3.5 Variation of concentrations of NaOH and stability of the derivatives in solution

The concentration of NaOH was also varied, using equal volumes of dihydroartemisnin in methanol and NaOH. Figure 4.14 shows that from 0.02M to 0.1M concentration, there was a sharp increase in the absorbance and then as the concentration of NaOH increased, the absorbance was becoming stable. This shows that concentrations of NaOH higher than 0.1M will result in similar results. Each solution was also left to stand and the absorbances were taken at time intervals of 20minutes for an hour. Figure 4.15 shows that even though the solutions were left standing (at room temperature), their absorbances kept increasing indicating that there is an incomplete reaction which is still taking place regardless of how much NaOH was in the solution.

5.3.6 Investigation of the addition of different concentrations of acetic acid to the derivative of Dihydroartemisinin and NaOH

Acetic acid reacts with NaOH to give sodium acetate which acts as a buffer when there is excess acetic acid. Dihydroartemisinin in methanol was heated with 1M NaOH for 30 minutes at 50^{O}C. 30 minutes after the absorbances were taken, different concentrations of acetic acid were added to the solutions. Figure 4.16 shows a drop in absorbance when 5ml of 1M acetic acid was added. The absorbance dropped below the initial absorbance showing that the acetic acid reacted with the excess NaOH and also the NaOH which had reacted with the dihydroartemisinin. Figure 4.16 shows that there was a little drop in the absorbance when 1ml of the 1M acetic acid was

added to the solution. Showing that the acetic acid was enough to react with the excess NaOH and just a little of the NaOH which had reacted with the dihydroartemisinin, but after a few minutes, the absorbance started rising again.

The same results was observed for Figures 4.18 and 4.19, but this time the rise in absorbances was very small compared to Figure 4.17. This shows that the right amount of acetic acid will form a buffer with the excess NaOH which gives the solution a particular pH where the derivative formed between Dihydroartemisinin and NaOH will have a stable absorbance when left standing.

5.3.7 Derivatisation of Dihydroartemisnin with ammonia

When 0.7M of ammonia slolution was added to DHA in MeOH and heated at 50^{O}C for 30minutes, it was observed from the absorption spectrum that the λ_{max} of 208.5nm had a specific absorptivity of 44.22 (Figure 4.20) compared to when the solution was heated at 60^{O}C which gave a λ_{max} at 235nm with a specific absorptivity of 74.11 (Figure 4.20) showing that heating at a different temperature affects the wavelength of maximum peak and its absorbance. From Table 4.22, the heating times were also compared and it was observed that heating for longer times did not affect the wavelength of maximum absorption but it showed an increase in absorbance.

From Table 4.25, it was observed that even heating at a higher temperature (70^{O}C) at 30minutes even gave a higher absorbance of 2.266 than when it was heated at 60^{O}C.

From Figure 4.24, when the solution was heated at 70^{O}C for 30minutes, it was left to stand and the absorbance was taken every 10minutes for an hour. It is observed that the absorbance kept on rising and did not become sTable showing that the reaction was not comlete just as in the case of the NaOH being used.

5.3.8 Derivatisation of Dihydroartemisninin with HCl

When the conc HCl was added to the solution of DHA dissolved in methanol, it was observed that there was a colour change when it was left to stand over a period of 120 minutes.. The change went from colourless to light pink which deepened over time and then finally turned to brown.

5.3.9 Variation of the volumes of HCL and the stability of their derivatives

When conc HCl was added to the solution of DHA dissolved in methanol, the maximum peak of absorption was at 254nm. It was observed that when 1ml and 2ml of conc HCl was added to 2ml of the stock solution, the absorbance kept on rising after 60minutes (Figures 4.23 and 4.24). This shows that the reaction had not been completed. When 3ml and 4ml was added to 2ml of the stock solution, the absorbances became constant from 30minutes to 60minutes. This shows that after 30minutes the reaction was complete (Figures 4.27 and 4.28). Therefore the solution of DHA needed more HCl to complete the reaction at a faster rate.

From Figure 4.29, the absorbances rises with the amount of conc HCl added to the stock solution. Even though the reaction completed after 30minutes for both solutions containing 3ml and 4ml of conc HCl, the absorbance of the one containing 3ml conc HCl was lower than the one with 4ml showing that more HCl in the system results in higher absorbances.

A calibration curve of absorbance against 2ml of different concentrations of DHA in MeOH and 3ml of conc HCl left for 30minutes was drawn (Figure 4.30). $y=310.17x + 0.0521$ and $R^2= 0.9974$.

5.3.10 Assay of Dihydroartemisinin containing tablets

The Tablets for the assay contained both DHA and Piperaquine phosphate. Since Piperaquine phosphate is a highly absorbing compound, it would interfere in the assay of DHA. Therefore the DHA had to be extracted out before it could be assayed. The solubilities of both compounds were considered and diethyl ether was chosen since DHA was soluble in it as compared to Piperaquine which was practically insoluble in diethyl ether. The percentege recovery was calculated was to be 90.36% for a known amount of DHA plus exciepients.

The residue was taken through the basic tests to confirm the presence of DHA. When the residue was dissolved in MeOH and reacted with conc HCl, and its spectrum determined (Figure 4.24) , it was found out that it had the similar UV absorption spectrum with a λ_{max} at 254.5nm as compared to the pure form (Figure 4.23), which had a λ_{max} at 255nm. The LOD was 1.09×10^{-4} %w/v and the LOQ was 3.31×10^{-4} %w/v.

The assay was done for three days (interday assay). Each assay was done for three different concentrations and for each concentration, the assay was repeated three times (intraday) and the average calculated. The mean percentage contents, standard deviations (SD) and relative standard deviations (RSD) were calculated. This was done for both Tablets A and B. From Tables 4.31 to 4.36, the standard deviations and the relative standard deviations were all below 2% which are all within the limits of the guidelines of ICH 2003.

