BIOPHARMACEUTICAL AND PHARMACOKINETIC STUDIES ON VARIOUS FORMULATIONS OF AMODIAQUINE IN ARTEMISININ COMBINATION THERAPIES

BY

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CERTIFICATION

This Is To Certify That The Thesis

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DEDICATION

This thesis is dedicated to Almighty **ALLAH, THE Most Exalted, The All-Knowing, The Most Great** and also for departed Soul of my father, **Pa Eniola** and my living mother, **Ayoka.**

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ABSTRACT

One of the objectives of the present studies is to investigate the possible interaction potential between ciprofloxacin and some representative antimalarial agents (chloroquine and halofantrine) utilizing in-vitro dissolution tests.

The in-vitro dissolution of ciprofloxacin (CP) (250 mg and 500 mg tablets) was studied using U.S Pharmacopoeia (USP) Apparatus 2 in the presence of varying concentrations of chloroquine (CQ) and halofantrine (HF). The dissolution media were 0.1N HCl and distilled water. The CQ concentration ranges from 0.69 – 3.47 M and HF 0.56 – 1.1M. The mean reduction in the maximal quantity of dissolved drug, $\mathbf{Q_{max}}$ in the presence of varying concentrations of CQ, was 47.8% and 45.4% with 500mg CP tablets in distilled water and 0.1N HCl respectively, and 43.4% and 37.6% with 250mg CP tablets in distilled water and 0.1N HCl respectively. The reduction in the presence of HF was 75% with 500mg CP tablets and 48.3% with 250mg CP tablets. The reduction in $\mathbf{Q_{max}}$ appears to be directly related to the quantity of CQ/HF present. The difference was significant with CQ ($p= 0.027$) and not with HF ($p = 0.2$).

We have inferred from our results that the release of CP is inhibited by the presence of CQ and HF.

The second objective is to determine the **biopharmaceutical** and **pharmacokinetic profile** of **Artesunate-Amodiaquine** formulations in both in-house and commercial formulations.

Evaluation of physicochemical characteristics *viz-a-viz* uniformity of weight, disintegration tests, assay and dissolution profile of amodiaquine and artesunate were carried out on artemisinin combination formulations.

The systemic uptake studies were conducted using sixty healthy volunteers after informed consent. The study adopted is a single-dose, open and parallel study.

The results obtained demonstrated that the physicochemical characteristic such as uniformity of weight, disintegration and assay of the formulations conformed to official (BP., 1998; I.P., (2005) and USP., 2004) stipulations. In both media, the percent release of amodiaquine component of the

formulations were found to be 94.6 -97.5 and 86.9 – 90% at 25 minutes in deionised water and (0.1, 0.05)N HCl respectively for product A, B, C and D. Product E and F which were in-house formulations showed release of 100% in less than 5 minutes. The artesunate was found to be structurally unstable in 0.1 and 0.05N HCl.

The in-vivo bioavailability showed that the peak plasma concentration varied from 0.22 to 1.05μg/ml (average 0.56-0.74 ± 0.21-0.27) at $\mathbf{T_{max}}$ 1.1 to 8.0 h (average 3.2 – 4.9 ± 1.3 -2.4). The $t_{i/2}$ of elimination ranges from 3.35 to 30.8 h while the quantum $\mathbf{AUC_{0\text{-}72h}}$ deviates from 125 to 230μg.hr/ml. The $\mathbf{AUC_{0\text{-}72}}$ obtained from this study agreed with similar study carried by Winstanley *et al.*, 1987 while the apparent terminal $\mathbf{t_{i/2}}$ of elimination of 5.2 ± 1.7 h was in contrast with our results. The $\mathbf{t_{i/2}}$ elimination (2.6 – 12 weeks) confirmed the long half-life associated with Amodiaquine.

It could be concluded that the release, absorption and disposition profile of amodiaquine in all the products evaluated were quite satisfactory. Also artesunate either alone or in combination has been found to be unstable in acidic medium.

The third main objective is to formulate deoxyribonucleic acid (DNA)/DAB16 polymer complexes (Dendriplexes-**Nano-medicine Technology and Gene delivery**); and characterize the formulations by determining the particle size and zeta potential using **Photon Correlation Spectroscopy** (PCS) and **Transmission Electron Microscope** (TEM). This is done with a view to be able to provide possible explanation for the particle variables which control in-vivo gene transfection.

The method of complex formulations is by electrostatic interaction between calf-thymus DNA and polycation polymer called **Polypropylenimine dendrimer 16**. Complexes were formulated based on total nitrogen on the dendrimer and anionic phosphate on the helical structure of the DNA.

The results obtained demonstrated that nitrogen to phosphate ratio in excess of 8 and / or a DNA concentration in excess of 250μg/ml leads to non-colloidal complexes. It also showed that higher levels of dendrimer to dendriplex leads to diminished dendriplex surface charge.

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 BACKGROUND

Malaria has been with mankind since the dawn of history. The anopheles mosquito vector is as old as man (Joy and Mu, 2003). Several scholars including Shakespeare had all variously and at different times described fevers attributable to malaria. Round about the 4th century BC, Hippocrates the great Greek physician recognized the distinct features of the disease and linked it to the proximity of stagnant waters, though he had no idea it was caused by a mosquito (Coker *et al.*, 2005).

Rome in Italy was one of the most malarious places in the world until the Pontine Marshes were cleared in the 1930s. In the early 1800s, the scientific community had attributed most diseases to bacterial origin. It was in 1880 that Charles Louis Alphonse Laveran, a French army physician working in military hospital of Constantine, Algeria identified the malaria parasite in a blood smear under the microscope. For this and later discoveries, he was awarded the 1907 Nobel Price for Medicine.

In 1898, the British microbiologist, Sir Ronald Ross, then attached to the Indian regiment proved that the malaria parasite was transmitted through the bite of a mosquito- a discovery that won him a Nobel Prize in Medicine in 1902. (Coker *et*

al., 2001). However, it was until 1948 when Shortt and Garnham described exoerythrocytic schizonts in the livers of infected monkeys or subsequently in humans, that the complete life cycle of the parasite was elucidated (Gardiner *et al.*, 2005). It was in 1976 that the first successful continuous *in-vitro* culture of human malaria parasite was achieved. Up till now the hope that the in-vitro culture could lead to quick discovery of a vaccine has not been realised. However, this development facilitates the discovery of new antimalarial drugs (Trager and Jense, 1976).

There are about 300 million acute cases of malaria each year globally, resulting in more than a million deaths (WHO, 1990). Nine out of every ten cases occurs in sub-Saharan Africa while about 90% of these deaths occur in Africa, mostly in young children. Malaria together with HIV/AIDS and Tuberculosis (TB) constitute major public health challenges undermining development in Nigeria in particular going by the high morbidity and mortality rates especially in children.

Malaria is not only associated with poverty but is also a cause of poverty and a major impediment to economic development in Nigeria and in Africa as a whole. It is responsible for potential medical complications such as low birth weight in infants, increase in unexplained sudden abortion and still births by pregnant women, resulting from malaria during pregnancy (Coker *et al.*, 2005).

Malaria disease, when unchecked may cause death, hamper children's schooling and social development through both absenteeism and permanent neurological disorders associated with severe episodes of malaria.

Several interventions have been instituted for prevention and or possibly for eradication of malaria. Some of the control measures are provision of mosquito nets and insect repellants, spraying of insecticides inside houses and draining of standing or stagnant water where mosquitoes lay their eggs.

Concern for this debilitating scourge galvanized some political commitment by African leaders for action on Malaria and this led to the founding of the Roll Back Malaria [RBM] global partnership in 1998. In the year 2000 African Heads of State met in Abuja Nigeria to further strengthen the RBM's goal of reducing the African malaria burden to less than 40% by the year 2010. The Abuja declaration signed in April 2000 endorsed a concerted strategy to tackle the problem of malaria across the entire Africa (WHO, 2003).

Tremendous progress has been made so far since the Abuja declaration.

Amongst steps taken by some countries such as Nigeria are:

- Reduction of tariffs on insecticide-treated nets [ITNs] to make them affordable

- Establishment of "Country Strategic Plans" [CSPs]. CSPs are all based on the four technical and pivotal points of the RBM set goals viz: evidence-based interventions prompt access to effective treatment, promotion of ITNs and improved vector control and prevention and management of malaria in pregnancy (Coker, 2005).

The treatment of malaria must take into consideration a proper diagnosis based on observed physical symptoms, clinical manifestations and investigative serological findings positive for parasitaemia.

Therefore, successful treatment outcomes are highly predicated on choice of antimalarial drugs employed and other palliative measures. The frontline antimalarial drugs employed for the treatment of acute uncomplicated falciparum malaria include chloroquine [CQ], sulphadoxine-pyrimethamine [SP], quinine, halofantrine (HF), mefloquine. Recently drug combinations such as SP-Mefloquine, Dapsone-proguanil have also been used for malaria treatment (Coker *et al.*, 2005).

Chloroquine has been the most effective drug against malaria disease for over 50 years. Statistics show that the efficacy of chloroquine in malaria management has dropped to below 40% in malaria sufferers, due to acquired resistance by the offending parasite, *P. falciparum*. Chloroquine resistant *P. falciparum* was suspected in Thailand in 1957 and found in patients in Colombia and Thailand in

1960. In Africa, it was first documented in 1979 in Tanzania (Menard *et al.*, 2005).

Results emanating from pilot studies carried out in the six geopolitical zones in Nigeria painted a very gloomy picture for chloroquine medication. Scientists have attributed the reason for the high level of chloroquine resistance to drug pressure and another possible explanation is population movements (Okonkwo *et al.*, 1999). The second line antimalarial medication SP combination was also reported to have suffered serious knocks. Introduction of newer chemotherapeutic agents have not helped much, and are plagued by one demerit or the other.

Halofantrine, a schizonticidal agent has demonstrated cardiotoxic potential by the prolongation of QT interval in the electrocardiogram of people administered with halofantrine (Karbawang and Na-Bangchang, 1994). Mefloquine is another effective suppressive antimalarial agent. However the pronounced extra pyramidal symptoms elicited in patients would suggest that caution is of the essence when administering mefloquine and mefloquine based combinations.

The Dapsone component of Lapdap carries a frightening associated side effect such as oxyhaemoglobinaemia (Coker *et al.*, 2001).

The problem of resistance and adverse side effects may have prompted the WHO to adopt Artemisinin Combination Based Therapies (ACTs) for combating the

debilitating malaria disease. Since 2001 WHO has recommended ACTs as first line treatment for uncomplicated malaria especially in areas experiencing resistance to older medications such as chloroquine, quinine etc. The discovery of quinghaosu (artemisinin) by the Chinese and subsequent identification of its unique sesquiterpene lactone endoperoxide structure heralded an important era in anti-malaria chemotherapy. Only this class of antimalarial drugs has not seen the development of drug resistance. Nonetheless, because of the short half-life of these drugs *in-vivo*, recrudescence of parasites has been noticed in patients undergoing short course treatment unless it is combined with another long acting drug. Therefore, drug combinations rather than monotherapy have proved to be the best solution for treating established malaria, and artemisinin based drug combinations have demonstrated cure rates similar to that of CQ 30 years ago (WHO, 2000; Maliga *et al.*, 2007). While numerous countries have adopted the change in their malaria health treatment policies, cost remains a major barrier to effective ACT implementation.

Before the emergence of chloroquine resistant *plasmodium falciparum* (CRPF), chloroquine had enjoyed wide usage in the malarial chemotherapy for over three decades. A number of studies aimed at improving the clinical outcomes associated with malaria chemotherapy (Wooden *et al.*, 1997; Fleck *et al.*, 1997) and solving

the problems of drug resistance, including the development of newer and more potent remedies abound in the literature.

Unfortunately very limited studies on drug-drug interactions with the most widely used antimalarials have been performed to date (Ette *et al.,* 1987; Adedoyin *et al.*, 1998; Fasunmon and Uwaifo, 1989), documentation on drug interaction studies appears scanty in the literature (James *et al.,* 1978; Hansten *et al.,* 1979). These limited studies also did not profile or position the antimalarials in their therapeutic environment. For example, in regions where malaria is endemic, common bacterial infections are equally rife and major antibacterial regimens are co-administered with antimalarials. Potential interactions between antimalarials and antibacterials have not been demonstrated. Chloroquine has been shown to be capable of inhibiting the activity of CYP2D6 *in-vivo* in human (Adedoyin *et al.,* 1998) a variant of the hepatic cytochrome P450 group of enzymes responsible for the metabolism of large number of drugs, thus suggesting the potential for drug-drug interaction.

Drug-drug interaction can be monitored both *in-vitro* and *in-vivo* using various methods. Dissolution testing has been utilized very well in *in-vitro* testing to predict interaction potentials of different quinolones (Rodriguez *et al.,* 1999). The quinolones are a class of synthetic antimicrobial agents, which were modeled after

nalidixic acid (Brenda, 1997). They were initially thought to have antimalarial effect by virtue of the basic quinolone moiety similar with the popular aminoquinolines which have enjoyed wide use as antimalarials (Alan *et al.*, 1988). This was shown not to be true (Coker *et al.*, 2001). However they have been proven to have a broad spectrum of activity against a plethora of bacteria and some other pathogenic microorganisms (Chukwuani *et al.*, 1998).

Several clinically significant drug interactions have been implicated with quinolones. These interactions occur by two common mechanisms: decreased gastrointestinal absorption or altered metabolism of drugs. Some interactions have resulted in fatal effects (Wijnands *et al.*, 1986; Raoof *et al.*, 1987; Staib *et al.*, 1987) while a good number of others lead to reduction in the bioavailability of some quinolones (Polk *et al.*, 1989; Akinleye *et al.*, 2007) as is the case with ciprofloxacin and theophylline. It has been reported that the reduction in bioavailability of fluoroquinolones by concurrent administration of some polyvalent ions is more striking for derivatives such as norfloxacin, ciprofloxacin and enoxacin (Mizuki, 1996; Adepoju-Bello *et al.*, 2007). This finding is considered interesting when viewed against the fact that the 4-aminoquinlones and 4-quinolones are structurally related. Failures of some chemotherapies have been attributed to inability of these medicinal agents to reach the site of

pharmacological action as a result of presystemic complexation reactions. As such drug regimen or design must be such as to avoid impediment to appropriate drug delivery.

Drug delivery systems have been the hallmark of pharmacy practice in view of the immense progress that has been made in dosage forms technology. From the days of extemporaneous dispensing, whereby drug delivery systems were primarily based on various formulations (liquid, solid, gas etc), it has now become a multidisciplinary science comprising drug design, physicochemical, biopharmaceutics, pharmacokinetic and pharmacodynamic studies.

Modern drug delivery systems have been defined as "The use of whatever means possible, be it chemical, physicochemical or mechanical to regulate the drug's access to the body's central compartment or in some cases directly to the involved tissues" with the aim of enhancing the efficacy of therapeutic agents (Vasant and Mannfred, 2003).

Research efforts on drug encapsulation have led to development of several agents that can serve as carriers for delivery processes. Of importance are the cationic lipids and some organic polymers. Various polymers with different architectures have been used for delivery of therapeutic agents (Duncan, 2003; Allen and Cullis, 2004; Choi *et al.*, 2005; Putnam 2006). Only a few of these polymers such as

poly-L-lysine based polymers, polycations such as polyethylenimine (PEI), DOTAP N-[(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium-methylsulphate, polyethylene glycol (PEG), palmitoylglycol chitosan including dendrimers have shown promise in clinical applications (Brown *et al.,* 2001; Uchegbu *et al.,* 2001; Singh and Florence, 2005) (**Fig. 1**).

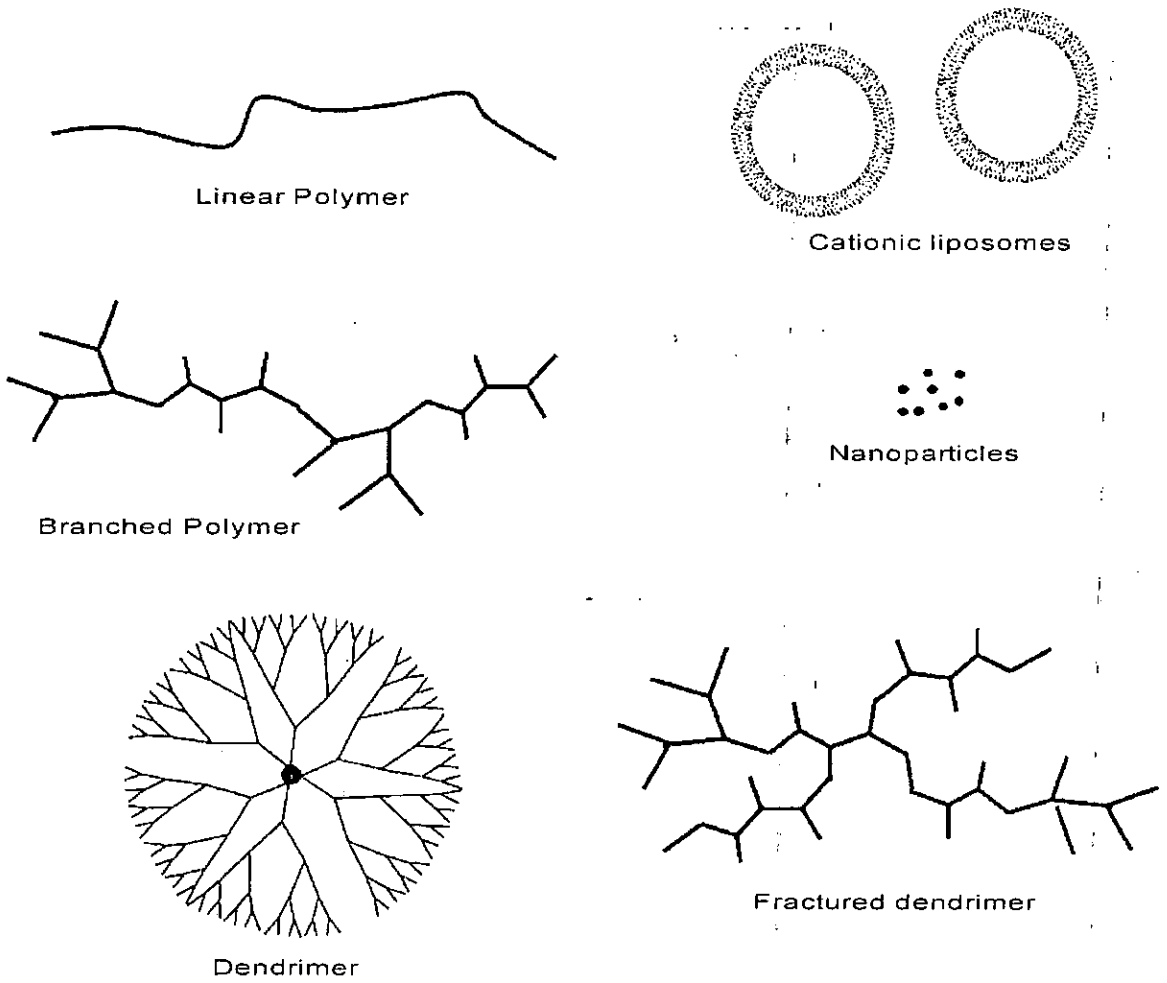


Figure 1: Examples of synthetic vectors

It has been postulated that constructs utilized as carriers in drug/gene delivery generally should be in the nanometer range and uniform in size to enhance their ability to permeate cell membranes and reduce the risk of undesired clearance from the body through the liver or spleen (Svenson and Donald, 2005).

The principles of drug and gene delivery have shown immense promise in clinical applications for delivery of anticancer drugs. Carrier-mediated anticancer drug delivery system is an approach that has been successful in cancer therapy. It is an approach providing sustained release of drug, protects the drug moiety from immediate dilution in the biological system, prolongs circulation time, enhances therapeutic index and finally decreases toxic effect of anticancer drugs (Khandare *et al.*, 2006). This concept of gene-polymer and drug-polymer complex delivery systems could also be applied to malaria chemotherapies with a view to improving drug-target specificity, reduction in resistance development and drug toxicity.