5.3.11 HPLC METHOD DEVELOPMENT

The absence of the chromophore system in Dihydroartemisinin makes it have a low absorbance. It absorbs between 210 and 220 nm. Methanol has a cut-off point of 205nm and when it was used as part of a mobile phase for this drug, it gave a very unstable baseline. Therefore acetonitrile was chosen as a replacement since it has a cut-off point of 190nm and therefore did not cause much interference (6.11). A wvelength of 220nm was chosen because, that is where Dihydroartemisinin absorbs and high concentrations of the solutions were used because low concentrations were not detected. Also, It was also realised that at a higher flow rate, DHA gave a double unresolved peak (Figure 6.12). The retention time for DHA was 3.054 minutes

DHA in both Tablets A and B was first extracted with diethyl ether and then the residue left when the ether was evaporated off was dissolved in the mobile phase for HPLC. Even though the DHA was extracted, other exciepients were also extracted and at a low wavelength of 220nm where so many compounds absorb, the chromatograms showed peaks of other exciepients along with the DHA giving unresolved peaks(Figures 6.13 and 6.14). This shows that the method was not specific to Dihydroartemisinin.

A calibration curve of peak area against concentration was drawn for DHA (Figure 4.31). y= 19.338x – 0.7136 and R2= 0.9993. The LOD was 5.35×10^{-3} %w/v and the LOQ was 3.12×10^{-2} %w/v.

5.3.12 Comparing the UV method to the HPLC method

The critical value for a two tailed f-test (f_{tab}) at p=0.05 is 39.30 and the calculated value for f_{cal} was 3.8885. This shows that there is no significant difference between the two variances and hence between the precisions of the two methods and the null hypothesis is accepted.

The critical value of $|t|$ (t_{tab}) for 7 degrees of freedom at p=0.05 is 2.36 and the calculated value for t_{cal} was 5.5900. This shows that there is a significant difference between the two means and hence between the means of the two methods and the null hypothesis is rejected. Therefore the UV method is preferred.

5.4 CONCLUSION

5.4.1 PIPERAQUINE PHOSPHATE

For the UV spectrometric assay of Piperaquine Phosphate in Tablets, $A_{(1\%,1cm)}$ at 349nm in 0.1%.v/v HCl was 287.21. From the calibration curve of a pure sample, y = 287.2x + 2E -5 and R^2 = 0.999. The LOD and LOQ were found to be 5.56 x 10^{-5} %w/v and 1.68 x 10^{-4} %w/v respectively.

The HPLC conditions were: ODS column, 250x4.60mm, with a mobile phase of acetonitrile and water with 0.1%v/v TFA (40:60) at a flow rate of 1.0ml/min, analyte monitored at UV wavelength of 349nm and an injection volume of 20µl which gave a peak of retention time as 9.183minutes. From the calibration curve of pure sample, y= 19.338x – 0.7136 and R2= 0.9993. The LOD was 5.35 x 10^{-3} %w/v and the LOQ was 3.12 x 10^{-2} %w/v.

According to the ICH guidelines, all the results for the respective parameters were met and therefore the methods can be used to analyse tablets containing Piperaquine Phosphate.

The null hypothesis was accepted for the comparison of the precisions but rejected for the comparison of means of the two methods. The HPLC method is preferred.

5.4.2 DIHYDROARTEMISININ

For the UV spectrometric assay of DHA in Tablets, the DHA was extracted with diethyl ether and evaporated off, dissolved in methanol and reacted with concentrated HCl for 30minutes. The λ_{max} was found to be 254nm. From the calibration curve of a pure sample, y=310.17x + 0.0521 and R^2= 0.9974 and the percentage recovery was 90.36%. The LOD was 1.09 x 10^{-4} %w/v and the LOQ was 3.31 x 10^{-4} %w/v

The HPLC conditions were: ODS column, 250x4.60mm, with a mobile phase of acetonitrile and water (70:30) at a flow rate of 1.3ml/min and analyte monitored at UV wavelength of 220nm and an injection volume of 20µl which gave a peak of retention time as 3.054minutes. From the calibration curve of pure sample, y= 19.338x – 0.7136 and R2= 0.9993. The LOD was 5.35 x 10^{-3} %w/v and the LOQ was 3.12 x 10^{-2} %w/v.

According to the ICH guidelines 2003, all the results for the respective parameters were met and therefore the method could be used to analyse tablets containing Dihydroartemisinin.

The null hypothesis was accepted for the comparison of the precisions but rejected for the comparison of means of the two methods. The UV method is preferred.

5.5 RECOMMENDATION

The HPLC conditions for the assay of DHA gave unresolved peaks of the Dihydroartemisinin and other excipients therefore making the method not good enough even though all the results for the ICH parameters were within the limits. It is recommended that better conditions should be developed which would give resolved peaks for the Dihydroartemisinin and other excipients which will be present.