1.2 Statement of Problem

- **Interaction potentials between CQ/CP and HF/CP**

In malaria endemic countries like Nigeria, bacterial infections, such as typhoid fever are equally wide-spread, thus it is a common practice for antibacterial and antimalarial agents to be co-administered given the dearth of proper diagnostic

tools. Chloroquine base has been co-administered with amoxicillin and the fluoroquinolones. These admixtures or polypharmacy may often lead to modification of either the therapeutic or toxic effects or even the bioavailability of either of the drugs. As a result of this, the interaction potentials of these drugs need to be thoroughly investigated.

- **Development of HPLC analytical Methods.**

In conducting drug-drug interaction studies using *in-vitro* dissolution method, one of the analytical challenges is to be able to assay precisely the various components of the dissolution mixtures. One of the most efficient ways is by the use of **High-performance Liquid Chromatographic (HPLC) method** which concomitantly elutes the drug substances. Currently no method capable of simultaneous elution of chloroquine and fluoroquinolones has been described in literature.

In view of the foregoing, extensive studies investigating the interaction potentials between ciprofloxacin and representative antimalarial (CQ & HF) using principle of dissolution testing are considered justified.

- **Biopharmaceutical and Pharmacokinetics of Amodiaquine in ACT formulations**

The emergence of multidrug resistant *plasmodium falciparum* (MDRPF) to some of antimalarial agents such as CQ, sulphadoxine-pyrimethamine (SP), halofantrine (HF) etc led to the recommendation by World Health Organization (WHO) that the Artemisinin combination based therapies (ACTs) be adopted. As such antimalarial drug resistance is becoming an increasingly important public health problem. The use of artemisinin combination therapies has been on the increase since its adoption by WHO. According to National Agency for Drug Administration and Control (NAFDAC) 13 Artemether-Lumefantrine, 40 Artesunate-Amodiaquine, 29 Artesunates, and 12 Dihydroartemisinin (DHA) have been registered which now abound in the Nigerian drug market (TWG, 2007) However, there exists little information regarding the disposition of amodiaquine in combination with other antimalarials such as Artesunate.

Evaluation of the active principles of these combinations both *in-vitro* and *in-vivo* therefore becomes critical and exigent for quality control assessment and appropriate healthcare delivery. Also variations in pharmacokinetic parameters in man are a possibility, thus underlying the fact that extrapolation to Africans of data obtained elsewhere should be carried out with some caution since pharmacogenetic and racial variations may affect the pharmacokinetic profile of

drugs that are extensively metabolized in humans e.g. amodiaquine. In view of the forgoing it becomes pertinent to establish appropriate disposition profiles of ACTs in Nigerians.

- **Nanomedicine**

The goal of nanomedicine technology towards the use of synthetic carriers to deliver genes or drugs (in a compact form) to the target cells/tissues/organs to avoid degradation in systemic circulation has not been optimally fulfilled.

Several efforts at formulating DNA-polycation and drug-polymer complexes using cationic liposomes or dendritic polymer surface functionality has resulted in the formation of complexes demonstrating limiting factors such as inconsistent particle size, aggregate/irregular shape and these culminated to poor drug-target interaction. There is then the growing need to investigate better complex formation and more efficient drug/gene delivery.

1.3 AIMS AND OBJECTIVES

1.3.1 General Objectives

The great variation in the chemical structures, the physical properties, and pharmacological effects of numerous compounds used as therapeutic agents suggests that any two drugs, administered concurrently might interact in virtually an endless number of ways (American Pharmaceutical Association, 1976). The interactions may occur both *in-vitro* and *in-vivo* leading to greater incidence of adverse, therapeutic or toxic effects. It has been reported that chloroquine, mefloquine, halofantrine, and some quinolones such as ciprofloxacin and sparfloxacin when co-administered can lead to pharmacodynamic interactions resulting in prolongation of QT interval which may result in elevated risk of ventricular arrhythmias including ventricular tachycardia and torsade de pointes (Lightbrown *et al.*, 2001). Therefore, the need to investigate the drug interaction potential becomes imperative given the frequency of such **polypharmacy** in the Nigerian hospital setting.

Traditionally, ACT was introduced into the drug market as a separate entities of the combined drugs but the advent of new technology led to co-formulation as fixed doses. Many of such formulations abound in Nigerian drug market. There is then a strong need to ascertain integrity of each active principle in the co-formulations for quality control assessment.

Wet granulation manufacturing procedures had been the general method for formulation of solid dosage forms such as ACTs in separate doses but direct compression method has now taken over, purposely to avert hydrolysis of endoperoxide linkage of the artemisinin component of the co-formulations. It has also been argued that co-formulation of Artesunate and amodiaquine using wet granulation could jeopardize the integrity of the Artesunate.

Although the field of gene therapy has experienced significant set backs and limited success. It is still one of the most promising and active research fields in medicine (Selkirk, 2004). To improve the delivery of DNA into cell, the DNA must be protected from damage and its entry must be facilitated. To these end new molecules such as cationic liposomes, dendrimers etc have been created and have the ability to protect the DNA from undesirable degradation during the gene transfection process. However, the formation of DNA-polymer complexes called polyplexes have been limited by formulation variables such as inconsistent particle size and shape hence the need for thorough investigation.

1.3.2 Specific Objectives

The specific objectives of the study are:-

(1) To evaluate physicochemical characteristics of various drug formulations and development of High Performance Liquid Chromatographic (HPLC) analytical method for specific quantification of chloroquine, ciprofloxacin and sparfloxacin concomitantly in aqueous solution.

(2) To investigate the possible drug-drug interactions between ciprofloxacin and chloroquine; ciprofloxacin and halofantrine *in-vitro* and biopharmaceutical release of Artesunate ad amodiaquine of both in-house formulations and other branded ACTS *in-vitro*.

(3) To investigate the biosystemic uptake of Amodiaquine substance in ACT formulations and evaluate pharmacokinetic parameters for amodiaquine in Nigerian subjects.

(4) To formulate DNA-DAB16 complexes (Dendriplexes), investigate the hydrodynamic size and zeta potential using Photon correlation spectroscopy & Transmission Electron Microscope (TEM) and provide a possible explanation for the particle variables which control *in-vivo* gene transfection.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 MALARIA

Epidemiology of malaria

Malaria coined from the Latin "mal-aria" or bad air, is the most prevalent of parasitic widespread disease of humans, affecting subjects mainly in tropical and subtropical areas. It is estimated that about 2073 million people (over 40% of the world's population) are exposed to the risk of malaria and that some 270 million of these are infected with malaria parasites. Global deaths emanating from this population are estimated at approximately 1 million a year (WHO, 1990).

Malaria is a vector-borne infectious disease caused by protozoan parasites of the genius *Plasmodium.* Over 40 species of plasmodia exist but only four types of the plasmodium parasite can infect humans. These are *Plasmodium falciparum, P. vivax, P. ovale, P. malariae.* Recent reports of *P. knowlesi* infections have been made in south east Asia. Most of the morbidity and almost all the mortality attributable to this scourge are caused by *P. falciparum.*

In Nigeria, malaria is epidemiologically holoendemic in occurrence. The main ecological zones in Nigeria include the coastal, forest, mangrove, savannah and sahel, and the ever-advancing quest for urbanization of the country's rural areas

may have helped obliterate the varying distinctions that once characterized these ecological zones resulting in almost uniform pattern of malaria transmission and disease states. Deforestation, population migration and changes in agricultural practice encourage ready transmigration of the anopheles vector from forest zone to other zones. In equal vein the seasonal rainfalls with attendant percolation of water are veritable support for vectoral life in the seeming dry sahel region.

The malaria infection depends largely on three main factors: the vector anopheles mosquito insect which feeds on human blood and thereby ingests the parasite gametocytes; the parasite plasmodium present in various forms and in different degrees in humans with malaria and the receptive humans, especially children and younger population with compromised immunity.

Other factors such as the environment, climate, temperature, and humidity have an appreciable influence on the anopheles vector and parasite plasmodium. The varying severity of transmission and prevalence of human malaria over the seasons are attributable to these factors. *P. falciparum* and *P. vivax* do not show any sporogonic activity at temperatures below 20^0C. Even at higher temperatures, sporogony tends to decrease with elevated temperatures. The aquatic development of the vectoral larval stage is inhibited by adverse temperature conditions. The life pattern and longevity of the anopheles mosquito are affected by relative humidity as the life span of the insect declines with decrease in humidity (Coker *et al.*,

2001). Surface waters, a veritable and viable medium for larval development, are a function of perennial rainfall. This explains why tropical areas experiencing rainfalls all year round show high and frequent malaria transmission rates. These various factors define the extent of malaria endemicity in these zones.

2.1.1 LIFE CYCLE OF MALARIA PARASITE

Figure 2.1 shows the life cycle of *P. falciparum*. Although infection in the human host begins when an infected female anopheles mosquito feeds on a human host and sporozoites are injected into the host's blood stream. These sporozoites rapidly (within minutes) invade hepatocytes where they multiply extensively to form exoerythrocytic schizonts, each containing up to 300000 merozoites. Six to 15 days after infection (depending on the species) the schizont infected cell ruptures releasing mature merozoites into the blood stream. These merozoites invade red blood cells and undergo a second round of replication that lasts 48-72 hours and produces 16-32 merozoites per infected red blood cell. The released merozoites invade new red blood cells to carry on the cycle (Gardiner et al., 2005). Later in the infection, some merozoites give rise to sexually differentiated forms (gametocytes). The trigger for this gametocytogenesis is unclear. When a female anopheles mosquito ingest the blood of a host containing malaria parasites the red blood cells and asexual stage parasites are digested while the gametocytes undergo

further development to form macrogametocytes (female), or microgametocytes (male). In the mosquito stomach, the male and female gametes fuse to form a ookinete that penetrates the gut lining and produces an oocyst in the gut wall. As the oocyst matures, it divides to produce sporozoites that move to the salivary glands and are able to infect a new host when the mosquito next takes a blood meal and the cycle continues. This developmental cycle in the mosquito host takes about 12 days depending on the parasite species and ambient temperature (Gardiner et al., 2005). Some *P. vivax* and *P ovale* do not immediately develop into exo-erythrocytic phase merozoites but instead produce hypnozoites that remain dormant for periods ranging from several months to as long as three years. After a period of dormancy, they reactivate and produce merozoites. Although the blood stage and mosquito stages of the malaria life cycle were identified in the 19th and early 20th centuries, it was not until the 1980s that the latent form of the parasite was observed. This discovery finally proffers explanation why people could appear to be cured of malaria but still relapse years after the parasite had disappeared from their blood streams.

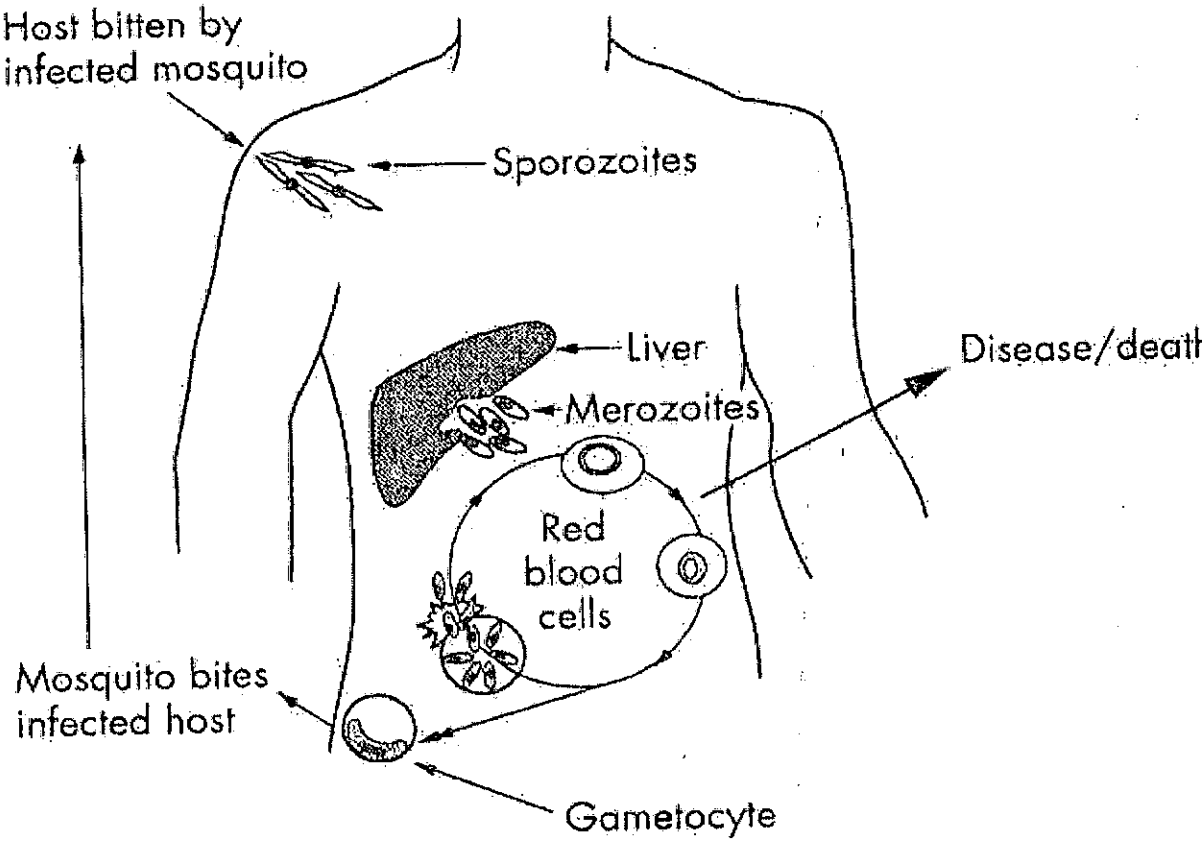


Fig. 2.1: Life cycle of malaria parasite (Gardiner *et al.*, 2005)

2.1.2 CLASSIFICATION OF ANTIMALARIAL AGENTS

A number of synthetic agents and natural remedies have been investigated for the treatment of malaria. These agents have been classified based on chemotherapeutic considerations.

2.1.2.1 Biological Classification.

This classification is based on chemotherapeutic effect on the pathways of plasmodial life cycle. The different stages of the life cycle of malarial parasites, as well as the different species, display different susceptibilities to various types of antimalarial drugs.

(a) **Tissue schizontocides** These agents act on the sporozoites well before the infection develops, i.e. exo-erythrocytic or liver stage and are used for causal prophylaxis to prevent invasion of the blood cells or as antirelapse drugs to produce radical cures of vivax and ovale malarias. Examples are primaquine, proguanil, pyrimethamine and sulphonamides.

(b) **Blood schizonticidal drugs:-** They are referred as suppressant drugs. They act on asexual erythrocytic stages of malaria parasites which are directly responsible for the clinical symptoms of the disease. The can produce a clinical cure or suppression of infection by susceptible strains of all four species of malaria parasite, but, since they have no effect on exo-erythrocytic forms, do not produce a radical cure of relapsing forms of ovale or vivax malarias (James,1996). Examples are quinine, mepacrine, chloroquine, mefloquine and artemisinin (James, 1996).

(c) **Gametocidal drugs:-** They exterminate sexual forms (gametocytes) of human malarial parasite of *P. vivax* and *P. malariae* but not the

gametocytes of *P.falciparum* to interrupt transmission of the infection to the mosquito vector. Examples are primaquine, quinine, mepacrine. Radical cure is achieved or possible with drugs that can destroy both exo-erythrocytic and erythrocytic forms of the parasites. Such agents are called secondary tissue schizontocides.

(d) **Sporontocidal drugs:-** These agents when given to a gametocyte carrier, prevent or inhibit the development of oocysts in mosquitoes feeding on the carrier i.e have no direct effect on gametocytes in the human host. They thus prevent the formation of sporozoites and multiplication of the parasites in the mosquitoes. Examples are proguanil, pyrimethamine and pamaquine.

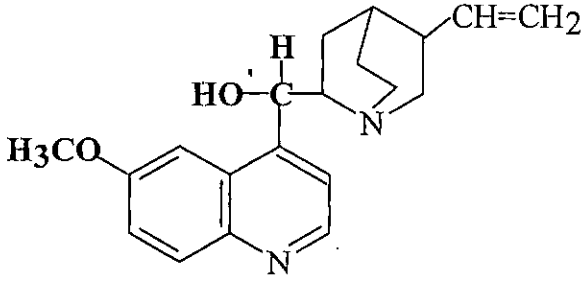
2.1.2.2 Chemical Classification

Malaria chemotherapeutics can also be classified based on structural similarities. A number of such classes of drugs have been investigated for the treatment of malaria. Majority of these drugs act on the erythrocytic stage of the parasite, and thus are used for the treatment of acute attacks of malaria. These include:-

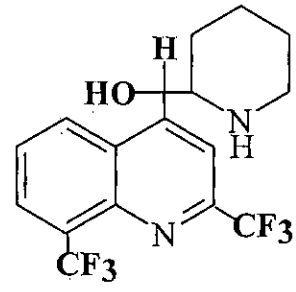
(a) QUINOLINE METHANOL

One of the first agents used for the treatment of malaria was the bark of the Cinchona tree, which was introduced from South America into Europe by the

Jesuits. It was not until 1820 that the active ingredient quinine was extracted from the bark, isolated and named by the French Chemists Pierre Joseph Pelletier and Joseph Bienaime Caventou. The active components contained in the bark include quinine [1], its diastereomer quinidine, and the desmethoxy distereomers cinchonine and cinchonidine. The structure of quinine was elucidated in 1908 and provided evidence that the quinoline nucleus could be a useful component of an antimalarial drug (Paul *et al.,* 1998). Mefloquine [2] which was later developed also belongs to this class.



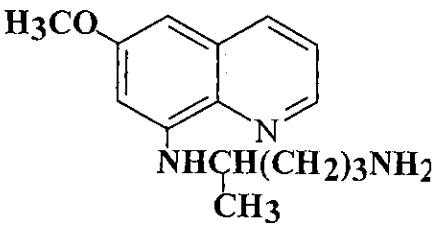
Quinine [1]



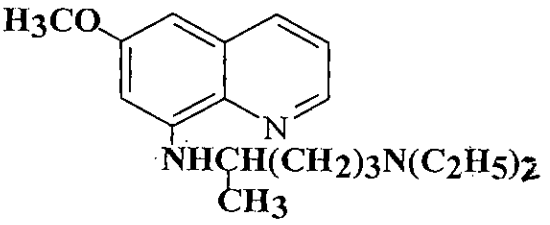
Mefloquine [2]

(b) 8-AMINOQUINOLINES

One of the first synthetic antimalarials to be investigated was the 8-aminoquinoline pamaquine [3]. During the 1920s, pamaquine was found to be more effective than



Primaquine [4]

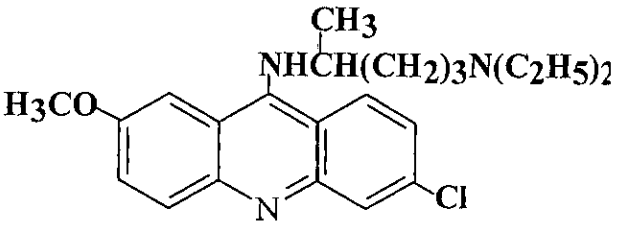


Pamaquine [3]

quinine in eradicating the liver stages of the malarial parasite in human. Primaquine [4] is also a good example.

(c) 9-AMINOACRIDINES

Further studies in the 1930's resulted in the synthesis of the 9-amino acridine drug mepacrine [5] which was used clinically until 1940.

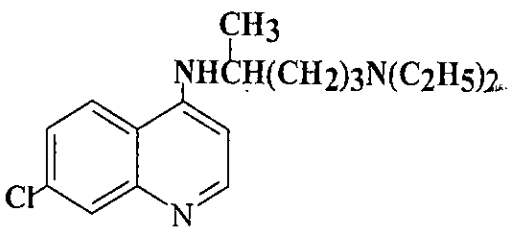


Mepacrine [5]

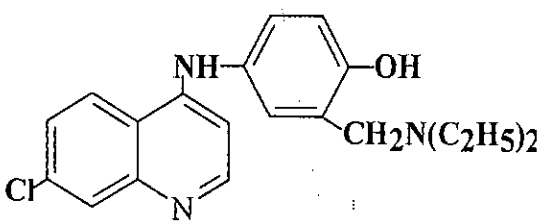
(d) 4-AMINOQUINOLINES

Until the development of resistance, perhaps the most effective of all antimalarial drugs was chloroquine [6], first produced in the 1930s. It was this drug in combination with vector control measures that initially produced the spectacular results seen in the first attempt to eradicate this disease in the 1950s and 60s (Gardiner *et al.*, 2005). However resistance to chloroquine emerged in Thailand in 1957 and resistance strains were isolated in patients in Thailand and Colombia in 1960. The first chloroquine resistance was discovered in Africa in Tanzania in 1979 (Menard *et al.*, 2005).

Further work on a wide variety of 4-aminoquinoline lead to discovery of amodiaquine [7] which was found to have an excellent activity profile (Steven and Alisa, 2005).



Chloroquine [6]

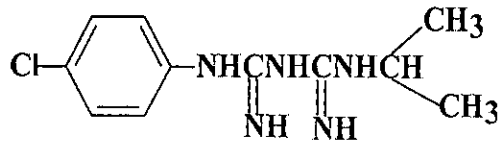


Amodiaquine [7]

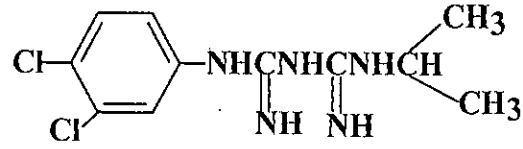
(e) BIGUANIDES

The observation by some scientists that sulphadiazine has some antimalarial effect led to the assertion that the pyrimidine moiety was important for

antimalarial activity (Olaniyi, 2005). It was later found out that compound with –NH group are inactive, this was later replaced with -guanido which then confers activity on the compound. Replacement of pyrimidine ring led to discovery of proguanil [11] and chlorproguanil [12].



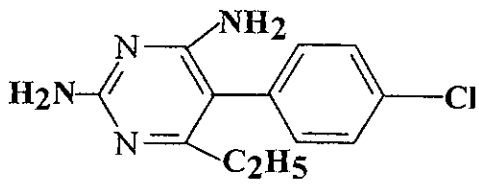
Proguanil [11]



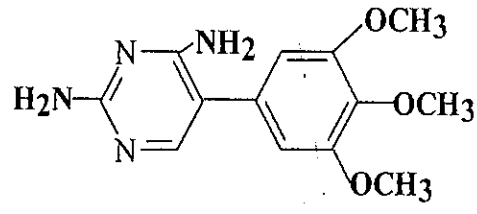
Chlorproguanil [12]

(f) DIAMINOPYRIMIDINES

Trimethoprim [13] was developed as an antibacterial agent but subsequently found to have antimalarial activity. Pyrimethamine [14] a dihydrofolate inhibitor also belongs to this group.



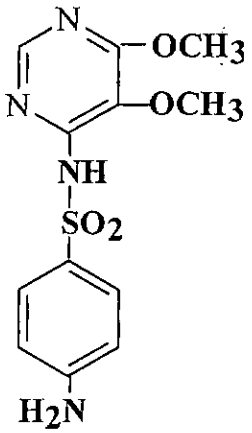
Pyrimethamine [14]



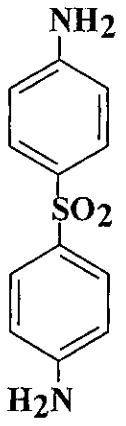
Trimethoprim [13]

(g) SULPHONAMIDES AND SULPHONES

Several medium and long-acting sulphonamides were introduced in 1958. Sulphadoxine [15], sulphamethoxypridazine, sulphadimethoxine and sulphalene were found to possess marked antimalarial activity. Sulphadoxine has been widely used for treatment and suppression of malaria, especially in association with pyrimethamine.



Sulphadoxine [15]



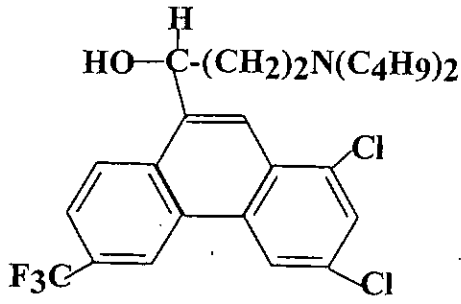
Dapsone [16]

Dapsone [16] a good example of sulphones has also been used in association with sulphadoxine and pyrimethamine for acute treatment of malaria attacks but its use has been limited by serious side effects such as allergic reactions.

(h) PHENANTHRENE-METHANOLS

Halofantrine [17] belongs to this class of antimalarial agent. It is a blood schizontocide whose use is contraindicated in pregnant women. High protein

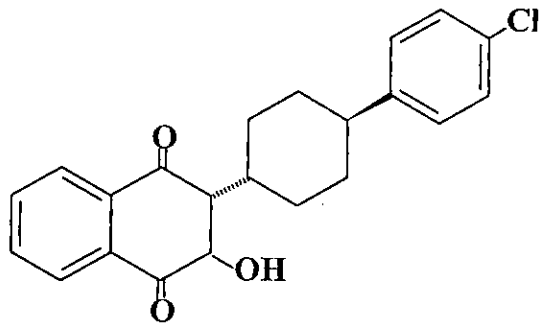
and high lipid food content may enhance it absorption. Its use has been precluded in patients with congenital prolongation of the QT interval and cardiac disease.



Halofantrine [17]

(i) NAPHTHOQUINONES

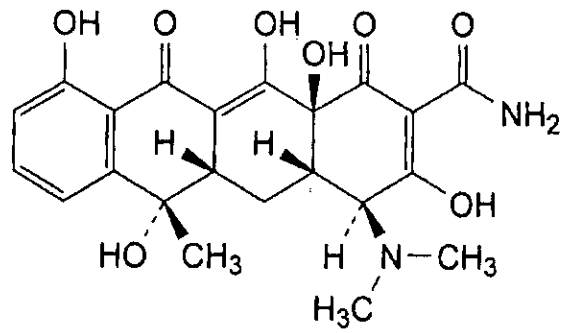
Atovaquone [18] is a synthetic hydroxynaphthoquinone developed in early 1980s. It is a highly lipophilic, water-insoluble analogue of ubiquinone 6, an essential component of mitochondria electron transport chain in micro-organisms. It has also been combined with proguanil for the treatment of uncomplicated multi-drug resistant *Plasmodium falciparum.*



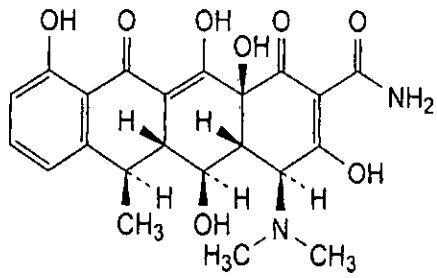
Atovaquone [18]

(j) ANTIBIOTICS

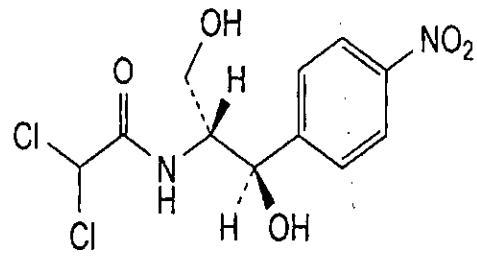
A number of antibiotics among which are tetracycline [19], doxycycline [20], chloramphenicol [21] have also shown some level of antiplasmodial activity. Some have been combined with other antimalarial agents for synergistic effect on the plasmodium. Doxycline, an important prophylactic drug cannot be used by young children or pregnant women.



Tetracycline [19]



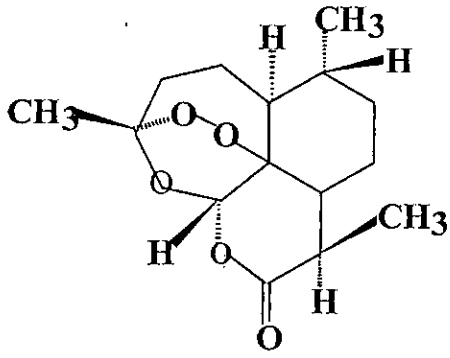
Doxycycline [20]



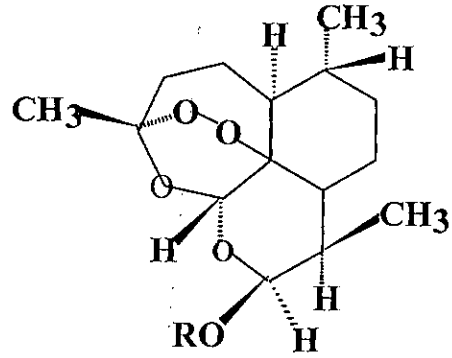
Chloramphenicol [21]

(k) SESQUITERPENE LACTONES

Artemisinin [22] was isolated from Chinese plant *Artemisia annua*. It is a sesquiterpene lactone with an internal peroxide linkage. Other active compounds derived from artemisinin are artemether [23], arteether [24], artesunate [25], and dihydroartemisinin [26]. It is only this class of drugs that has not seen the development of drug resistance. Nonetheless, because of short half-life of these drugs *in-vivo*, recrudescence is associated with a short course treatment.



Artemisinin[22]



R= CH_3 Artemether[23]
R= C_2H_5 Arteether[24]
R= $-CO(CH_2)_2COONa$ Artesunate[25]
R= H Dihydroartemisinin [26]

2.1.3 MALARIAL DRUG RESISTANCE

WHO defined antimalarial drug resistance as the ability of a parasite strain to survive and/ or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject (WHO, 2003).

Resistance to antimalarial drugs arises as a result of spontaneously-occurring mutations that affect the structure and activity at the molecular level of drug target in the malaria parasite or affect the access of the drug to that target. Therefore antimalarial drug resistance is becoming one of the greatest challenges in malaria treatment.

P. falciparum multidrug resistance gene 1 (**pfmdr 1**) has been implicated in resistance to diverse antimalarials, such as the aminoquinolines chloroquine and amodiaquine; mefloquine and halofantrine. **Pfmdr 1** encodes the protein P. glycoprotein homologue (**Pgh**), which belongs to the ATP binding cassette family of transporters. These proteins use energy derived from ATP to transport various structurally related substrates out of the cell. **Pgh** localises to the parasite's food vacuole membrane where another protein associated with chloroquine resistance, *P. falciparum* chloroquine resistance transporter (**pfcrt**), is also found. This transporter protein probably interact to alter parasite susceptibility to antimalarials (Kremsner and Krishna, 2004).

Various factors relating to drug, parasite and human host interactions contribute to the development and spread of drug resistance. The molecular mechanism of drug action is a critical element in the speed at which resistance developed. In addition, drugs with a long terminal elimination half-life enhance the development of resistance particularly in area of high transmission. Similarly, increased drug pressure is a significant contributor to drug resistance. As increased amounts of a drug are used, the likelihood that parasites will be exposed to inadequate drug levels rises and resistant mutants are more readily selected.

Parasite factors associated with resistance include the *Plasmodium* species concerned and the intensity of transmission. Human host factors include the widespread and / or irrational use of antimalarial drugs and possible the level of host immunity. Because of the widespread incidence of drug resistance, advocacy for multidrug therapies has increased as this principle has been used successfully in tuberculosis, cancer therapy and in HIV management.

2.1.4 WHO REQUIREMENTS FOR IDEAL ANTIMALARIAL DRUGS

These include the following properties:

- High efficacy in providing both clinical and parasitological cure
- Very safe, especially in infants and in pregnancy

- Guaranteed against parasite resistance
- Effective against all malaria species
- Have applications in intermittent preventive treatment
- Potent anti-gametocyte (transmission blocking) activity
- Available in fixed-dose combinations
- Simple regimen- ideally as single dose
- Long shelf-life (at least 3 years)
- Available in paediatric formulations and course-of-therapy packaging (WHO, 2003).

2.1.5 ANTIMALARIAL DRUG COMBINATION THERAPY

Combination therapy with antimalarial drug is the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and different biochemical targets in the parasite. Combination therapies can be either fixed-combination medicinal products, in which the components are co-formulated in the same tablet or capsule, or multiple-drug therapy, in which components are co-administered in separate tablets or capsules.

Some drug combinations such as the use of an antimalarial drug with a non-antimalarial drug that may enhance its action (e.g. chloroquine plus

chlorpheniramine) and the use of a blood schizontocidal drug with a tissue schizontocidal or gametocytocidal drug (e.g. chloroquine plus primaquine) are not classified as combination therapy (WHO, 2000).

Likewise, certain medicinal products that fit the criteria of synergistic fixed-dose combinations are considered as single synergistic products in that neither of the individual components in itself would be given alone for antimalarial therapy. Examples include:

- Sulphadoxine-pyrimethamine
- Chlorproguanil-dapsone
- Atovaquone-proguanil

The rationale for the use of fixed combination and multiple-drug therapies are found in the synergistic and additive potential of individual drugs. The aim is to improve efficacy, retard the development of resistance to individual components of the combination and finally shorten duration of treatment which in turn increases compliance.

2.1.5.1 Sulphadoxine/Pyrimethamine (SP)

Pyrimethamine was discovered during World War II. It has a good oral bioavailability, is absorbed in few hours in children with malaria and has an elimination half-life of about 80 h. Sulphadoxine also has good bioavailability and

an elimination half-life of about 120 h in children with malaria. WHO by its definition of drug combinations considered sulphadoxine-pyrimethamine a single drug rather than a combination in which each component acts independently. Although pyrimethamine inhibits dihydrofolate reductase (DHFR) and sulphadoxine inhibits dihydropteroate synthase (DHPS). Inhibition of both enzymes prevents synthesis of folic acid in parasites. After chloroquine, SP was the most commonly used antimalarial treatment in almost all endemic areas between the 1960s and the 1980's (Wernsdorfer, 1994; Mordmuller *et al.*, 1998)

2.1.5.2 Sulphadoxine/Pyrimethamine/Chloroquine

The combination of chloroquine with SP has been sporadically tried in the past. The results of a review, which summarized the findings from five trials of SP plus 4-aminoquinolines, showed a quicker resolution of symptoms with the combination than with SP alone (McIntosh and Greenwood, 1998).

2.1.5.3 Sulphadoxine/Pyrimethamine/Amodiaquine

Amodiaquine, like chloroquine, leads to annoying pruritus, especially in Africans. Additionally, it causes agranulocytosis and liver damage in white people taking the drug for chemophylaxis (Kremsner and Krishna, 2004).

2.1.5.4 Sulphadoxine/Pyrimethamine/Quinine

Quinine needs to be administered on 7 consecutive days when given as monotherapy, which is not always achievable because of cinchonism (tinnitus, nausea) and the very bitter flavour of most salts. However, apart from artemisinin, quinine is the only drug used to treat severe malaria. Its combination with SP has been used in several trials to improve efficacy mostly in areas where parasites are still sensitive to SP (Kremsner and Krishna, 2004).

2.1.5.5 Sulphadoxine/Pyrimethamine/Mefloquine

This single dose, fixed, triple combination formulation has never been extensively used in most countries, and was introduced onto the market when the efficacy of SP against parasites was already impaired (Kremsner and Krishna, 2004).

2.1.5.6 Atovaquone/Proguanil

Atovaquone acts by inhibiting the parasite's mitochondrial complex bc_1 and disrupting the membrane potential. Because of the high frequency of resistance associated with atovaquone monotherapy (Looareesuwan *et al.*, 1996), it was combined with proquanil, a biguanide. Atovaquone-proguanil has been developed for therapy and prophylaxis of malaria, and shows good safety and tolerability in

children and adults, with high efficacy against *P. falciparum* malaria when used in a 3-day regimen (Kremsner and Krishna, 2004).

2.1.5.7 Chlorproguanil/Dapsone

Dapsone is a sulpha drug, with elimination half-life of about 30 h, which has been combined with chlorproguanil (which has an elimination half-life of about 20 h). Results of chlorproguanil-dapsone combination indicate that the combination is well tolerated and efficacious. However, there was a high incidence of serious haematological adverse events after its use. The safety of this combination is, therefore, a major concern, particularly since dapsone can cause methaemoglobinaemia and haemolysis in individuals (Kremsner and Krishna, 2004).

2.1.5.8 Quinine/Tetracycline

Tetracycline is an antibiotic that probably acts by inhibiting the binding of aminoacyl tRNA to the ribosome. It acts fairly slowly, is well absorbed, and has elimination half-life of 8 h, which is similar to that of quinine. A major limiting factor in compliance has been symptoms of cinchonism. Because the use of

tetracyclines is contraindicated in children and pregnant women, use of this combination has always been restricted (Kremsner and Krishna, 2004).

2.1.5.9 Quinine/Clindamycin

It has never been widely used, although several studies show both good efficacy and sound safety profiles in adults, children and pregnant women.

2.1.5.10 Artesunate/Amodiaquine

WHO developed Artesunate-amodiaquine for treatment of malaria in African children through a private-public partnership. In a multicentre phase III trial conducted in Gabon, Kenya, and Senegal, this combination drug showed a better overall efficacy than amodiaquine alone (Adjuik *et al.*, 2002). However, 6% of patients in both groups developed neutropenia. Despite this side effect, WHO still recommends this regimen for treatment of uncomplicated *P. falciparum* malaria in Africa children (Kremsner and Krishna, 2004).

2.1.5.11 Artesunate/Suphadoxine/Pyrimethamine

Artesunate plus SP has also been assessed in African children. It gave promising results in the Gambia, where it was as well tolerated and efficacious as SP alone (Seidlein *et al.*, 2000).

2.1.5.12 Artemether/Lumefantrine

This is the only fixed-dose artemisinin-containing formulation registered after international recognized guidelines. It seems safe and well tolerated in children as well as in adults. Like atovaquone, lumefantrine absorption is enhanced with food. Lumefantrine is structurally related to halofantrine and has a half-life of several days, unlike halofantrine it does not cause cardio toxicity. Like artesunate-mefloquine, there is a considerable mismatch in elimination half-lives of artemether and lumefantrine.

2.1.5.13 Artesunate/Mefloquine

A 3-day regimen of this combination has been used successfully in Thailand for the treatment of malaria. It is safe, well tolerated and highly effective. The disadvantage of this regimen includes its price and the pharmacokinetic mismatch

leading to long-term exposure of parasites to low doses of mefloquine (Kremsner and Krishna, 2004).

2.1.5.14 Dihydroartemisinin (DHA)/Piperequine/Trimethoprim.

This combination has also found to be active against malarial parasite and safe with no serious side effects. It has been shown that the cure rates for this combination are high. In addition, piperaquine/DHA might be given when chemoprophylaxis or treatment including long-half-life drugs such as chloroquine or mefloquine has failed (Kremsner and Krishna, 2004).

2.1.6 Artemisinin based Combination Therapies

The emergence of multi-drug resistant *Plasmodium falciparum* (***mdrpf***) led to WHO recommendation of artemisinin based combination therapies (ACTs) as against monotherapy. The features of ACTs relate to the unique mode of action of the artemisinin component, which includes the following:

- Rapid and substantial reduction of the parasite biomass
- Rapid parasite clearance
- Rapid resolution of clinical symptoms
- Effective action against multidrug-resistant *P. falciparum*

- Reduction of gametocyte carriage, which potentially reduces transmission of resistant alleles.

Because of the short half-life of artemisinin derivatives, their use as monotherapy requires daily doses over a period of 7 days. Combination of one of these drugs with a longer half-life partner antimalarial drug allows a reduction in the duration of antimalarial treatment while at the same time enhancing efficacy and reducing the likelihood of resistance development.

In most of the artemisinin based combinations currently in use or being evaluated, e.g. Artesunate-amodiaquine, the partner drug is eliminated slowly. The partner drug is therefore unprotected once the artemisinin has been eliminated from the body and operates a selective pressure on new infections. The implications of this "**pharmacokinetic mismatch**" are not fully understood at present, particularly in areas of high transmission in Africa. The safest approach is to use a drug partner that has a residual short half-life while still enabling parasite clearance within 3 days. However, this is difficult to achieve given the limited range of antimalarial drugs available (WHO, 2003).