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APPENDIX

Table 6.1: Average absorbance against concentration for Piperaquine phosphate

Concentration (%w/v)	Average absorbance
0.0004	0.142 ± 0.0010
0.0008	0.266 ± 0.0017
0.0016	0.551 ± 0.0010
0.0021	0.717 ± 0.0010
0.0025	0.843 ± 0.0015
0.0033	1.1 ± 0.0010
0.0041	1.364 ± 0.0015

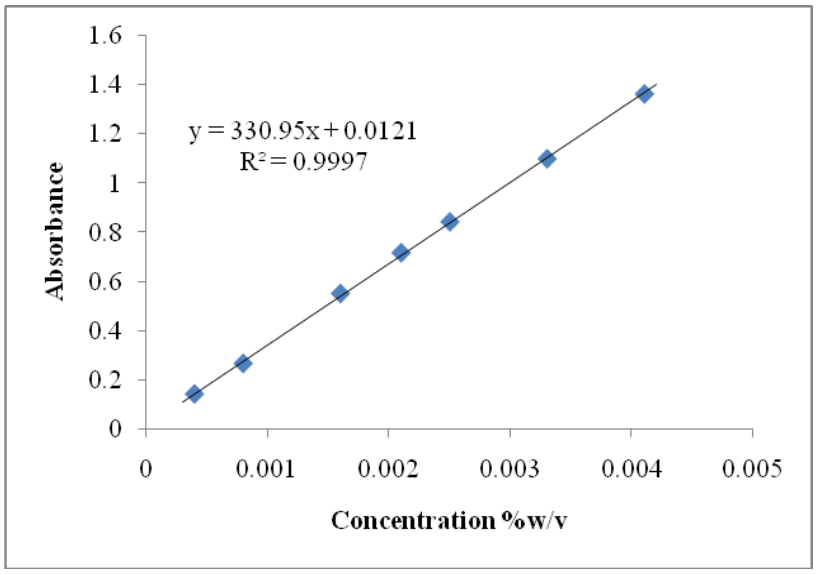

Figure 6.1: UV spectrometry Calibration curves for Piperaquine phosphate (assay of Tablets)

Table 6.2: Average absorbance against concentration for Piperaquine phosphate

Concentration (%w/v)	Average absorbance (n=3)
0.0004	0.140 ± 0.0012
0.0008	0.267 ± 0.0012
0.0016	0.554± 0.0010
0.0021	0.719 ± 0.0015
0.0025	0.845± 0.0012

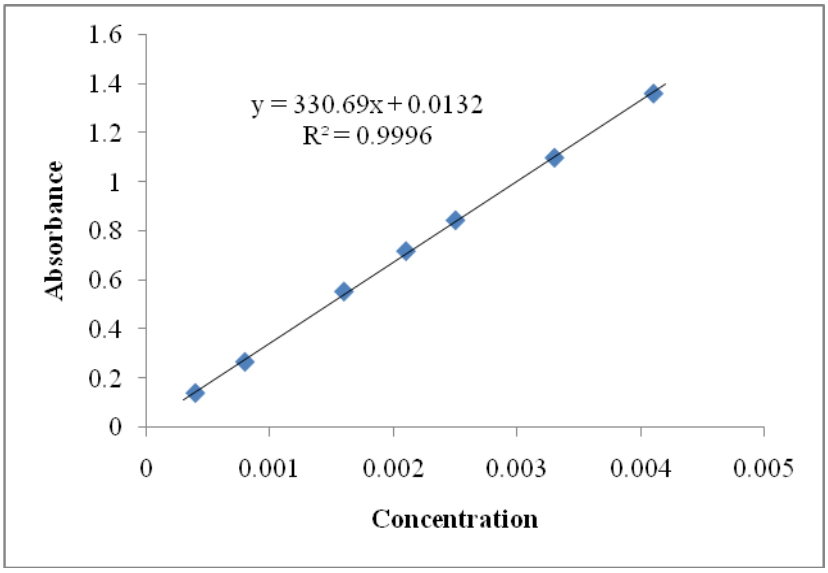

Figure 6.2 UV spectrometry Calibration curves for Piperaquine phosphate (assay of Tablets)

Table 6.3: Average absorbance against concentration for Piperaquine phosphate

Concentration (%w/v)	Average absorbance (n=3)
0.0004	0.139 ± 0.0012
0.0008	0.263 ± 0.0010
0.0016	0.551± 0.0012
0.0021	0.721 ± 0.0015
0.0025	0.844± 0.0012
0.0033	1.098 ± 0.0010
0.0041	1.362 ± 0.0010

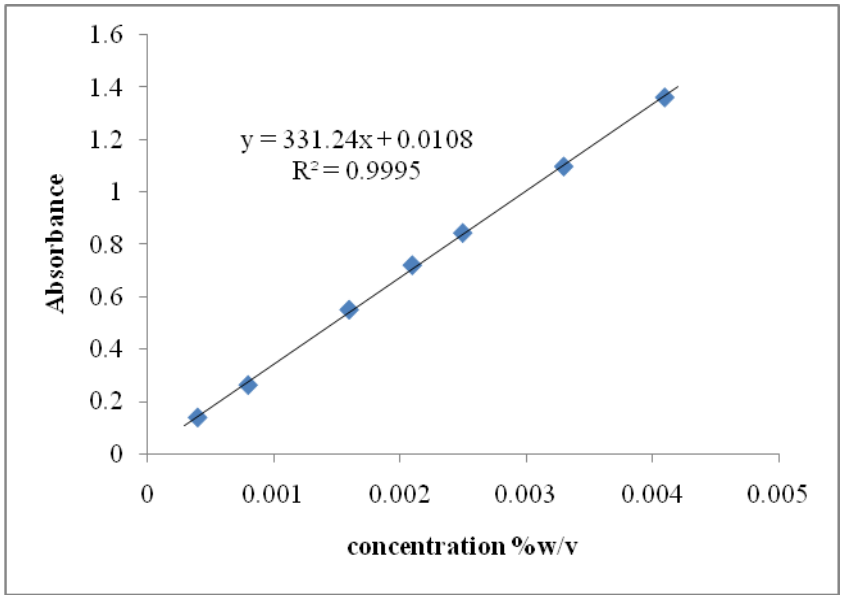

Figure 6.3 UV spectrometry Calibration curves for Piperaquine phosphate (assay of Tablets)