2.1.6.1 AMODIAQUINE

Amodiaquine is a 4-aminoquinoline antimalarial agent structurally related to chloroquine and has been around for almost 60 years. It is chemically named 4-[(7-Chloro-4-quinolyl) amino]-2-(diethylaminomethyl) phenol. It was developed during World War II by the US Army-sponsored program as alternative to quinine. It is a very effective blood schizontocidal drug. Blood parasite clearance is completely effected within 24-48 hours of amodiaquine 600mg base administration. The drug was widely used between 1948 and 1990 before being temporarily removed from the essential drug list after reports of serious adverse events in travelers using it as chemoprophylactic agent. Nevertheless, in the mid-1990's, amodiaquine was re-introduced into WHO Treatment Guidelines and recent studies have shown good safety and efficacy in children with uncomplicated malaria, alone (Olliaro *et al.*, 1996) or in combination with Artesunate (Adjuik *et al.*, 2002) or with sulphadoxine-pyrimethamine (Hwang *et al.*, 2006).

It is similar to chloroquine in that both contain quinoline ring system with a tertiary terminal nitrogen function that is charged at physiological pH.

2.1.6.2 Synthesis and Disposition

Its synthetic pathway involves series of steps as shown in **Figure 2.2.**

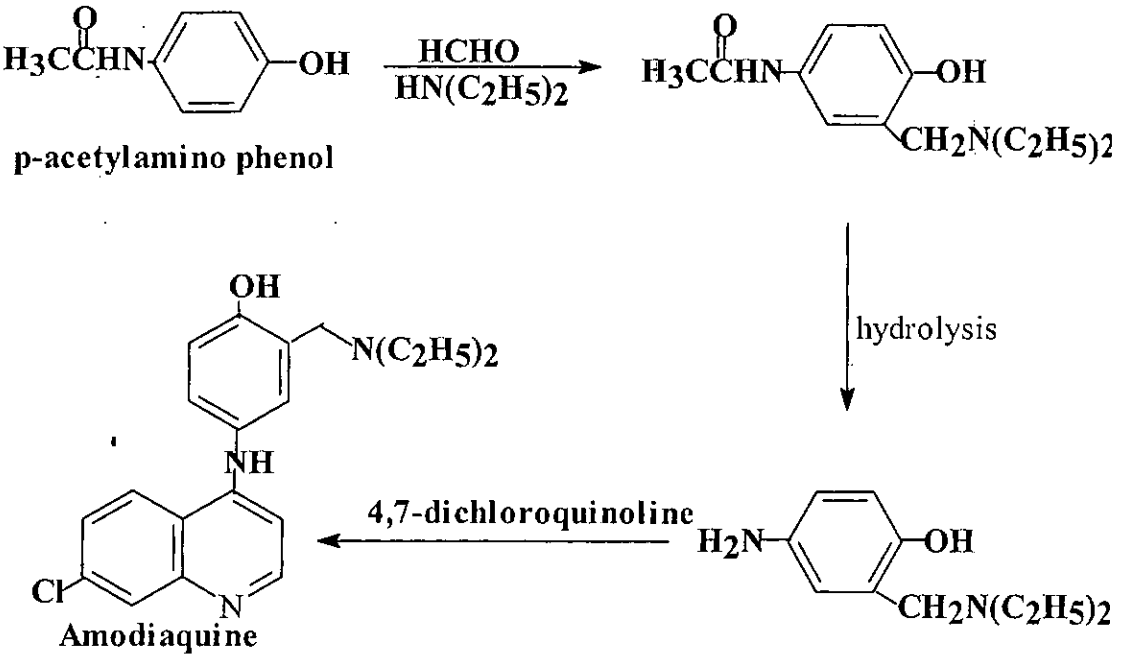


Figure 2.2: Synthesis of amodiaquine

Amodiaquine differs chemically from chloroquine in that it contains a 4-hydroxyanilo function in its side chain, although it is important to note that amodiaquine and chloroquine both have four carbon atoms separating the secondary and tertiary nitrogen atoms. Molecular modelling studies have stressed the fact that the internitrogen separation is approximately 8.3Å in both chloroquine and amodiaquine (Koh *et al.*, 1994)

The metabolism of amodiaquine in human has been widely studied. Although it has a high absorption profile from the gut, due to a large first pass effect,

amodiaquine has a low bioavailability (White *et al,* 1987). The principal plasma metabolite is its monodesethylamodiaquine (DesAMQ). This metabolite has been shown to be equipotent to amodiaquine *in vitro* against chloroquine-sensitive parasites. The DesAMQ also undergoes further metabolism by de-ethylation into bis-desethylamodiaquine (Laurent *et al.,* 1993).

The clinical use of amodiaquine has been limited by the side effects such as hepatotoxicity (Neftel et al., 1986) and agranulocytosis (Winstanley, 1990 & Park *et al.,* 1990). The toxicity of amodiaquine has been associated with oxidation of its hydroxyanilino moiety under three distinct conditions; auto-oxidation, peroxidase-catalysed oxidation and hydroxylation (**Fig. 2.3**) to form a chemically reactive quinoneimine (Vermeulen *et al.,* 1992) and hydroxylamine group which binds to cellular proteins group. However, large doses of amodiaquine have been reported to produce syncope, spasticity, convulsions and involuntary movements (Jaeger, 1987).

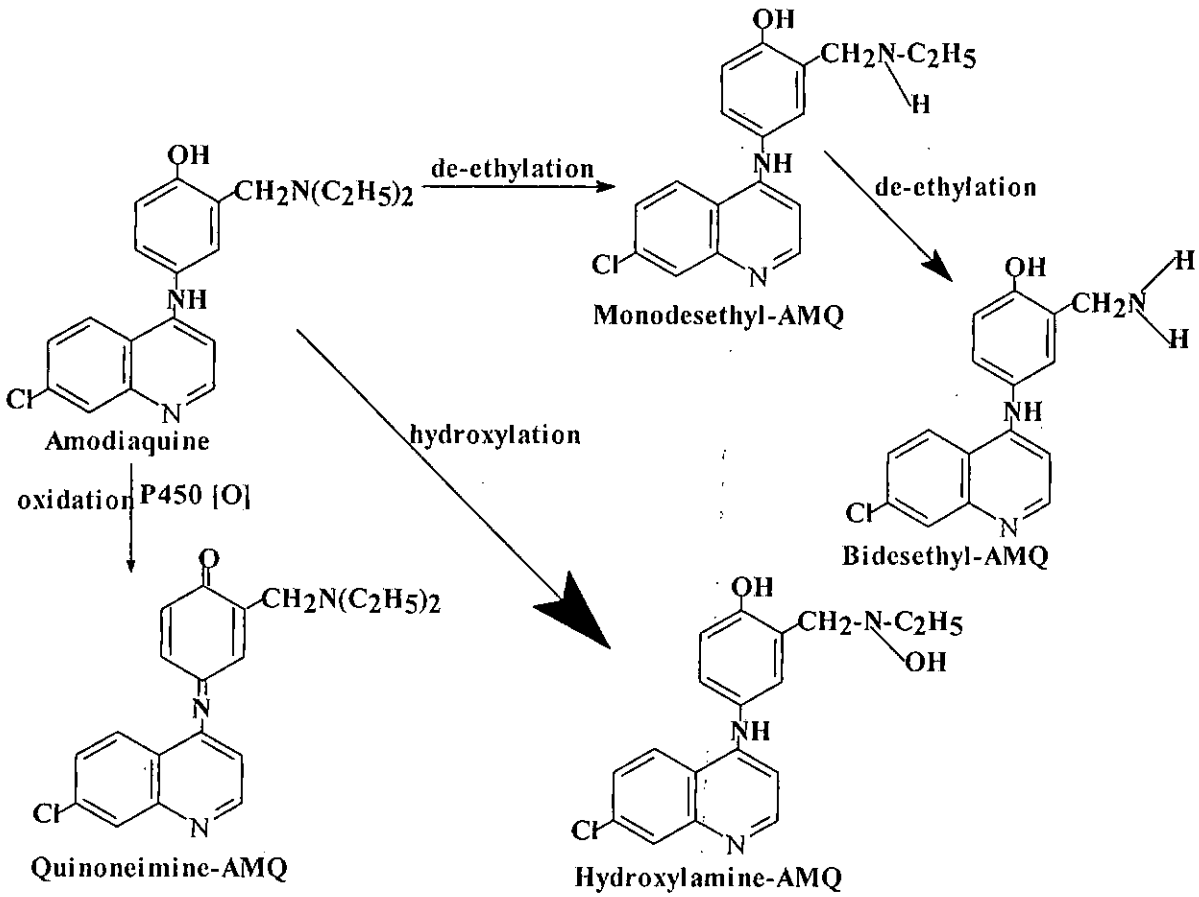


Figure 2.3: Structure of amodiaquine and its metabolites.

2.1.6.3 Parasitological Considerations of mechanism of action of 4-aminoquinolines

Although the definitive mechanism(s) of action of these compounds is /are still unknown, a number of facts relating to the drugs' action are now widely accepted. The 4-aminoquinolines are able to accumulate to such a high concentrations within the acid-food vacuole compartments of the malaria parasite (Aikawa, 1972; Yayon *et al.,* 1984). This acidic organelle is thought to be the site at which the drug

exerts its antimalarial effects. It is clear that the accumulation of the 4-aminoquinolines is an important part of their ability to inhibit parasite growth.

It has been demonstrated that the 4-aminoquinoline antimalarials were able to interact with both mammalian and malarial parasite DNA *in-vitro* (Perker and Irwin, 1952).

This hypothesis evolved from initial observations of some scientists who also showed that chloroquine was able to inhibit DNA and RNA synthesis in both mammalian and bacterial cells. Also in support of this hypothesis, it had been shown that exposure to *P. knowlesi* to chloroquine resulted in a breakdown of the parasite's ribosomal RNA (Warhurst and Williamson, 1968; Warhurst, 1969). These findings suggested that the mode of action of chloroquine/amodiaquine was related to inhibition of DNA replication and RNA transcription in the parasites (Peters, 1970). The molecular mechanism is thought to be by intercalation of the quinoline ring in the DNA in the parasite and the basic group forming an ionic link with phosphate groups across the double helical strands of the DNA (**Fig.2.4**). The quinoline-DNA complexation inhibits "thermal melting" (i.e. natural separation of the DNA helical strands) during the replication phase. This interference with a natural process of maturation of the parasite results in its death.

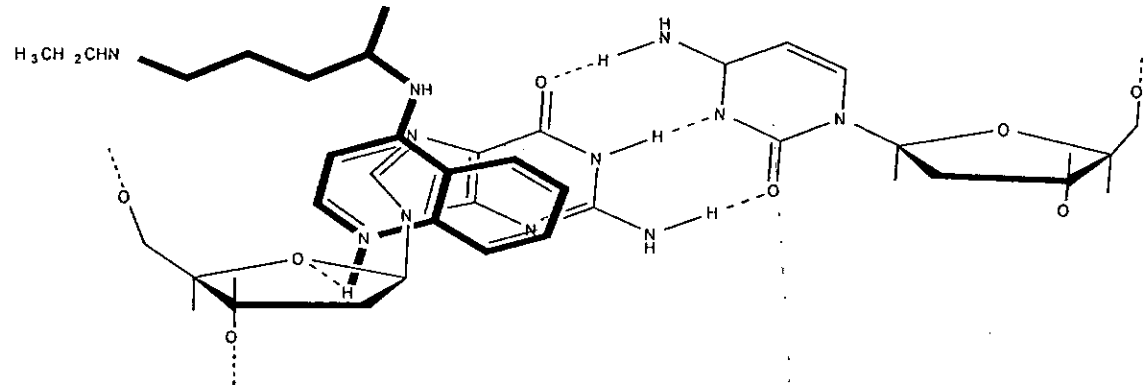


Figure 2.4: 4-aminoquinoline-DNA complex

However, development of multidrug resistant *P. falciparum* has precluded the use of these antimalarials as monotherapy.

2.1.6.4 ARTEMISININ AND ITS DERIVATIVES

Artemisinins are derived from a plant *Artemisia annua* commonly called sweet wormwood (or sweet Annie). In China, where they were first discovered, the antipyretic properties of the plant which is locally called "qinghao" were observed as far back as 1500 years ago. In 1971, a highly active chemical from qinghao, known as qūinghaosu was obtained and is now called artemisinin (Quinghaosu Antimalarial Co-coordinating Research Group, 1979). Since this initial discovery, an array of semi-synthetic oil and water artemisinin derivatives have been developed (**Fig. 2.5**). The recrudescence rate associated with artemisinin when

used in monotherapy largely depends on the dose administered, the duration of treatment and severity of disease but not at present on parasite resistance.

2.1.6.5 Chemistry and synthesis

Artemisinin is comparatively easily purified by crystallization after extraction from *Artemisia annua* plants but is very difficult to synthesize *de novo* (Woodrow et al., 2005). Artemisinin is a sesquiterpene lactone structure in which antimalarial activity is linked to endoperoxide trioxane moiety (Haynes, 2001). It is stable in neutral solvents heated up to 150°C. Liquid solvent extraction with toluene, n-hexane, chloroform or petroleum ether is the most currently applied technique for artemisinin extraction with extraction times ranging from a few minutes to several hours (Christen and Veuthey, 2001). The drug is the parent compound for semi-synthetic derivatives that have been chemically modified at the C-12 position to produce artesunate, artemether, arteether, dihydroartemisinin (**Fig. 2.6**). These compounds have been variously formulated for oral, rectal and parenteral administration.

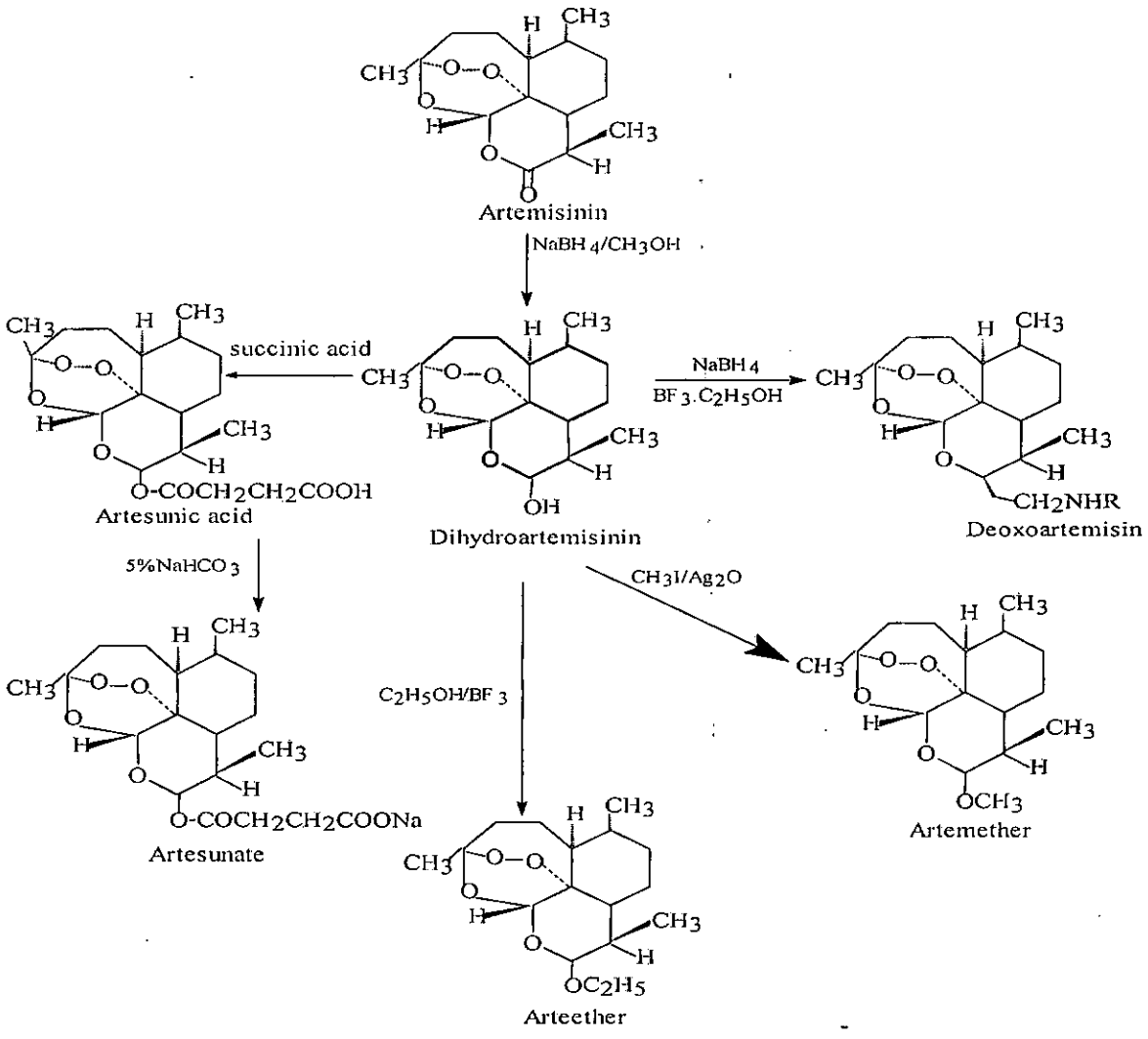


Figure 2.5: A scheme outlining the methods of obtaining suitable derivatives from naturally occurring artemisinin (Olaniyi, 2005).

2.1.6.6 Mechanism of action of Artemisinin

In-vitro studies have suggested an uptake of artemisinin by both healthy and malaria infected red-blood cells (Asawamahasakda *et al.*, 1994). It is known that artemisinin binds to haem, either in haemoglobin (inside red blood cells) or haemozoin (stored haem within the malaria parasite) (**Fig. 2.6**). Through an iron-

mediated cleavage of the peroxide bridge artemisinin free radicals are formed. These free radicals (**Fig. 2.7**) are destructive to parasite membranes thereby exterminating most forms of the parasite (Asawamahasakda *et al.,* 1994; Cumming *et al.,* 1998). The reactivity of the endoperoxide function of artemisinins with haem iron is considered to be key factor for pharmacological/parasitological activity of antimalarial endoperoxide drugs.

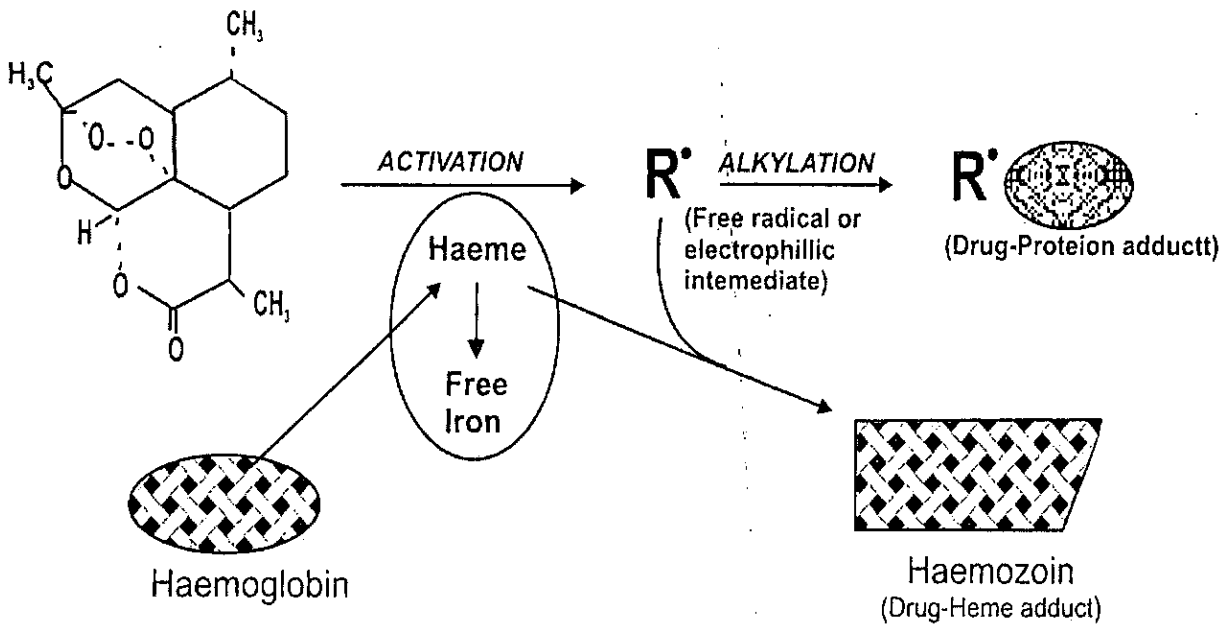


Figure 2.6: Mechanism of action of artemisinin and its derivatives. How artemisinin produces its antiplasmodial and cytotoxic activities.