Table 6.4: Weight uniformity test, deviation and % deviation

Weight of Tablets (g)	Deviation	% Deviation
0.5582	0.0005	0.089654
0.5613	0.0036	0.645508
0.5522	-0.0055	-0.98619
0.5579	0.0002	0.035862
0.5575	-0.0002	-0.03586
0.5581	0.0004	0.071723
0.5586	0.0009	0.161377
0.5547	-0.003	-0.53792
0.5573	-0.0004	-0.07172
0.5572	-0.0005	-0.08965
0.558	0.0003	0.053792
0.5542	-0.0035	-0.62758
0.5606	0.0029	0.519993
0.5591	0.0014	0.251031
0.562	0.0043	0.771024
0.5546	-0.0031	-0.55585
0.5543	-0.0034	-0.60965
0.5535	-0.0042	-0.75309
0.56	0.0023	0.412408
0.5604	0.0027	0.484131

Mean weight = 0.5575g

Table 6.5: Weight uniformity test, deviation and % deviation Tablet B

Weight of Tablets (g)	Deviation	% Deviation
0.2065	-0.0029	-1.38491
0.2063	-0.0031	-1.48042
0.2065	-0.0029	-1.38491
0.216	0.0066	3.151862
0.212	0.0026	1.241643
0.2065	-0.0029	-1.38491
0.2177	0.0083	3.963706
0.2158	0.0064	3.056351
0.2043	-0.0051	-2.43553
0.2181	0.0087	4.154728
0.2061	-0.0033	-1.57593
0.2074	-0.002	-0.95511
0.2061	-0.0033	-1.57593
0.2053	-0.0041	-1.95798
0.2084	-0.001	-0.47755
0.1997	-0.0097	-4.63228
0.2043	-0.0051	-2.43553
0.2063	-0.0031	-1.48042
0.2178	0.0084	4.011461
0.2174	0.008	3.820439

Mean weight = 0.2094g

Table 6.6: Average absorbance against concentration for Piperaquine phosphate

Concentration (%w/v)	Average absorbance (n=3)
0.00041	0.222 ± 0.0010
0.00082	0.326 ± 0.0010
0.00122	0.456 ± 0.0017
0.00164	0.587 ± 0.0015
0.00205	0.693 ± 0.0010

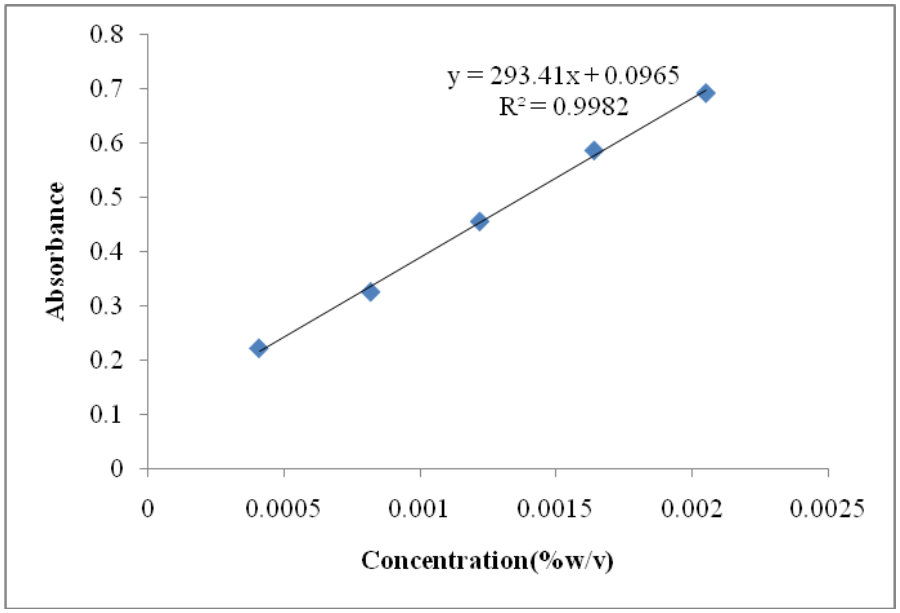

Figure 6.4 UV spectrometry Calibration curve for Dihydroartemisinin (assay of Tablets)

CHROMATOGRAMS OF PIPERAQUINE PHOSPHATE

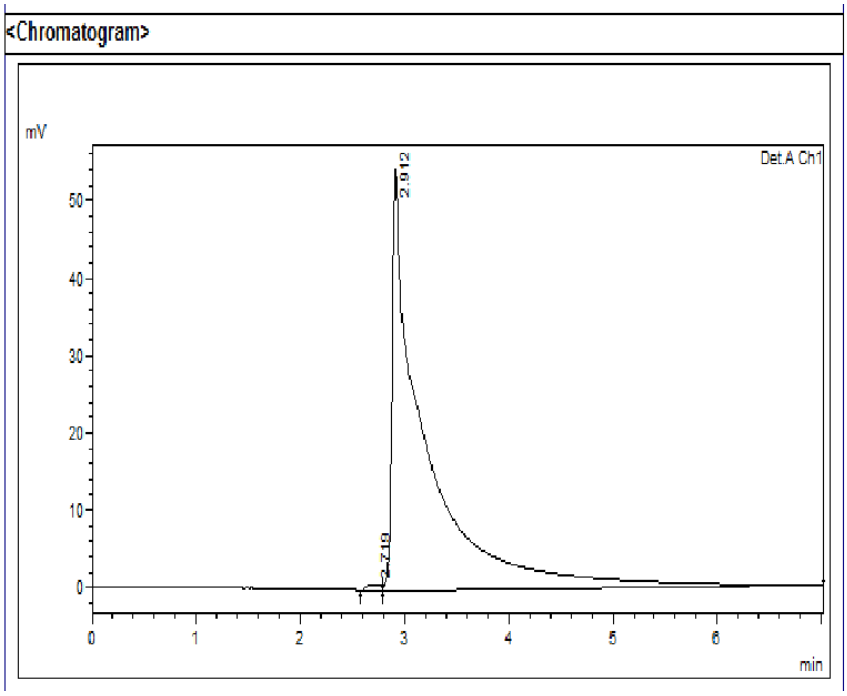

Figure 6.5 Mobile phase- Acetonitrile : Water with 0.1%v/v TFA (50: 50)

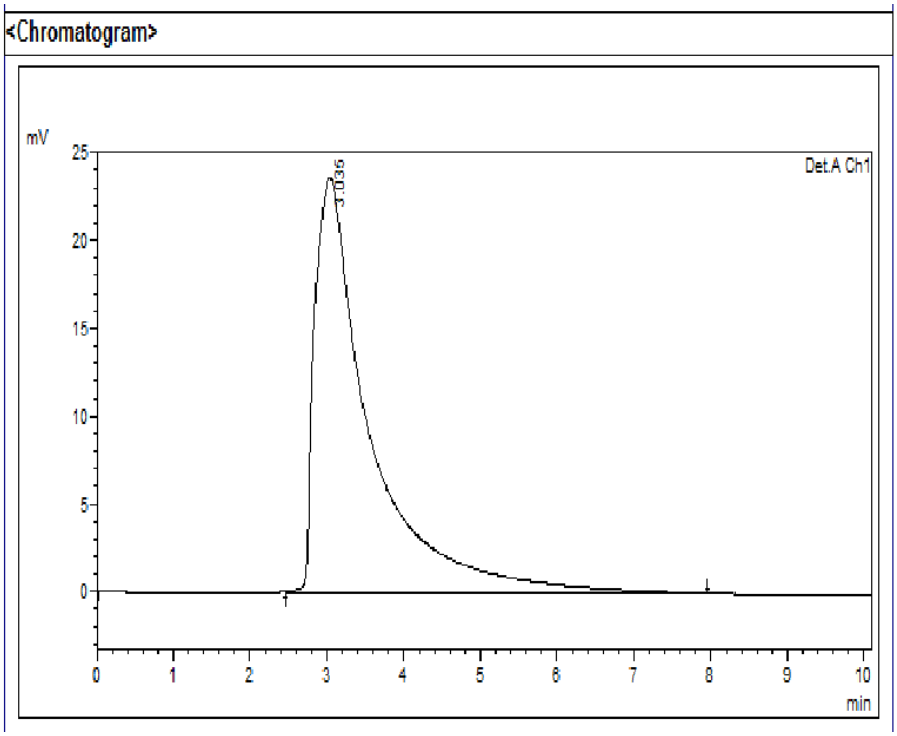

Figure 6.6 Mobile phase- Acetonitrile : Water with 0.1%v/v TFA (60: 40)

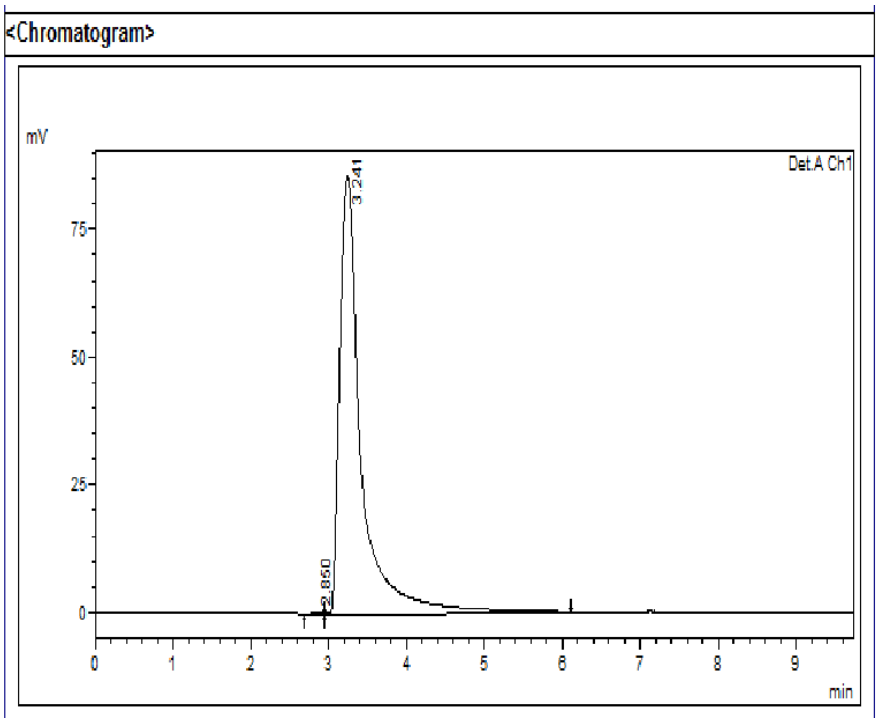

Figure 6.7 Mobile phase- Acetonitrile : Water with 0.1%v/v TFA (40: 60)