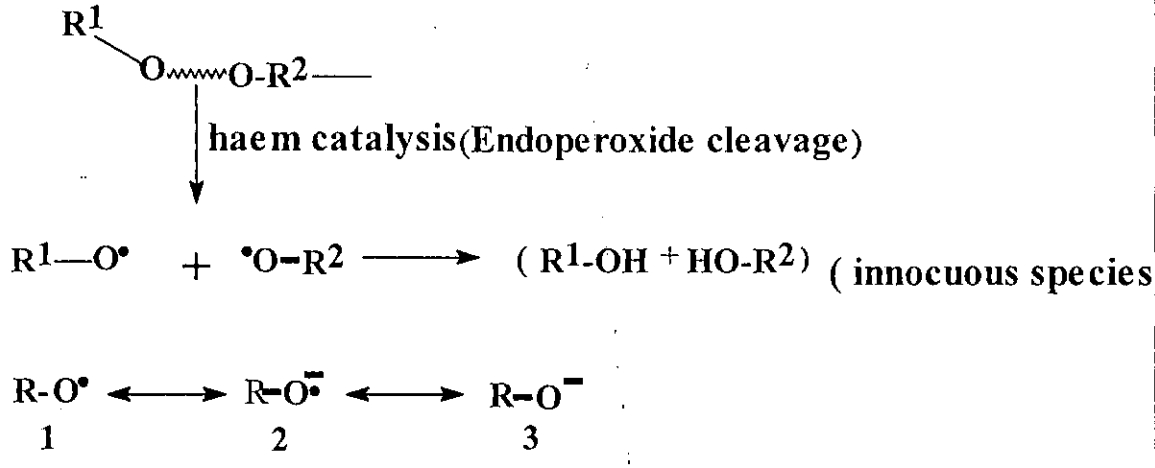


Figure 2.7: A scheme showing resonance stabilized oxygen centered free radical species.

This provides a quantum **parasiticidal** effect on all the forms of plasmodium – trophozoites, merozoites, schizonts, gametes, that is, leads to an intensely seeming "pharmacodynamic" (cytotoxic) synergism, though short-lived. However, all these can be quenched by a pinch of **antioxidants** (e.g. Vitamin C) or excess cationic species such as Fe^{2+}.

2.2 DRUG INTERACTION

The increase in the number of new drugs has contributed immensely to modern drug therapy, but like all progress it has also created new problems. A problem of increasing concern is the greater incidence of adverse effects when two or more drugs are used concomitantly. It is clear that either the therapeutic or toxic effect

of a drug can be greatly modified by interactions with other drugs, foods, environment substances.

Many other less dramatic, but clinically significant drug interactions have been reported. It is difficult to estimate how often drug interactions have contributed to increased toxicity or decreased therapeutic efficacy. A possible correlation has been noted between the significant increase in adverse effects and the use of multiple drug therapy. Although information on the number of patients involved in drug interactions is limited, there are many reports on potential drug interactions. Long lists of such interactions have been compiled. Many of these reports are based on insufficient data, a limited number of patients, or animal data alone.

2.2.1 Drug Interactions *In-vitro*

In-vitro interactions may be defined as those interactions which occur outside the body. Therefore this category includes interactions between drugs due to incompatibility (e.g. drug-drug interactions in an intravenous infusion), due to interaction of a drug either its containers or packaging (e.g. drug binding on an infusion bag), due to loss of drugs during laboratory analyses (e.g. drug binding to laboratory equipment) or due to changes in the bioavailability of drugs when the formulation is altered (Griffin and D'arcy, 1997).

Currently much information is appearing in the literature usually from pharmaceutical research sources on drug interactions *in-vitro.* Much of this information in the past has been interactions between drug and drug; drug and fluid in intravenous infusions. More recent work have concentrated on interactions between specific drugs, notably chloroquine, cyclosporin, insulin and pharmaceutical packaging materials (glass and plastics), and the mechanisms by which these *in-vitro* interactions occur. Drug interaction research has also been focused on drug-excipient interactions. In the past, it has not always been appreciated that the so-called "inactive" excipient used in product formulations are physiologically active substances which are often quite capable of interacting or producing complexes with drug substances in the formulation. They can cause changed absorption and altered bioavailability. The often quoted outbreak of phenytoin intoxication in Australia during 1968-9 was caused by increased bioavailability due to a simple change in capsule filler from calcium sulphate to lactose (Tyrer *et al.,* 1970).

2.2.2 Drug interaction *in-vivo*

The *in-vivo* interaction usually results in enhancement or reduction of drug efficacy or increase in drug toxicity, whereas *in-vitro* interactions invariably result in reduced bioavailability (drug efficacy) during dosage.

Most drug interactions in humans are detected by qualitative observations during clinical use of several drugs concurrently which result in either increased toxicity or decreased therapeutic effects (Wijnands *et al.,* 1986; Raoof *et al.,* 1987; Staib *et al.,* 1987).

Co-administration of several drugs to patients, prevalent in clinical practice, is fraught with problems. Each agent may have been chosen rationally, but when co-administered, the outcome can be unpredictable. Cimetidine, for example, devoid of anticoagulant activity, potentiates the hypoprothrombinemic effect of the oral anticoagulant, warfarin. Possible causes of this kind of effect are many. Often, such drug interactions involve a change in pharmacokinetics. Thus, a drug interaction occurs when either the pharmacokinetic or pharmacodynamic of one drug is altered by another. Some drugs stimulate drug metabolizing enzymes and hasten drug loss; others inhibit these enzymes. Many others displace a drug from plasma and tissue binding sites or interfere with its absorption (Tyrer *et al.,* 1970).

2.2.3 PHARMACOKINETIC DRUG INTERACTIONS

2.2.3.1 Interactions affecting drug absorption

Interactions during the absorptive phase result in either or both of the following potentially clinically significant effects:

(1) Increase or decrease in the relative rate of absorption (ka)

(2) Increase or decrease in the amount of drug absorbed (F).

A decrease in the fraction of drug absorbed is equivalent to a decrease in dose given, with the obvious clinical implications. Although a drug may eventually be completely absorbed, it may be absorbed slowly that: (a) it may never reach effective serum concentrations, (b) the rate of onset may be greatly delayed when prompt relief of acute symptoms, such as pain, is needed, or (c) the slow rate of absorption may act to sustain release and unduly prolong an effect. However, it is important to note that the rate of absorption is usually not important for compounds that are given in multiple-dose regimens to achieve a constant serum level such as antibiotics, sedatives, or tranquilizers. The reason for this is that the average steady-state serum level in a multiple-dose regimen is affected by the fraction of drug absorbed (F) but not usually by the relative rate of absorption.

The effect of ionization on drug transport across cell membranes is also an important factor to be considered in interaction affecting drug absorption. Most drugs are either weak acids or bases, the unionized species has sufficient lipid solubility to pass rapidly through the membrane, while the polar ionized species are impermeable. Thus, the ionization tendency of the drug indicated by pKa and the pH of the aqueous environment affect the rate of transfer by changing the fraction unionized. Transfer of acids across biological membranes will be favoured by low pH and transfer of bases will be favoured by high pH. The pH of the gastrointestinal (GI) lumen fluids can be varied greatly, which may affect the transport of drugs in some cases.

The effect of pH changes on ionization and dissolution of drug during absorption have been unnecessarily exaggerated. The frequent generalization that absorption of acidic drugs will be decreased by antacids which raise intragastric pH is not always warranted and is an example of the danger in extrapolating from animal studies, carried out under special experimental conditions, to clinical situations where additional factors may be involved (William, 1976).

Most drugs are administered orally as solid dosage forms (capsules, suspensions or tablets) and the slowest (rate limiting) step is usually the rate at which the drug goes into solution (dissolution rate). Although ionization decreases passage of drug across membranes, it increases the rate of dissolution. Even though a large

amount of the drug in solution is ionized, the rate of reversible proton transfer is so rapid that as soon as the unionized fraction in the solution is absorbed, it is almost immediately replenished. As such the rate of dissolution in gastric fluids and intestinal fluids is dependent on the properties of the drug and dosage form; the conditions at the site of dissolution including pH, interfering substances and motility and residence time at sites of dissolution which is dependent of gastric emptying and intestinal transit time(Tyrer *et al.*, 1970).

Physicochemical Interactions during Absorption

Several substances can bind or solubilize drugs which may alter absorption. Kaolin reduces the absorption of lincomycin. Cholestyramine, a quaternary ion-exchange resin used to bind intestinal bile salts and reduce serum cholesterol levels, also binds with many drugs. Antacids containing polyvalent cations (aluminium, calcium and magnesium ions) chelate with Tetracycline and some fluoroquinolones.

2.2.3.2 Interactions Affecting Drug distribution

Drugs are reversibly bound to tissue, plasma proteins, and the receptor. The ratio of free drug to bound drug at each of these sites depends on the properties of the drug, the patient, and the presence of other substances. Displacement of

anticoagulants from protein binding sites by Phenylbutazone analogs has led to serious bleeding episodes (William, 1976).

2.2.3.3 Interactions Affecting Drug Elimination.

Any drug that changes the rate of elimination of another drug may affect these two important pharmacokinetic parameters such as elimination rate constant and the biological half-life. Half-life determines the overall rate of elimination and the amount of drug remaining in the body at a given time. It also determines the average serum concentration after steady-state levels is reached during multiple dosing. Its implication is that a drug interaction that doubles the half-life will double the average serum level while drug interactions that decrease the half-life by 50% will reduce the steady state serum levels to one-half of their original value.

2.2.3.4 Interactions Affecting Drug Metabolism

Changes in drug metabolism may be by enzyme induction or inhibition. Although there have been fewer documented reports of enzyme induction in humans, there is no doubt that enzyme induction occurs and that it is quite important clinically. Butabarbital, Phenobarbital, Secobarbital, and other barbiturates induce the metabolism of dicumarol and warfarin, reducing the anticoagulant effect. An

increase of the dose is then required to obtain the same clinical effect. A danger occurs when the inducer is withdrawn; metabolism then returns to normal and serum levels may reach toxic levels.

Disulfiram is used therapeutically for its ability to inhibit aldehyde dehydrogenase enzyme, leading to accumulation of acetaldehyde and unpleasant symptoms when alcohol is ingested. It also inhibits the metabolism of Phenytoin leading to toxic symptoms and has also been reported to enhance the anticoagulant effects of warfarin (Rothstein, 1968).

2.2.3.5 Interactions Affecting Renal Excretion

Interactions that affect renal excretion of drugs will be clinically significant only when the drug or its active metabolite is appreciably eliminated by the renal route. The effect of probenecid on increasing serum levels of penicillin is well known. Probenecid is said to decrease the renal clearance of indomethacin (William, 1976).

2.2.4 PHARMACODYNAMIC DRUG INTERACTIONS

Interactions resulting from the use of two drugs that have opposing or similar pharmacological effects often occur in clinical practice. An excessive central

nervous system depressant effect, resulting from the concurrent use of two or more drugs exhibiting a depressant action, represents one of the most frequently encountered drug-related problems. Other pharmacodynamic interactions are interactions at receptor sites, alteration of electrolyte levels, alteration of gastrointestinal flora etc.

2.3 PHARMACOKINETICS

Pharmacokinetics is a biological science concerned with the characterization and mathematical description of the absorption, distribution, metabolism and excretion of medicinal agents, their metabolites, and other substances of biologic interest in a whole animal.

It includes consideration of the relationship of these processes to the time course of pharmacologic effects (Evans *et al.,* 1995). The biological, physiological and physiochemical factors which influence the transfer processes of the drug in the body also influence the rate and extent of administration, distribution, metabolism and excretion of those drugs in the body.

In many cases pharmacological action, as well as toxicological action is related to plasma concentration of drugs. Consequently, through the study of pharmacokinetics, one will be able to individualize therapy for the patients.

These considerations have led to the extensive use of therapeutic drug concentration monitoring and to the development and rapid growth of a new discipline, called clinical pharmacokinetics.

Clinical pharmacokinetics is a health sciences discipline that deals with the application of pharmacokinetics to optimize the pharmacokinetics management of individual patients.

The application of clinical pharmacokinetics principles to the individualization and optimization of drug dosing regimens is a rational process. It begins with a clear formulation of the therapeutic problem (in the form of questions such as: Why does this patient not respond to a population – average drug dosing regimen? What is the most appropriate drug dosing regimen for this elderly patient with renal failure? Leading to a provisional decision (the dosage adjustment to be made or the initial dosage regimen to be used) followed by the assessment of the outcome of

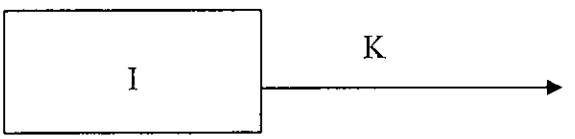
that decision (drug concentration in plasma and therapeutic response) (Evans *et al.,* 1995).

2.3.1 PHARMACOKINETICS MODELS

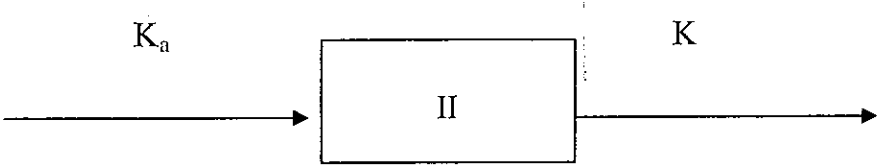
Drugs are in a dynamic state within the body. In biological system drug events often happens simultaneously. In order to describe a complex biologic system, simplifying assumptions are made concerning the movement of drugs. A hypothesis or model is conceived using mathematical terms, which are a concise means of expressing quantitative relationships. Various mathematical models can be derived to simulate the rate processes of drugs absorption, distribution and elimination. These mathematical models make possible the development of equations to describe drug concentrations in the body as a function of time. The different types of models are:

2.3.1.1 Compartmental Models: The body can be depicted by a series or systems, of compartments that communicate reversibly with

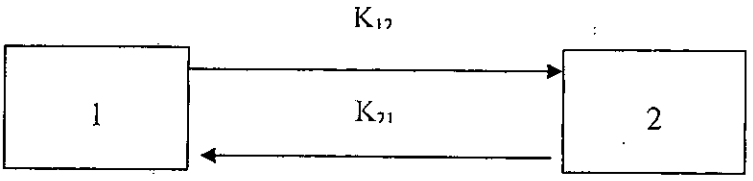
each other. A compartment is not a real physiologic or anatomic region but is considered as a tissue or group of tissues, which have similar blood flow and drug affinity. Within each compartment the drug is considered to be uniformly distributed. These models are based on linear assumptions using linear differential equations. The dynamic entry and exit of drugs are represented by the rate constants of the movement. This model is an open system since drug elimination is possible.



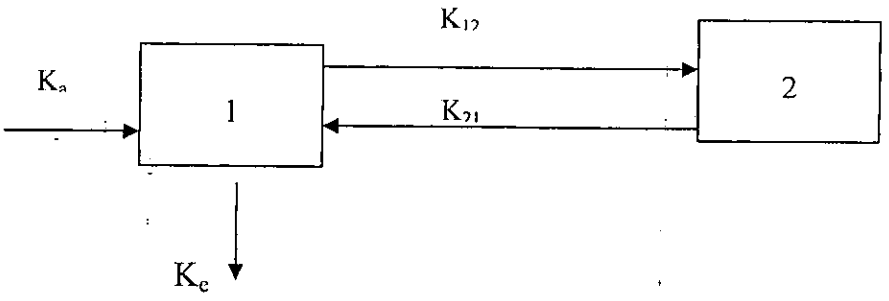
Model I: One compartment open model I.V injections



Model II: One compartment open model with first order absorption



Model III: Two – Compartment open model I.V injection

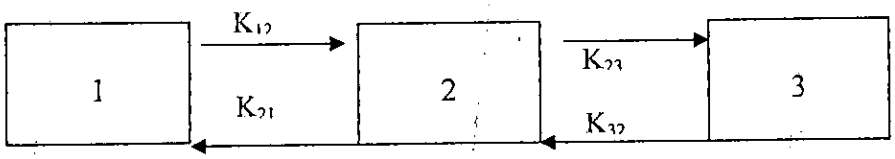


Model IV: Two – Compartment open model with 1st Order Absorption

2.3.1.2 Mammillary Model: This is the most common compartment model used in pharmacokinetics. The model consists of one or more peripheral compartments connected to a central compartment. The central compartment is assigned to represent plasma and highly perfused tissues which rapidly equilibrate with drug. The mammillary model is considered as a strongly connected system since one can estimate the amount of drug in any

compartment of the system after drug is introduced into a given compartment. Elimination of drug occurs from the central compartment since the organs involved in drug elimination primarily kidney and liver are well perfused tissues.

2.3.1.3 Cartenary Model: This model consists of compartments joined to one another like the compartments of a train. So the cartenary model does not apply to the way most functional organs in the body are directly connected to the plasma, it is not used as often as mammillary model.



2.3.1.4 Flow or physiologic model: Figure 2.8 is also referred to as blood flow or perfusion models. This is a based on known anatomic and physiologic data. This requires no curve fitting, drug concentrations in the various tissues are predicted by organ tissue

size, blood flow. Physiologically based pharmacokinetics models can be applied to several species and with some drugs human data may be extrapolated. This is not possible with the compartment models (Leon *et al.,* 1945).

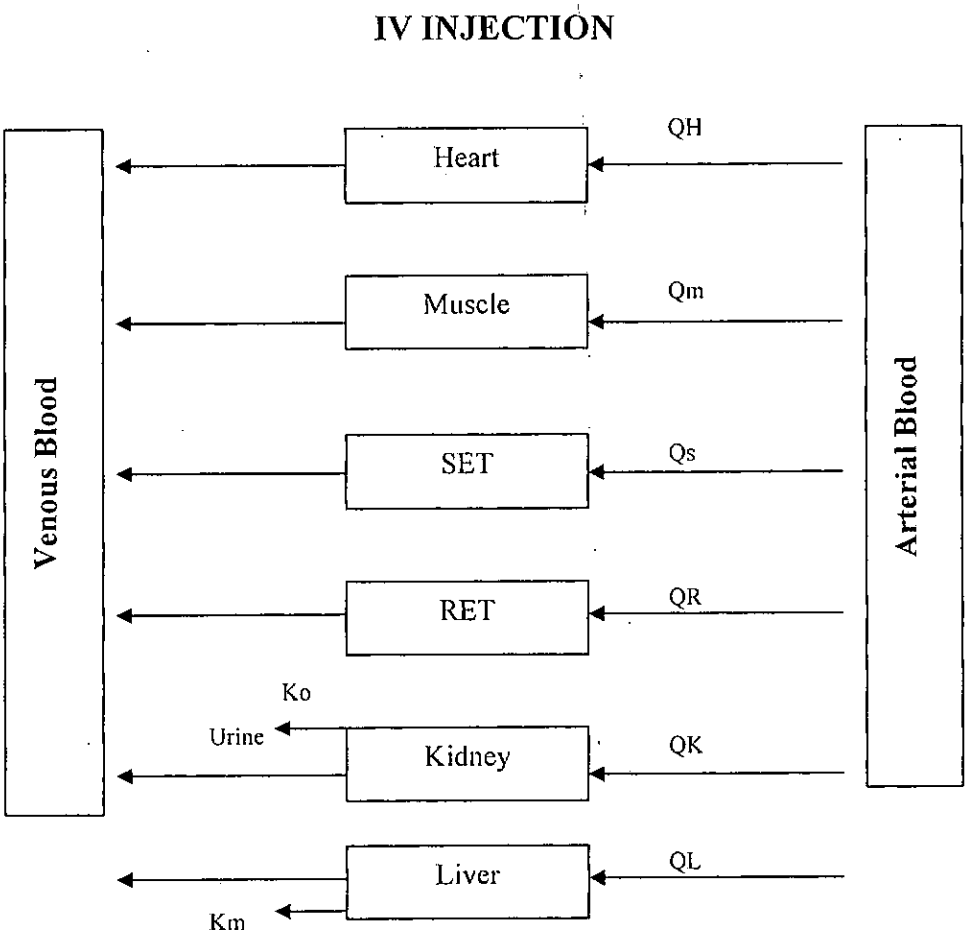


Fig. 2.8: Pharmacokinetics model of drug perfusion.