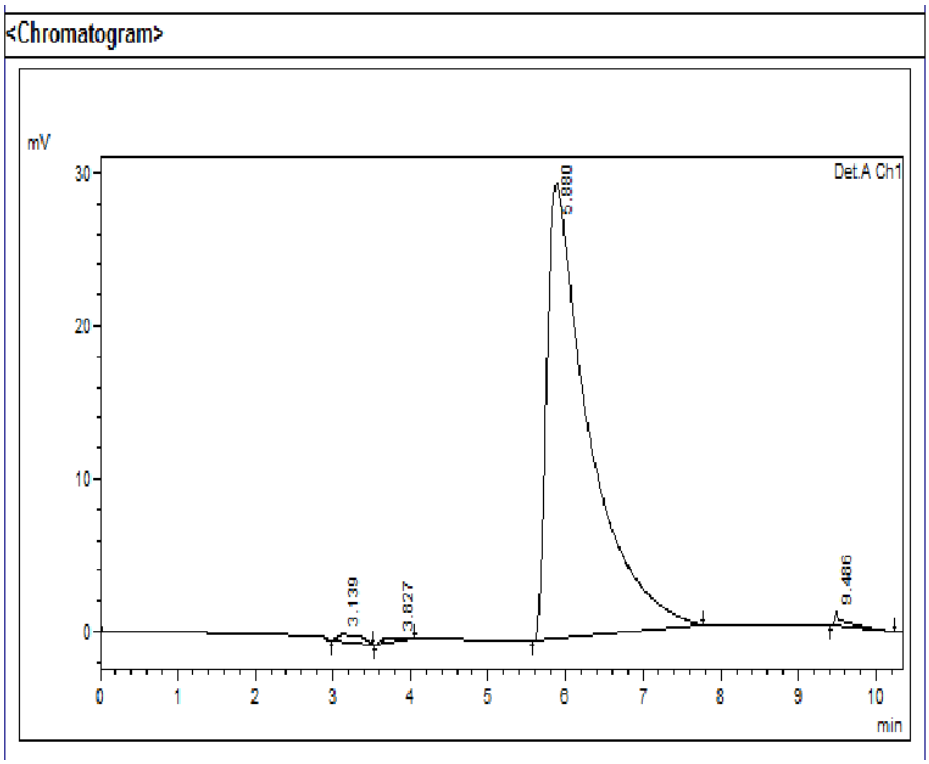

Figure 6.8 Mobile phase- Acetonitrile : Water with 0.1%v/v TFA (20: 80)

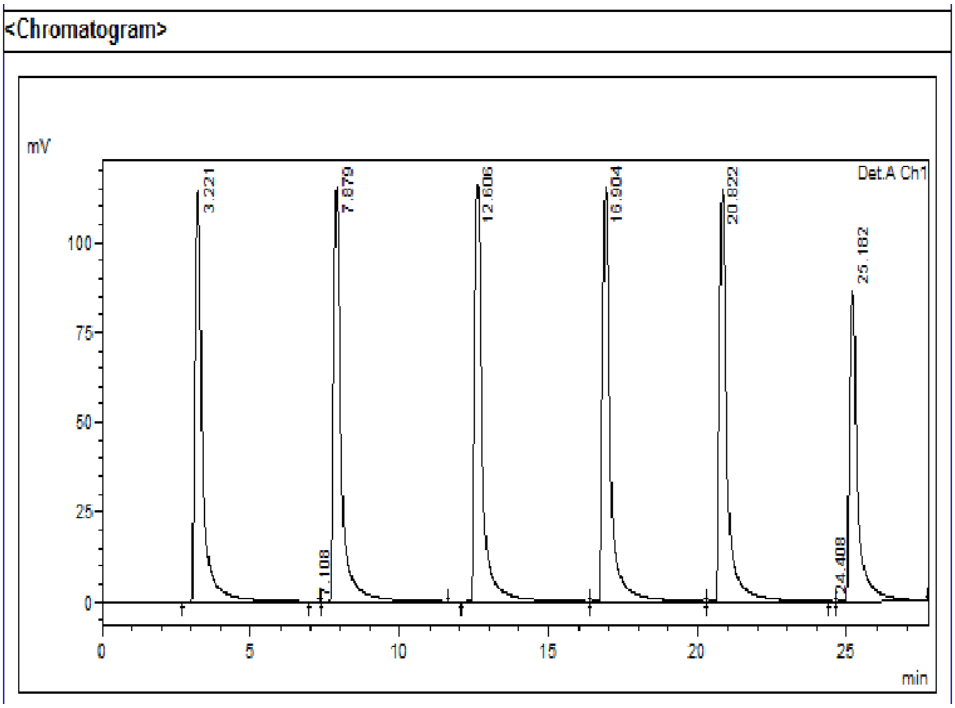

Figure 6.9 Mobile phase- Acetonitrile : Water with 0.1%v/v TFA (40: 60)