The K's represent kinetic constants; K_o is the 1st order rate constant for urinary drug excretion and k_m is the rate constant for hepatic elimination. Each box represents a tissue compartment.

RET as rapidly equalizing tissue; **SET** –as slowly equilibrating tissue

2.3.2 GUIDELINES FOR COLLECTION AND ANALYSIS OF PHARMACOKINETICS DATA

These guidelines provide an overview of major components of experimentally applied pharmacokinetics.

In the total design and implementation of pharmacokinetics studies, an ideal and complete assay of experimental data should include a number of considerations:

(1) The dosage form should be pre-analyzed: Most commercial dosage forms are inexact, and content uniformity should be assayed. Injectable, vials and ampoules contain some overages require analysis. Solid dosage forms are required to yield an average of the stated quantity of drug with limited variability.

(2) Accuracy in administration of the dose should be confirmed. All doses should be timed exactly for starting time and duration of administration. Mackichan *et al.*, (1979), found immediate loss of about 50% of a dose of intravenous Diazepam by absorption during passage through the plastic tubing of an infusion set.

(3) Attention to methods and sites of blood collection is needed. Blood samples should be collected by direct venipuncture in clean glass tubes without anticoagulant (serum) or with anticoagulant (plasma). One of the major assumptions employed is that venous blood collected from one site adequately reflects circulating arterial blood concentrations (Chiou *et al.*, 1981).

(4) Serum (or blood) concentration data following intravenous injection (bolus or infusion) provides partial characterization of drug disposition properties.

(5) Serum (in blood) concentration data following oral doses of the drug in solution and common dosage forms provides additional pharmacokinetics parameters related to absorption and intrinsic clearance.

(6) Urinary excretion rates of drug (as a function of time, dose and route of administration) should be measured to accompany the above studies. Urinary excretion is often a major route of drug elimination, and analyses permit quantitation of renal clearance. Collection of other excreta or body fluids (feces, bile, milk, saliva) may permit determination of other relevant elimination or distributional pathways.

(7) Many drug metabolites are either pharmacologically active or otherwise of pharmacokinetics interest. Their measurement will allow evaluation of AUC and mean residence time (MRT) and perhaps permit quantitation of metabolite formation and disposition clearance.

(8) Multiple dose and steady state experiments are necessary if therapeutic use of drug relies on steady-state concentrations.

(9) Tissue analyses add reality and specificity to drug distribution characteristics.

In the absence of *in-vivo* data, it is generally impossible to make valid conclusions about bio-availability from the dissolution data. The use of various tests methods makes it even more difficult to interprete dissolution results since there is no simple correlation among dissolution results obtained with various methods.

2.3.3 DEFINITION OF PHARMACOKINETIC PARAMETERS

2.3.3.1 ABSORPTION

Most drugs administered to patients are administered extra-vascularly. Although intramuscular and subcutaneous routes are used, the oral route accounts for most of the extravascular drug administered. The absorption process depends on the drugs disintegrating from its dosage form, dissolving in gastro-intestinal fluids and then diffusing across biological membrane barriers into the blood stream. The rate and extent of drug absorption may vary considerably depending on the nature of drug itself (e.g. Solubility; pka etc) and on the physiological environment (e.g. pH, gastro-intestinal motility, vascularity).

The fraction of a drug that is absorbed into the systemic circulation is referred to as its bioavailability. The bioavailability (f) of a given drug is usually calculated by comparing, in the same subjects, the area under the plasma concentration-time curve (AUC) of an equivalent dose of the intravenous form and oral form for drugs to be orally useful. An exemption is when the site of action of the drug is at the lumen then low bioavailability would then be considered advantageous. Some drugs are greatly absorbed from the lumen but have extensively being metabolized in

the liver (1st pass effect) such are referred to have a high hepatic extraction rate. Formulations that provide sustained release permit drugs taken orally to be taken at less frequent intervals.

$$F = \frac{\text{AUC oral}}{\text{AUC i.v}} \quad \text{..... equation 1}$$

Condition that may influence the rate and extent of drug absorption include abnormal GI motility, diseases of the stomach as well as the small and large intestine, GI infections, radiation, food and interaction with other substances in the gastro-intestinal tract. Drugs such as antacids, kaolin, sucralfate, when co-administered with others could hinder absorption (James, 1996).

2.3.3.2 DISTRIBUTION

After a drug enters the vascular compartment, it interacts with various blood constituents and is carried by various transport processes to different body organs and tissues. The overall process is referred to as distribution. The factors determined the distribution pattern of a drug are binding of the drug to circulating blood components, binding to fixed receptors, passage of the drug through membrane barriers, and the ability to dissolve in structural or storage lipids molecular weight. It is generally believed that only the free fraction of the drug is available for distribution and

elimination, to cross cellular membranes or to interact with the drug receptor to elicit a biologic response. Therefore changes in the protein binding characteristics of a drug can have a profound influence on the distribution and elimination of a drug as well as on the manner in which steady state concentrations are interpreted

2.3.3.3 METABOLISM

The rate of the enzymatic process to metabolize a drug is usually characterized by the Michaelis – Menten equation

$$-\frac{dc}{dt} \quad \frac{V_{max} \; X \; C}{K_m + C} \qquad \text{..... Equation 2}$$

where V_{max} is the maximum velocity of the reaction; Km the Michaelis Menten constant, is the drug concentration at which the rate of metabolism is one half of the maximum and C is the concentration of drug in blood. The liver is the principal organ responsible for xenobiotic metabolism. One of its major roles is to convert lipophilic non-polar molecules to more polar water – soluble forms.

The drug molecule can be modified by phase I reactions which alter chemical structure by oxidation, reduction, or hydrolysis or by phase II

reactions, which conjugate the drug (glucuronidation or sulfation) to water – soluble forms.

Most drug metabolism takes place in the microsomal fraction of the hepatocytes, where many environmental chemicals and endogenous biochemicals (xenobiotics) are also processed and by the same mechanism. Enzymes of the hepatic microsomal system can be induced or inhibited. Enzymes induction and inhibition have greatest significance for drugs with low to moderate hepatic extraction fractions microsomal enzyme induction leads to an increase in the activity of enzyme present, most commonly through increases in the quantity of the oxidizing enzymes. There are many isoenzymes of cytochrome P450 which affected variable by different enzyme-inducing drugs.

2.3.3.4 EXCRETION

Excretion of drug or chemicals from the body can occur through biliary, intestinal, pulmonary or renal routes. Each of these represents a possible mechanism of drug elimination; renal excretion is a major pathway for the elimination of most water-soluble drugs or metabolites. Kidney function, in contrast to liver function, is readily and reliably evaluated by estimation of creatinine clearance. Creatinine is a metabolic product of muscle

metabolism and is produced at a constant rate by the body. It is primarily eliminated from the body by the kidneys through the glomerular filtration mechanism. Renal clearance of creatinine at 120ml/min approximates the glomerular filteration rate of 90 to 130ml /min. Therefore measurement of creatinine clearance on a routine basis provides as effective tool to evaluate kidney function.

2.4 DRUG DELIVERY SYSTEMS

Pharmaceuticals have primarily consisted of simple, fast-acting chemical compounds that are dispensed orally (as solid pills and liquids) or as injectables. In the past decades, formulations that control the rate and period of drug delivery (i.e., time-release medications) and target specific areas of the body for treatment have become increasingly common and complex. Understanding of complexity of human body systems have made it possible for the discovered bioactives and gene therapies to play a vital role in drug delivery.

Some of the problems encountered are many drugs potencies and therapeutic effects are limited or otherwise reduced because of the partial

degradation that occurs before they reach a desired target in the body. Further more injectables could be made less expensive and administered more easily if they could be administered orally. However, this improvement in drug formulation cannot happen until methods are developed to safely encapsulate drugs through specific areas of the body such as stomach, where low pH can degrade a medication or through an area where healthy bone and tissue might be adversely affected.

Pharmaceutical preparations for oral administration are required to have sufficiently water solubility to enable dissolution within the GIT prior to absorption. Limited dissolution within the GIT often reduces the bioavailability of hydrophobic drugs. To improve GIT dissolution, certain non-aqueous solvents such as polyelectrolyte nanosystems (Polyethylenimines) are often used in the form of emulsions and micro-emulsion.

The ability of these polyelectrolytes to disperse a hydrophobic drug within aqueous media and promote the oral absorption of same also exemplified the principle of drug delivery. Several soluble polymers bearing pendant amphiphilic or hydrophobic groups, commonly known as Polysoaps (Quartenary ammonium palmitoyl glycol Chitosan have been studied and found applicable based on exploiting their solubilisation capacity for

hydrophobic molecules (Uchegbu et al., 2001). The goal of drug delivery systems therefore is to deploy medications intact to specifically targeted part of the body through a medium that can control the drug administration by means of chemical trigger. Some of these agents that have shown tremendous effectiveness in enhancing drug targets specificity, lowering systemic toxicity, improving treatment absorption, rates and preventing systemic biodegradation include: – polymers, dendrimers, etc.

Recently, three routes leading to production of particles that will meet some of these requirements have been widely investigated. The first route takes advantages of the ability of amphiphilic molecules (i.e., molecules consisting of a hydrophilic and hydrophobic moiety) to self-assemble in water above a system specific critical micelle concentration (CMC) to form micelles. However size and shape of these micelles depend on the geometry of the constituent monomers, intermolecular interactions, and conditions of the bulk solution (i.e., concentration, ionic strength, pH, and temperature). Micelles have the ability to encapsulate and carry lipophilic actives within their hydrocarbon cores. Depending on the specific system, some micelles either spontaneously rearrange to form liposomes after a minor change of solution conditions, or when exposed to external energy input

such as agitation, sonication, or extrusion through filter membranes (Svenson, 2004).

The second route relies on engineering the well-defined particles through processing protocols e.g. homogenization of oil-in-water (o/w) emulsions or w/o/w double emulsions to produce stable and monodisperse droplets (Svenson, 2004).

Currently a new third route to create well defined, monodisperse, stable molecular level nanostructure is being studied based on the "dendritic state" architecture (Tomalia, 2004) generally referred to as dendrimers **(Figure 2.9).**

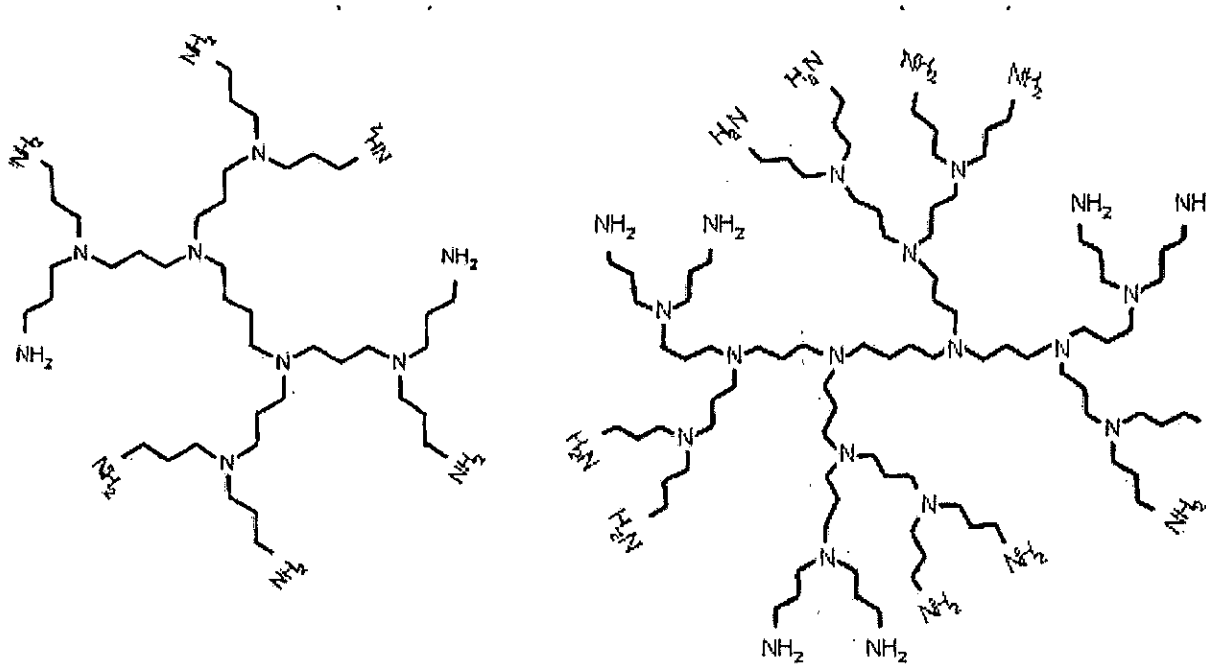


Figure 2.9: (A) Polypropylenimine (PPI) dendrimer generation 2 with 8 terminal amino groups {G2-PPI dendrimers [DAB8]}; (B) polypropylenimine dendrimer generation 3 with 16 terminal amino groups {G3-PPI dendrimers [DAB16]}

2.5 GENE THERAPY

Gene therapy is the insertion of genes or nucleic acids into an individual's cells and tissues to treat diseases and hereditary diseases in which a defective mutant allele is replaced with functional one to restore normal function and produce therapeutic effect.

Although the field of gene therapy has experienced significant set backs and limited success. It is still one of the most promising and active research fields in medicine (Selkirk, 2004). Our knowledge of human genome and the technological development of molecular biology have made the concept and the development of gene therapy clearer than ever.

There are a number of obstacles limiting successful gene therapy but the most difficult to surpass has been the inability to transfer the appropriate gene into a target cells/tissues/organs without affecting healthy ones such that an amount of the gene product is delivered to correct the disease (*Mark et al.*, 1997).

In view of this, two general approaches for delivering genetic materials exist: *in-vivo* and *ex-vivo*. The in-vivo strategy involves the direct injection or infusion of deoxyribonucleic acid [DNA] (usually via a viral or non-viral vector) to resident cells of the target tissue. This has to satisfy the basic requirements of accessibility of target cell to injection or infusion and secondly that the transfer vector readily and specifically infects, integrates and expresses the transgene in target cells

(Mark *et al.*, 1997). The ex-vivo approach involves removal of cells, infecting the surrogate cells with a replication deficient virus (or genetically modifying the cells) followed by transplantation of the cells or organ system.

As such the success of gene therapy largely depends on the development of suitable vectors or vehicles that can deliver gene(s) to specific tissues with minimal toxicity (David *et al.*, 2002). Currently, there are two major classes of vehicles for gene transfer: Viral and Non-viral vectors.

2.5.1 Viral Vectors for Gene Therapy

Majority of on-going clinical trials in gene therapy or DNA vaccines use retroviruses, adenoviruses, adeno-associated viruses, herpes simplex viruses, pox viruses, poli-viruses, baculoviruses and Sindbis viruses to deliver transgene expression. The use of these methods has various inherent disadvantages such as safety concerns (Immunogenicity) and scale-up difficulties (Zinselmeyer *et al.*, 2002).

Despite several hurdles associated with viral vectors, they still provide potential gene delivery vehicles for the treatment of many human diseases including cancer and various inherited disorders(Zinselmeyer *et al.*, 2002).

2.5.2 Non-viral or Synthetic Carrier Systems

A number of studies have examined the possibility of delivering genes for the treatment of genetic diseases (Brown *et al.*, 2001) and quite a number of non-viral carriers for gene transfer have been synthesized including cationic liposomes, polylysines, polypeptides, polymeric vesicles, recombinant histones, lipopolyplexes and other reagents. Such systems tend to be comparatively less efficient than viral system but have inherent advantage of flexibility and safety (Schatzlein, 2001).

However, the transfection/expression efficiency of these non-viral systems remains insufficient especially in the presence of several physiological barriers (intracellular and extracellular) to overcome for effective systemic delivery of DNA. Hence the ideal vector must be stable in the systemic circulation, escape the reticuloendothelial system, and be able to extravasate tissues, enter the target cell, escape lysosomal and endosomal degradations and transport DNA to the nucleus to be transcribed (Schatzlein *et al.*, 2001).

With increasing understanding of the physicochemical properties essential to overcome the various barriers, it is possible to apply rational design for the cationic carriers (Fegner *et al.*, 1994).

It is of note that the biodistribution of current carrier systems is being influenced to a large extent by intrinsic physicochemical properties such as charge and size. The

positive charge of the complex has resulted in greater accumulation at lung or tumour site. Interaction of these charged nanoparticles with the body, result in modified biodistribution. Strategies such as coating of the DNA carrier complex with hydrophilic polymers have been used to mask these intrinsic targeting effects and avoid non-specific interaction (Schatzlein, 2003).

A number of poly-amino acids (Kleoeckner *et al.*, 2006; Brownlie *et al.*, 2004), cationic block co-polymers (Antonietti *et al.*, 2003), dendrimers (Singh *et al* 2005; Yamamoto *et al.*, 2006), cyclodextrin, polyglycerol-chitosan (Chan *et al.*, 2007). Several modifications *viz-a-viz* increasing the number of amino groups (Wheeler *et al.*, 1996) thereby increasing the size of the complex; incorporation of DOPE, cholesterol (Hong *et al.*, 1997) to facilitate endosomal escape; diversion of lipoplex from the first capillary bed encountered i.e. the lung endothelium using polyethylene glycol lipid; coupling of lipoplex-PEG complex, poly-L-Lysines based polymers with targeting ligands such as asialoorosomucoid (Wu *et al.*, 1987), transferring (Cotton *et al.*, 1990), folate (Mislick *et al.*, 1995) monoclonal antibodies (Chen *et al.*, 1994) and some lysosomotropic or endosomolytic agents (e.g. chloroquine, quinacrine) (Cheng *et al.*, 2006). All these have led to protection of DNA in the complex and improvement in transgene expression of the transfecting non-viral target vectors.

2.6 DENDRIMERS

Since their introduction in 1985, dendrimers have attracted much attention because of their fascinating structure and unique properties (Newkome *et al.*, 1986; Frechet *et al.*, 2001). Dendrimers (from the Greek "dendron": tree, and "meros": part) are globular, size monodisperse macromolecules in which all bonds emerge radially from a central focal point or core with a regular branching pattern and with repeat units that each contributes a branch point. They act as the root from which a number of highly branched, tree-like arms originate in an ordered and symmetric fashion. These hyperbranched molecules were also called "arborols" (from the Latin "arbor" also meaning tree)

A higher generation dendrimer comprises three distinct topological regions, namely surface, dendritic branching, and central core (Newkome *et al.*, 1996; Vogtle 1998 and 2000). This makes these polymers attractive candidates as carriers in both drug and gene delivery applications. Drug delivery can be achieved by coupling a drug to dendrimer through one of two approaches. Firstly, hydrophobic drugs can be complexed within the hydrophobic dendrimer interior to make them water soluble. Secondly, drugs or genes can be covalently coupled onto the surface of the dendrimer (Patri *et al.*, 2005).

Not all regularly branched molecules are dendrimers because properties of dendritic state, such as core encapsulation (Hawker *et al.*, 1993; Hecht *et al.*, 2001) and unusually low intrinsic viscosity in solution (Mourey *et al.*, 1992) are reached only when globularity is achieved at a certain generation or size threshold.

2.6.1 Synthesis and Functions

Two distinct synthetic methodologies have been used for the preparation of dendrimers: the divergent approach in which growth starts at the core and proceeds radially outward toward the dendrimer periphery, and the convergent approach in which growth starts at what will become the periphery of the dendrimer receeding inward (**Fig. 2.10**) (Tomalia *et al.*,1985 and 1986).

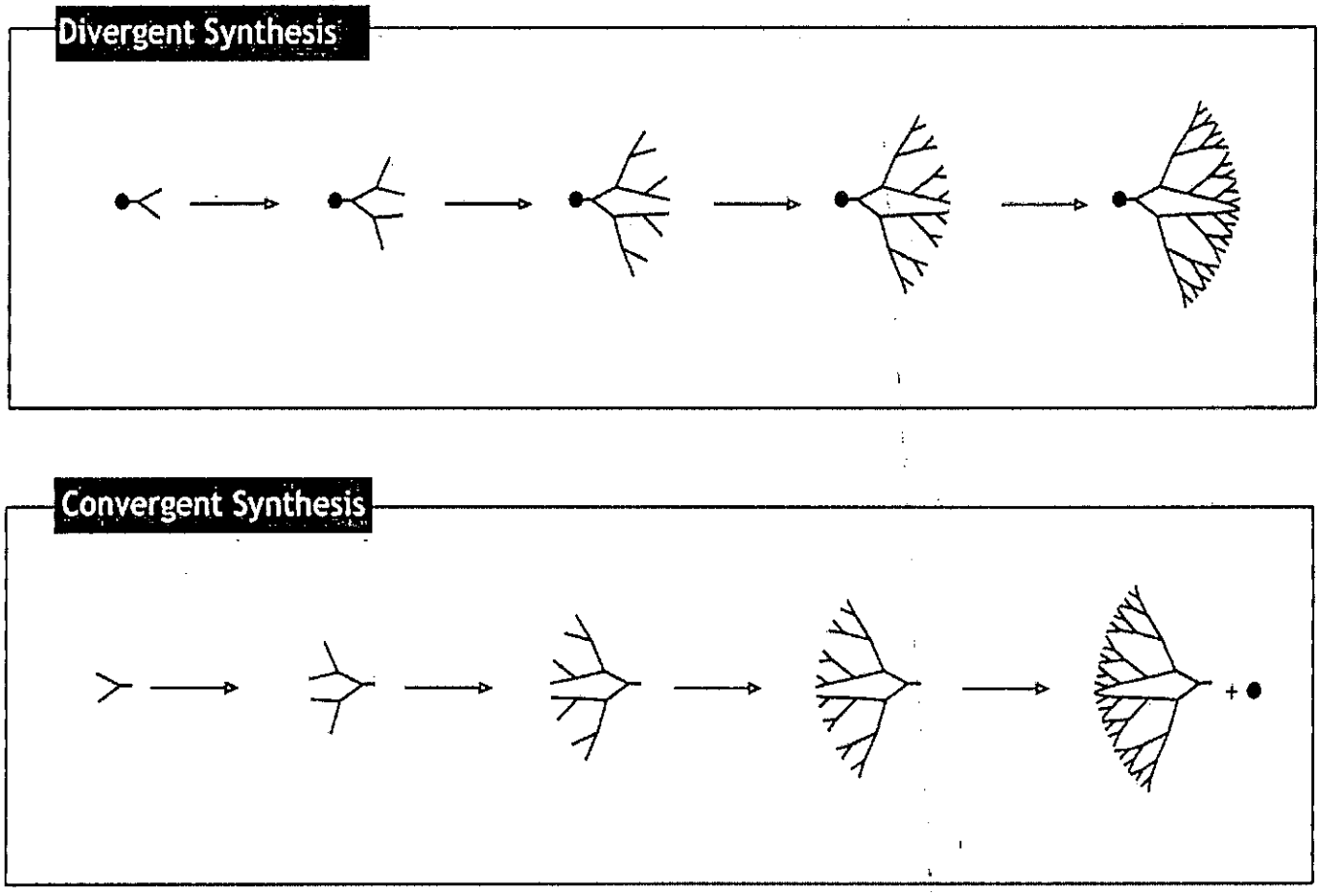


Figure 2.10: Schematic diagram of divergent and convergent synthesis of dendrimers.

In general, the convergent approach provides better overall structural control, in part as a result of its enhanced potential for purification at intermediate stages of growth, and in part, as a result of its innate ability to introduce differentiated functionalities at the focal point and the periphery of the dendrimer. In contrast, purity and structural uniformity are harder to maintain in the divergent approach, because the number of reactions to be completed at every stage increases exponentially thereby requiring large excess of reagents (Tomalia *et al.*, 1985).

Although the majority of the dendrimers prepared up to date have been built of covalent bonds, many noncovalent dendrimers have also been prepared by a variety of self-assembly processes involving, for example hydrogen bonding or coordination chemistry.

The first exploration of dendrimers as molecules for gene delivery focused on the Polyamidoamine dendrimer (PAMAM) (Tomalia *et al.,* 1985). The PAMAM dendrimers are normally based on an ethylenediamine or ammonia core with four and three branching points.

The other commercially available dendrimer with relevance for drug and gene delivery is based on polypropylenimine (PPI) units with butylenediamine [DAB] used as the core molecule. The repetitive reaction sequence involves Micheal addition reaction of acrylonitrile to a primary amino group followed by hydrogenation of nitrile groups to primary amino groups (Loup *et al.,* 1999). These dendrimers are frequently referred to as DAB-x, or DAB-Am-x, with **x** giving the number of surface amines (**Fig.2.11**).

Other dendrimers are phosphorous containing dendrimers, oligonucleotide dendrimers (dendrimer in which the oligonucleotide to be delivered becomes part of an anionic dendrimer) (Li *et al.,* 2004) and Poly-L-lysine dendrimers (PPL) which have been used as fluorescent dendrimers as a nanoprobe of cell transport (Al-Jamal *et al.,* 2006).

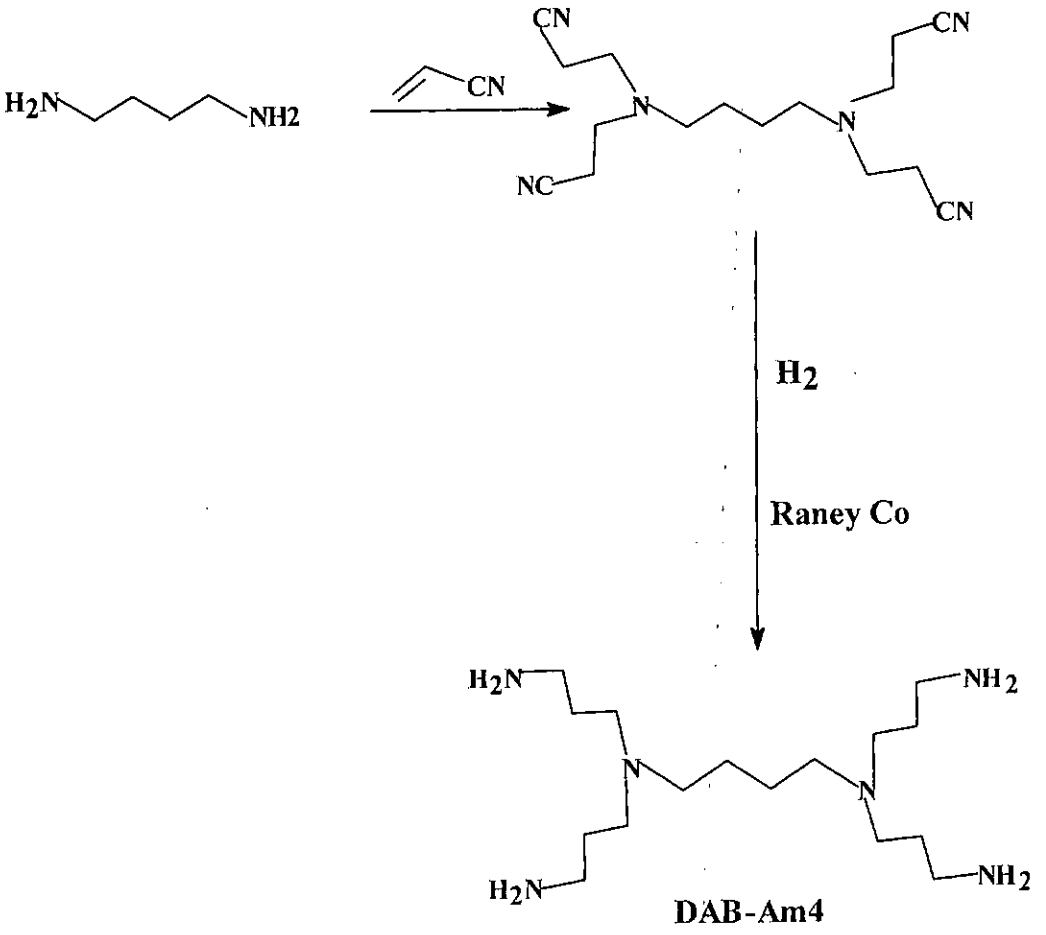


Figure 2.11: Synthesis of Polypropylenimine Dendrimer (DAB)

2.6.2 Dendrimer-nucleic acid interaction

The complexation process between dendrimers and nucleic acids is similar to that of cationic polymers which have high charge density. Dendrimers interact with various forms of nucleic acids, such as plasmid DNA or antisense oligonucloetides, to form complexes which protect the nucleic acid from biodegradation (Bielinska *et al.*, 1997; Abdelhady *et al.*, 2003). The interaction

between dendrimer and nucleic acids is based on electrostatic interactions (Tang *et al.*, 1997). During the complexation reaction the topological structure of plasmid DNA is altered and a more compact configuration is achieved, with cationic dendrimer amines and the anionic nucleic acid (NA) phosphate reaching local charge neutralization and the formation of NA-dendrimer complexes ("dendriplexes") (Tang and Szoka, 1997).

The nature of the complex is not only dependent on the stochiometry and concentration of the DNA phosphates and dendrimer amines but also on the bulk solvent properties (e.g. pH, salt concentration, buffer strength) and even the dynamics of mixing. The medium in which complexes are formed not only affects their morphology but also modifies other properties and even stability *in-vivo* (Tang and Szoka, 1997). High ionic strength, i.e. increased amounts of NaCl, interferes with the binding process (Kabanov *et al.*, 2000).

2.6.3 Dendrimers in drug delivery

Dendrimers have been utilized to carry a variety of small molecule pharmaceuticals including DNA. Encapsulation of the well known anticancer drug cisplatin, methothrexate and 5-fluorouracil within PAMAM dendrimers (**Fig.2.12**) gave conjugates that exhibited slower release, higher accumulation in solid tumours and lower toxicity compared to free cisplatin (Malik *et al.*, 1999).

The guest drugs or molecules were retained within the dendritic branching clefts by hydrogen bonding with interior protonated amide groups. Also, the effect of PAMAM dendrimer generation size and surface functional group on the aqueous solubility, and therefore, bioavailability of nifedipine has been studied (Devarakonda *et al.*, 2004).

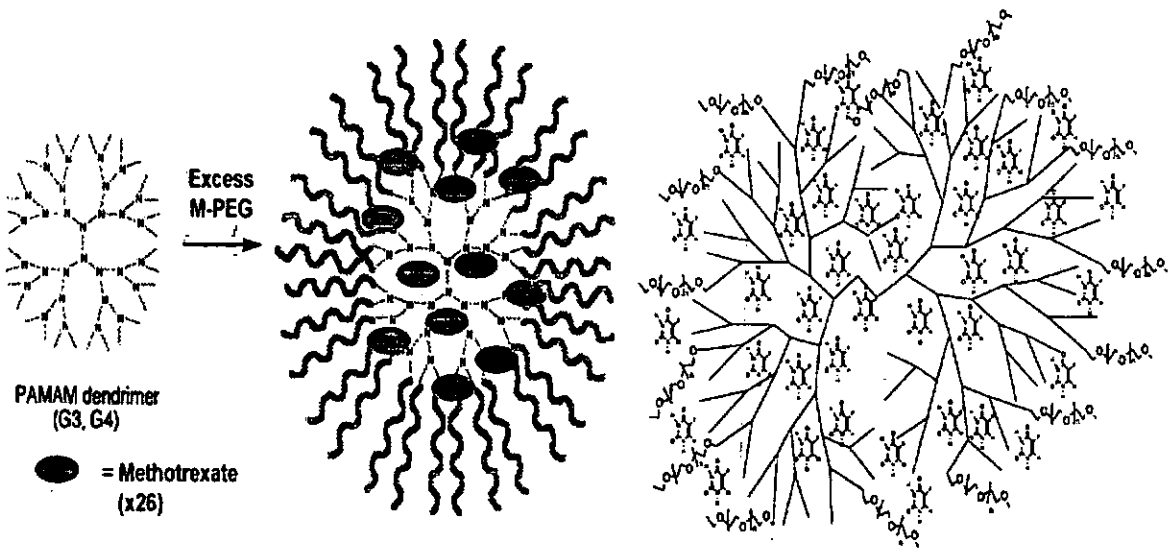


Figure 2.12: Schematic presentation of the encapsulation of anticancer drugs methotrexate (left) and 5-fluorouracil (right) into PEGylated generation 3 and 4 PAMAM dendrimers.

The solubility enhancement of nifedipine was higher in the presence of ester-terminated dendrimers than their amino-terminated analogues possessing the same number of surface groups. The nifedipine solubility increased with the size of the dendrimers (Devarakonda *et al.*, 2004).

CHAPTER THREE

EXPERIMENTAL

3.0 MATERIALS AND METHODS

3.1 MATERIALS

Product Formulations:

ACTs

A (Macsunate®)-containing Amodiaquine 200mg + Artesunate 50mg in fixed Dose combination.

B (Diasunate®)-containing Amodiaquine 400mg + Artesunate 100mg in fixed Dose combination.

C (Erimal®) -containing Amodiaquine 300mg + Artesunate 100mg in fixed Dose combination

D (Dart®) -containing Amodiaquine 600mg + Artesunate 200mg in separate Dose.

E (In-house)-containing Amodiaquine 400mg + Artesunate 100mg in separate Dose.

F (In-house)-containing Amodiaquine 200mg + Artesunate 100mg in separate Dose

Tablets:- Nivaquine® [chloroquine sulphate (Courtesy of May and Baker Nig. Plc]

Ciprotab® (ciprofloxacin) 250mg & 500mg (Courtesy of Fidson Nig Ltd)

3.2 METHODS

3.2.1 Evaluation of the Physicochemical Characteristics of Ciprofloxacin (CP) tablets.

Some of the physical parameters assessed were hardness, uniformity of weight and disintegration test.

3.2.1.1 Hardness

Ciprofloxacin tablets [Ciprotab®] (10) were subjected to hardness test using Schleuniger hardness tester.

3.2.1.2 Uniformity of Weight

The sample tablets (20) were weighed together and the average weight determined. Each of the tablets was again weighed and the percentage deviation from the average weight was calculated and compared with the BP (1998) specification.

3.2.1.3 Disintegration times of CP tablets in different media.

Ciprofloxacin tablet (one) was placed in each of the six tubes of the basket of the disintegration tester (Erweka®) and the instrument was operated using deionized water, 0.1N HCl acid, O.1M Acetic acid and Phosphate buffer pH 7.4 as disintegration media respectively. The time for complete disintegration of the tablets were taken.

3.2.1.4 Assay for CP content in CP tablets (Ciprotab®)

Ciprofloxacin tablets (20) were weighed and average weight determined. The tablets were crushed and triturated into fine powder. The equivalent of 50mg of active ingredient was calculated, weighed into 100ml volumetric flask, dissolved in about 50mls of mobile phase which consisted a mixture of acetonitrile and tetrabutylammonium bromide (20:80) adjusted to pH 2.0 with orthophosphoric acid, made up to volume with the same solvents and filtered with 0.45μm Millipore filter. The filtrate was serially diluted to give 100μg/ml.

Ciprofloxacin standard (50mg) was weighed and transferred into 100ml volumetric flask and made up to 100ml mark with mobile phase and further diluted to get equal concentration of both the sample and standard. 20μl of the sample and the standard were injected into HPLC system (**waters®**) separately in six replicates. The percentage purity was calculated comparing the peak area values of both the standard and the sample.

3.2.2 Development of Analytical methods for Drug Quantification.

3.2.2.1 UV spectrophotometric analysis.

Two analytical methods namely Ultra-violet spectrophotometric (UV) and High-performance liquid chromatographic methods (HPLC) were employed for the assay of samples.

Effect of chloroquine and halofantrine on the dissolution profile of ciprofloxacin were evaluated in deionized water and 0.1N Hydrochloric acid (HCl) in order to determine the level of interaction using in-vitro dissolution method.

The stock solutions (100µg/ml) of CQ, CP and SP were diluted to produce Ciprofloxacin, 10µg/ml (CP), Chloroquine, 10µg/ml (CQ) and Sparfloxacin, 10µg/ml (SP) standards which were scanned separately using UV spectrophotometer with scanner in order to determine the maximum wavelength of absorption of individual drug. CP/CQ, CP/SP, CQ/SP and CQ/CP/SP mixtures in equal concentrations (10µg/ml) were also scanned to determine if there was any spectra overlap, possible shift in maximum wavelength of absorption when in combination and then determined common wavelength for HPLC method.

Appropriate preliminary dissolution samples were generated for CP alone and in the presence of CQ using dissolution apparatus as described in the US Pharmacopoeia, 2004. A one in 100 dilution of dissolution samples was done and

finally subjected to UV spectrophotometric determination. Calibration plot was done using CP standard.

3.2.2.2 In-House development of high-liquid chromatographic method for concomitant elution of chloroquine, ciprofloxacin, halofantrine and sparfloxacin.

Preparation of standards in aqueous solution

Fresh Stock solutions of ciprofloxacin HCl and chloroquine sulphate standards, 500µg/ml were prepared by dissolving 50mg of each drug respectively in 100ml mobile phase. The CP and CQ concentrations in the range of 0.2 to 10µg/ml were then prepared from the stock solution.

A stock solution of Sparfloxacin (internal standard) 500µg/ml was prepared by dissolving 50mg of the drug in 100ml of mobile solvent and further diluted to give 25µg/ml. A mixture of CP and CQ standards were prepared in the above concentration range and in the presence of 25µg/ml of internal standard. The sample tablets were powdered and extracted in the mobile solvent containing 25µg/ml of internal standard, mixed, filtered and sonicated before injection into the HPLC system.

Instrumentation and Chromatographic conditions

The HPLC analysis was performed with a Waters® Liquid chromatograph equipped with M510 pumps, UV waters® 486 Turnable absorbance detector and a

Rheodyne injection valve supplied with a 20µl loop. The analytical column was a stainless steel 250 x 4mm I.D packed with Lichrospher 60RP, 5µm particle size, Merck 50984. It was coupled with guard column 10µm, 4 x 4mm I.D and the system was maintained at ambient temperature. The data collection was performed with waters 746 data module integrator.

The mobile phase was a mixture of acetonitrile and 0.005M Tetrabutylammonium bromide [TBAB] (ion pairing agent) (20:80) adjusted to pH 2.0 with orthophosphoric acid. The mobile solvent was filtered under vacuum using sintered glass funnel and degassed by sonication. The flow rate of 1.5ml/minute was employed with 20µl injection each time while the detection wavelength was 254nm. The attenuation, chart sped and AUFS were 8; 0.25 and 2 respectively.

Precision and Recovery studies

Five different concentrations of CP and CQ standards in the presence of internal standard were prepared in the mobile phase in replicates of 5-8. The concentrations used were 0.2, 1.0, 5.0, 10.0 and 100µg/ml. Peak area ratios were calculated and the coefficients of variation (CV) of the peak area ratios which were calculated from ratios of standard deviation (SD) to the mean were used for the assessment of precision. The absolute recovery was determined by comparing the peak area ratios of the extracts with those obtained by direct injection of the standards.

Limit of detection and quantification

The limit of detection (LOD) was defined as the concentration with a signal-to-baseline noise ratio of 3.0 while the limit of quantification (LOQ) was defined as the lowest concentration measured with signal to ratio of 10:1.

Application of the method

The method was applied for the assay of various formulations of chloroquine and ciprofloxacin tablets, capsules and suspensions. Quantification of CQ, HF and CP in the dissolution samples generated from the in-vitro dissolution studies was done using this method.

3.2.3 Interaction potentials of (a) Ciprofloxacin and Chloroquine; and (b) Ciprofloxacin and Halofantrine by means of in-vitro analysis.

3.2.3.1 Interaction between CP and CQ

The dissolution profiles of ciprofloxacin tablets (250 & 500 mg) were determined in 900 mls of deionized water and 0.1N HCl acid using US Pharmacopoeia (2004) apparatus II (7-Vessel Sontax® dissolution tester). The temperature and the degree of agitation were set at 37^0C ±0.5 and 50 revolutions per minute respectively. The study was conducted in two phases. One involved determination of ciprofloxacin tablets alone while the second phase was evaluated

in the presence of varying concentrations (0.69 – 3.47moles/L) chloroquine phosphate standard.