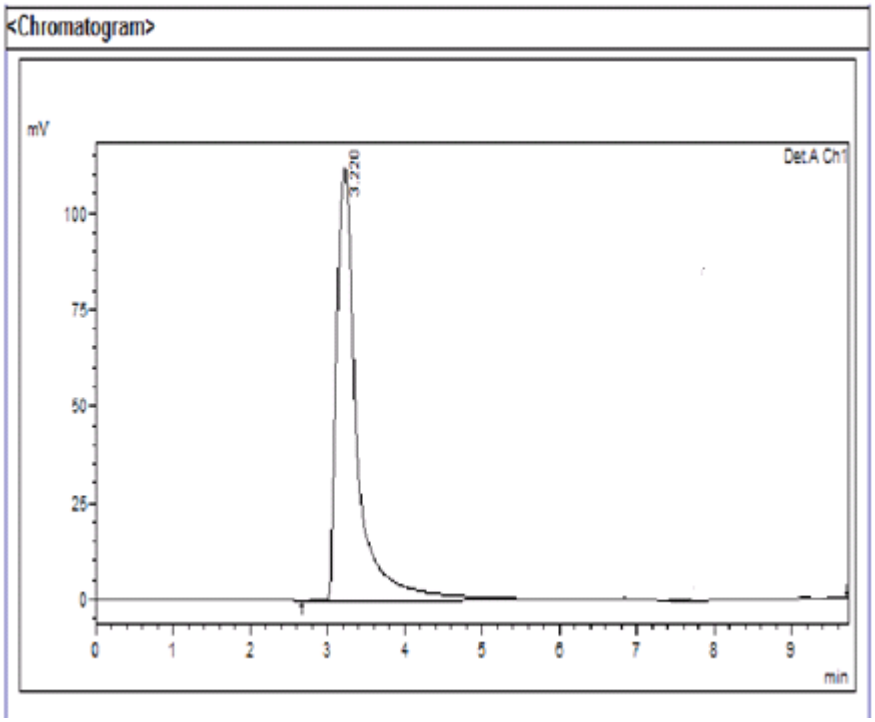

Figure 6.10 Peaks of Piperaquine phosphate in Tablet A

CHROMATOGRAMS OF DIHYDROARTEMISININ

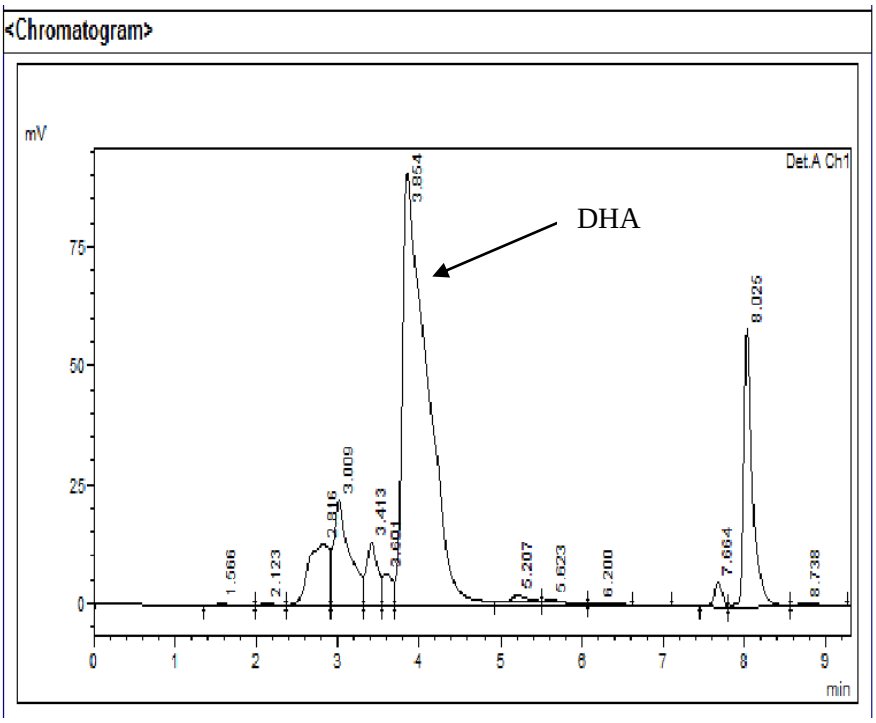

Figure 6.11 Mobile phase- Acetonitrile : Water with 0.1%v/v TFA (90: 10)

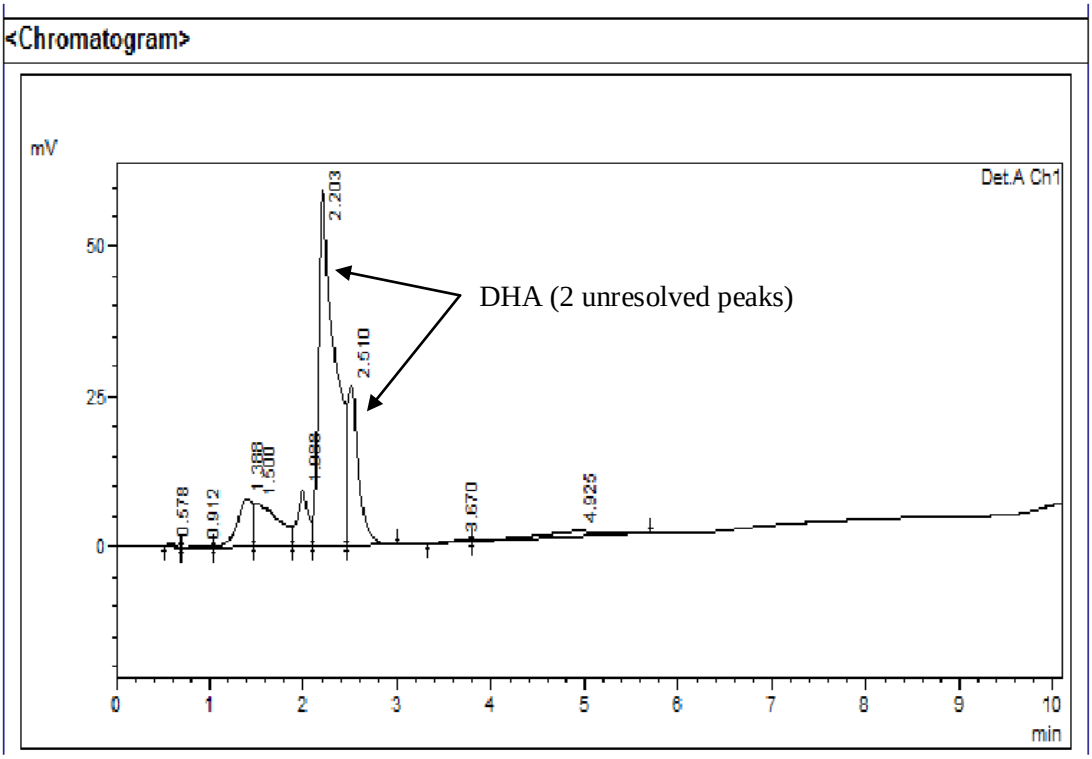

Figure 6.12 Mobile phase- Acetonitrile : Water with 0.1%v/v TFA (90: 10)

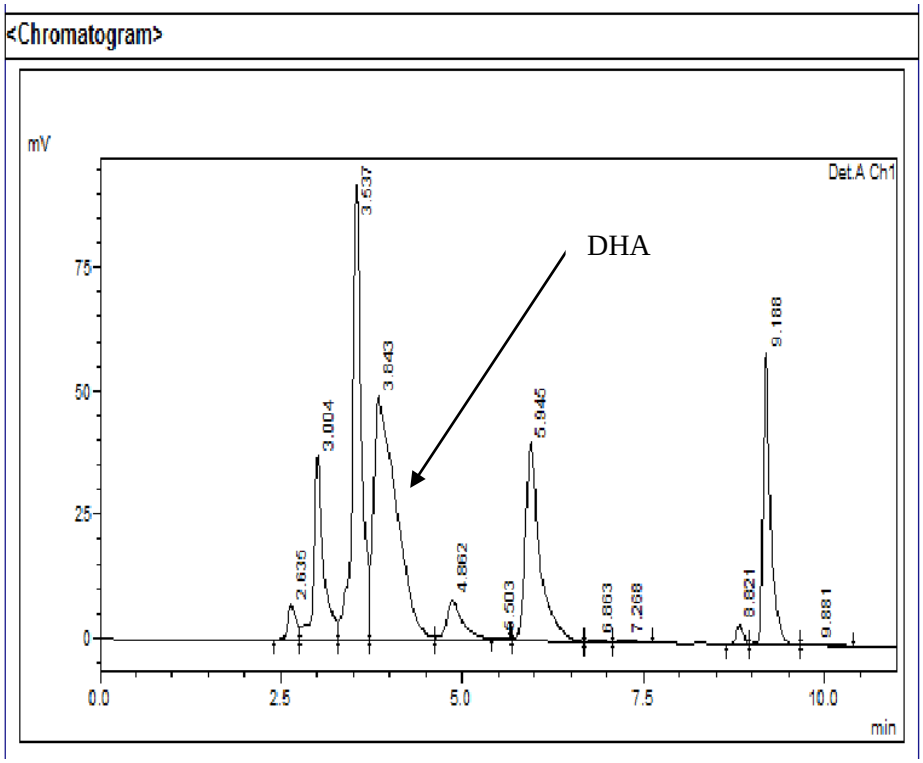

Figure 6.13 Dihydroartemisninin in Tablet A

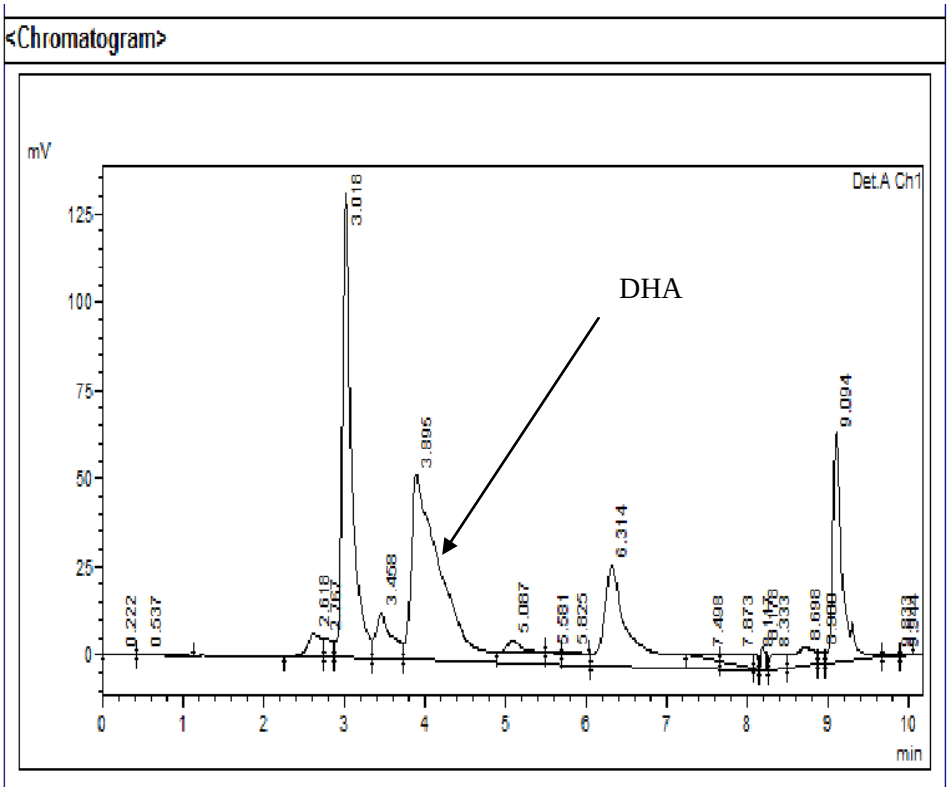

Figure 6.14 Dihydroartemisninin in Tablet B

Figure 6.15: TLC chromatogram for DHA and Piperaquine phosphate pure samples compared with Arthemether mixed with Lumefantrine pure samples.

Table 6.7: Rf values for Arthemeter, Lumefantrine, DHA and Piperaquine phosphate.

Spot number	Drug pure sample	Rf values
1	Arthemether/ Lumefantrine spots	Arthemether – 0.3491 Lumefantrine – 0.7547
2	DHA spot	DHA – 0.4906
3	Piperaquine phosphate	Piperaquine phosphate – 0