Sample (1.0ml) was collected at pre-determined time intervals (5, 10, 20, 30, 40, 50, 60, 80 and 120 minute). One-ml internal standard (250μg/ml) added and diluted to 10ml with mobile phase, mixed, filtered and sonicated prior to injection. One-ml of the dissolution medium was replaced for the 1-ml sample taken. The assay was done using the method above (**3.2.2.2**). The pH of CQ concentrations alone and after addition of ciprofloxacin (250mg) tablets were also measured using a pH meter.

3.2.3.2 Interaction between CP and halofantrine (HF).

US Pharmacopoeia apparatus II as described above with its fixed conditions (Temperature 37oC, 50 r.p.m) was utilized for evaluating the dissolution profile of ciprofloxacin tablets alone and in the presence of 0.56 – 1.11 moles/L halofantrine in 0.1N HCl.

Sample (1.0ml) was taken at time intervals (5, 10, 20, 30, 40, 50, 60, 80 and 120 minute) and quantification was carried out as described in **3.2.3.1**

3.2.3.3 Qualitative method of investigating complexation by pH titrimetry.

Sodium hydroxide solution (0.05M) was titrated against ciprofloxacin (25ml, 10μg/ml) standard solution and equal concentrations of ciprofloxacin and halofanrine HCl (25ml, 10μg/ml) standard mixtures. The pH of resulting solutions was measured using a pH meter until there was no change in pH.

3.2.4 Evaluation of the Physicochemical Characteristics of Artemisinin Combination therapies (ACTs).

3.2.4.1 Uniformity of Weight of Co-formulated Tablets & Capsules

The ACT branded formulations (Coded A-F) were used in this study. On different occasions 20 tablets/capsules of each brand were weighed together and the average weight determined. Each of the tablets/capsules was again weighed and the percentage deviation from the average weight was calculated and compared with the BP (1998) specification.

3.2.4.2 Disintegration Test

Six tablets each of samples (Coded A-F) was subjected to disintegration test using deionized water as the medium.

3.2.4.3 Assay for Artesunate and Amodiaquine in ACT tablets/capsules [A, B, C, D, E and F] using slightly modified International Pharmacopoeia HPLC method (2004).

The calibration procedure using reference standards (Artesunate and Amodiaquine)

Amodiaquine (50 mg) and Artesunate (50 mg) standards were weighed and mixed in 10 ml volumetric flask with 10 ml methanol to produce a stock solution of 5000 µg/ml respectively. This solution was serially diluted to produce concentrations of 100-500µg/ml of both amodiaquine and Artesunate. 20 µl of the concentrations were injected into the HPLC system and the results were used to plot calibration curves for the standards (**Fig. 4.5a and b**).

Amodiaquine/artesunate co-formulated (A-F) (20) were weighed and average weight per tablet/capsules was determined. The tablets were then crushed, powdered and triturated properly to form a fine powder. Equivalent weight of 10mg artesunate/40mg amodiaquine was weighed from the powder and mixed with 5mls of methanol in 10ml volumetric flask. The resulting solution was made up to 10ml mark with methanol. About 5ml of the supernatant was filtered using a microfilter 0.45µm size. 20µl of the filtrate was injected into the HPLC system. Concentrations of AMQ and AT were determined from the linear calibration

equations derived from the plots. The percentage contents (AMQ & AT) in the formulations were then calculated.

3.2.4.4 Dissolution Profile Studies on Oral Fixed-dose ACT Tablets

The dissolution profiles of amodiaquine and artesunate in the co-formulated tablets/capsules were determined in 900 mls of 0.05N HCl and deionized water respectively using US Pharmacopoeia dissolution Apparatus II- the paddle (7-compartment Sontax® dissolution tester). The temperature and the degree of agitation were set at 37°C ± 5 and 50 revolutions per minute respectively. Sample (5.0ml) was collected at pre-determined time intervals 0, 4, 8, 25, 40, 50 and 60 minutes. An equal volume, 5 ml, of the dissolution medium was replaced after every sampling in order to maintain sink condition. The samples were filtered with syringe filter pore size 0.45μm to remove insoluble excipients. This was then filled into Water Empower® HPLC auto sampler bottles and the system was programmed to inject 20 μl in triplicates. Slightly modified International Pharmacopeia HPLC method of analysis was used for quantification of dissolution samples.

3.2.5 Systemic Uptake (Bioavailability) Studies on Amodiaquine

Sixty healthy volunteers aged between 28 and 40 years and with body weights were selected for the study after informed consent. The subjects were all male. They were divided into six different groups (Group I, II, III, IV, V and VI to products A, B, C, D, E, and F respectively). All volunteers were healthy as assessed by physical examination and laboratory investigations.

The study adopted is a single dose, open and parallel study because of the long terminal half life of amodiaquine in the co-formulations (9-18days) (Pussard *et al.*, 1987)

3.2.5.1 Drug Administration and Subject Management:

All volunteers were instructed to embark on overnight fast. That is, each subject had its last meal 7.00 p.m. the previous day prior to commencement of study.

Each volunteer was administered with 600mg Amodiaquine and 200mg Artesunate formulation with water and taken orally.

Venous blood samples (5 ml) were taken from each subject through the forearm vein during the following periods : 0hr, 15min, 30min, 1hr, 2hr, 3hr, 4hr, 6hr, 8hr, 12hr, 24hr, 48hr, 72hr, 7days, 14 days, 21days, and 28days into heparinized tubes while the plasma samples were retrieved by centrifuging blood at 3000 rpm and then stored in sterile bottle at -20°C.

Amodiaquine, the object drug was quantified according to the slightly modified method of **Gitau *et al.*, 2004** as described below.

3.2.5.2 Chromatographic conditions.

The chromatographic elution was performed under isocratic conditions at ambient temperatures. An Agilent® HPLC fitted with Rheodyne valve injector (20µl loop) coupled with a variable wavelength UV/VIS detector (340nm), and data anlyzed by Chem-station software. The column was a reversed-phase (4.6 mm x 250 mm; Zorbax C8, particle size, 5µm).

The method involves extraction with diethyl ether with an acidic (pH 2.8) **mobile phase** 0.025M KH_2PO_4-Methanol (80:20% v/v) containing 1% (v/v) triethylamine and adjusted to pH 2.8 with orthophosphoric acid. The flow rate was 1.5ml/min, generating an operating pressure of 191 bars. Peak area ratios (drug/internal standard) were plotted as a function of the known concentrations of amodiaquine. The monodesethylamodiaquine metabolite which was also eluted **[Appendix 11]** was not monitored because of non-availability of the primary standard. However, same was identified going by literature reports.

3.2.5.3 Preparation of standard solutions

A stock solution of AMQ (100μg of the base/ml) was prepared by dissolving the dihydrochloride salt in 100ml of mobile phase. Working solution (10μg/ml) was prepared by diluting 1.0-ml of the stock solution to 10ml mobile phase. Internal standard stock (100μg/ml) was prepared by dissolving chloroquine phosphate in mobile phase while 2 in 10 dilution of the stock gave a working concentration of 20μg/ml.

3.2.5.4 Sample Preparation and Calibration Curve

Amodiaquine standard (20-200μl) of the working solution was spiked in one-ml drug-free (blank) plasma containing known concentrations of AMQ (200 – 2000ng/ml). These gradient concentrations were subjected to extraction procedure below. The extracted samples were injected into HPLC to obtain corresponding peak area ratios. These ratios were plotted against the gradient concentration to arrive at the linear equation (**Fig. 4.8**).

3.2.5.5 Extraction Procedure.

One-ml plasma was spiked with 20μl of internal primary standard, chloroquine (20μg/ml), and one-ml of 1M NaOH. The mixture was vortex-mixed for 2 min, diethylether (5ml) was added and the mixture tumbled for 15min followed by

centrifugation (1500g, 10min). The upper organic layer/phase was transferred to a clean 15ml glass centrifuge tube and evaporated to dryness under a gentle stream of nitrogen gas. The residues from the mixtures were reconstituted in mobile phase (100µl) and 20µl aliquot injected onto the chromatographic system. This procedure was done in duplicate with three injections per sample.

3.2.5.6 Recovery Studies

Two different concentrations (200 ng/ml and 800 ng/ml) of Amodiaquine in the presence of 400ng/ml chloroquine (internal standard) were spiked into plasma. The drugs were extracted as described earlier. The residues were reconstituted in 100µl mobile phase whirl-mixed before 20 µl was injected onto the HPLC. The stock solutions of amodiaquine was diluted in mobile solvent to obtain 200ng/ml and 800ng/ml in the presence of internal standard (400ng/ml chloroquine). Peak area ratios of amodiaquine were compared with those obtained by direct injection of the primary standards in mobile phase.

3.2.6 Characterization of DNA/DAB Formulations

3.2.6.1 Preparation of DNA (10 & 1mg/µl) & DAB16 (10 & 1mg/ml) solutions.

The sodium salt of Calf Thymus DNA (100mg) was procured from Sigma-Aldrich (UK). The sample of the DNA (100mg) was transferred into a 10 ml volumetric

flask, mixed and made up to volume with deionised water to arrive at concentration of 10 mg/ml.

One ml of the stock solution was also made up to 10 ml with water to produce a concentration of 1mg/mL. Samples of DAB16 (10mg and 100mg) were separately dissolved in 10ml of dextrose (5%) to obtain 1mg/mL and 10mg/mL concentrations of the polymer respectively.

3.2.6.2 Preparation of DNA/DAB formulation based on Nitrogen to Phosphate (N:P) ratio

Stock solutions of DAB16 (1 and 10mg/mL) and DNA (Calf thymus) [1 and 10mg/mL] concentrations were prepared separately as shown in sections 3.2.6.1. Complexes (DNA/DAB16) were formulated as described in **Table 3.0**. These complexes were formed by addition of DAB solutions to DNA solutions and the complexes left undisturbed for about 3 minutes before mixing or stirring. This procedure is very important and crucial in order to avoid precipitation of DNA, most especially at DNA concentrations of 250μg/ml and above. The complexes were allowed to equilibrate for 15 minutes for a single point sizing while same used for the time-dependent assay were measured immediately after stirring at various time intervals over 24hrs at 37^0C. The table and calculations below show the formulation based on N:P.

DAB16 Mw 1600g → 30 Nitrogen DNA 1 Mw of PO_4^- → 340 Daltons (g)

53g → 1 "

53g total Nitrogen (DAB16) → 340g PO_4 of DNA

0.16g " → 1g "

i.e. An N:P ratio of 1 requires 53g of DAB16 to complex with 340g DNA or alternatively an N:P ratio of 1 may be obtained if 0.16g of DAB16 is complexed with 1g of DNA.

Table 3.0 : Formulation ratio of DNA/DAB in µg/ml

		FORMULATIONS					
			DNA/DAB16 µg/ml Ratios				
N : P							
2	25/8	50/16	100/32	250/80	400/128	500/160	1000/320
3	25/12	50/24	100/48	250/120	400/192	500/240	1000/480
5	25/15	50/40	100/80	250/200	400/320	-	-
6	25/24	50/48	100/96	250/240	400/384	-	-
8	25/32	50/64	100/128	250/320	400/512	-	-
10	25/40	50/80	100/160	250/400	400/640	-	-
12	25/48	50/96	100/192	250/480	400/768	-	-
20	25/80	50/160	100/320	250/800	400/1280	-	-
30	25/120	50/240	100/480	250/1200	-	-	-
60	25/250	50/500	100/960	250/2400	-	-	-
180	25/750	50/1500	100/2880	250/7200	-	-	-

3.2.6.3 Determination of Hydrodynamic diameter measurement

The formulations were prepared in 5% dextrose solution (as dispersion medium) in a total volume of two millilitres. This was placed in a polystyrene cuvet and all measurements were performed at 25^0C and analyzed using the CONTIN mode of analysis. Before each measurement standards (polystyrene latex beads, Sigma Co., UK) were run. The average diameter of DAB16/DNA complexes formed were sized by dynamic light scattering using a Malvern Zeta sizer 3000 HAS (Malvern Instrument Ltd., UK). Triplicate samples were prepared on day 1 and 2. All measurements were carried out in triplicate.

For the time-dependent (kinetic studies) particle size assay, the sample particle size was determined only once as assay time per instrument run was 15 minutes.

3.2.6.4 Determination of Zeta potentials of formulations

Before the start of each measurement, a manufacturer's zeta potential standard (Malvern Zeta potential Transfer standard, DTS 1050) was filled into the in-built cuvet for analysis after cleaning with soapy solution, followed by de-ionized water and finally rinsed in 70% ethanol. All z-average data obtained conformed to manufacturer's specifications.

Sample (2ml) was filled slowly into the Zeta-sizer using a 2ml syringe to avoid formation of bubbles which could interfere with measurements. The sampling time was set at 30 seconds with 3 measurements per sample. The equipment stabilized at 25^0C. Replicate samples were prepared and measured on day 1 and 2.

3.2.6.5 Transmission Electron Microscopy

Transmission electron microscopy of the particles was performed to visualize the morphology and size of the dendriplexes.

A drop of formulation was placed onto a copper grid (300 mesh, Taab Laboratories, UK) that had a Formvar/carbon support film. Excess sample was removed with filter paper (No.9 Hardened) then a drop of stain (1% aqueous Uranyl acetate) added for contrast. The prepared grid was then examined under the Transmission Electron Microscope, TEM (FEI CM120 BioTwin) (Eindhoven, Netherlands). Images were captured with an AMT (Stowmarket, UK) Digital Camera.

3.2.7 Statistical Analysis

$\%Q_{max}$, the dissolution time points profile and the AUC of CP tablets alone were compared by t-test with CP-CQ and CP-HF. The dissolution time profile data of CP alone and in the presence of CQ/HF were also analyzed by estimation of a similarity factor, f_2 (FDA recommendations) using dissolution time profile of CP alone in water and 0.1N HCl as reference while CP-CQ/CP-HF as test and presented in the following equation,

$F_2 = 50\log \{[1 + 1/n\sum^{n=1}(R_t - T_t)^2]^{-0.5} \times 100\}$ where R_t and T_t are percent dissolved at each time point for reference and test respectively. Using the f_2 values, dissolution profiles were considered dissimilar if these values were less than 50 (Thomas *et al.*, 1998).

Kinetica ™, a biopharmaceutics software programme was used to analyse dissolution parameters such as $\%_{Qmax}$.

The maximum plasma amodiaquine concentration (C_{max}) and the time to reach C_{max} (T_{max}) were determined by visual inspection of the individual semilog plots and data. The terminal elimination rate constant, λ and elimination half life ($t_{i/2}$, $0.693/\lambda$) were obtained by least-squares regression of the log of plasma drug concentrations in the log-linear phase. The plasma amodiaquine AUC from time zero to 28days (AUC0-28) and to infinity (AUC0-∞) were calculated by the linear trapezoidal rule with extrapolation to infinity using Ct/λ, where Ct is the last

measured concentration. Graph pad® prism version 4.0 software program, Microsoft Excel Analytical Tools, Microcal® Origin 6.0 and WinNonLin Pro ® Version 2.1 pharmacokinetic software (Courtesy Univ. of Manchester) were applied. In all cases, a value of $p \leq 0.05$ was considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Physico-chemical properties of Ciprofloxacin (CP) Tablets

The mean hardness of CP 250 mg and 500 mg tablets were found to be 4.4 ±0.7 and 5.6 ± 0.5. The sample tablets conformed to the BP (1998) specification for uniformity of weight. None of the tablets deviated from the average weight by more than ±5 percentage. The tablets disintegration time as shown in **Table 4.1,** in all the media conformed to the BP, 1998 specification for tablets. The assay of ciprofloxacin tablets gave percentage purity of 101 ± 1.5%. Ciprofloxacin conformed to both BP 1998 (98 – 105%) and USP, 2004 (90 – 110%) assay purity level.

Table 4.1: Disintegration time of CP tablets

MEDIUM	DISINTEGRATION TIME (Minute)	
	CP 250mg	CP 500mg
0.1M Acetic acid	3.8 ± 0.2	2.4 ± 0.1
0.1N HCl	1.6 ± 0.1	3.0 ± 0.1
Deionized water	2.5 ± 0.3	2.0 ± 0.1
Phosphate buffer 7.4	3.5 ± 0.3	2.2 ± 0.1

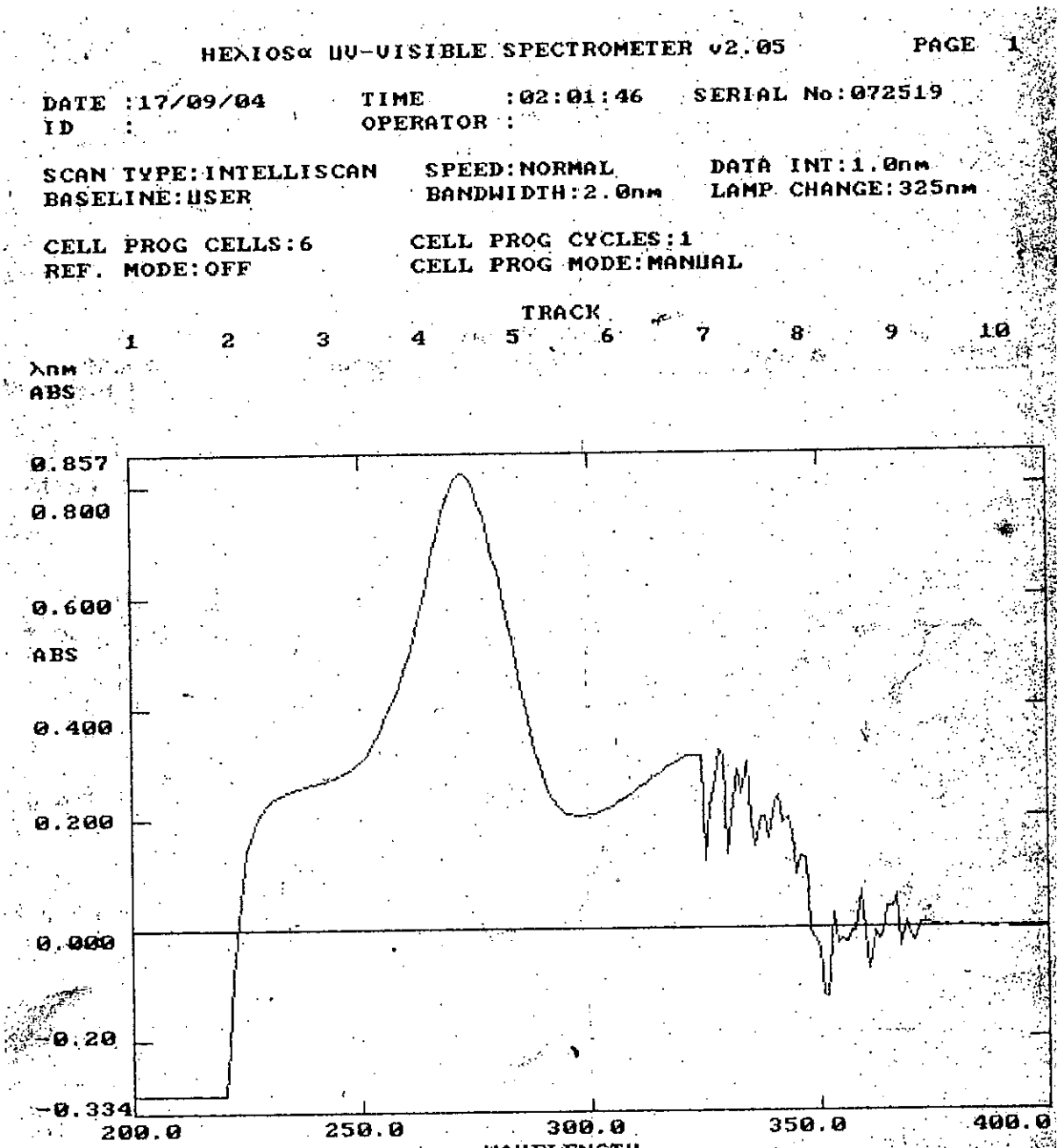


Fig. 4.1a: UV scan of Ciprofloxacin standard with λ_{max} 274nm

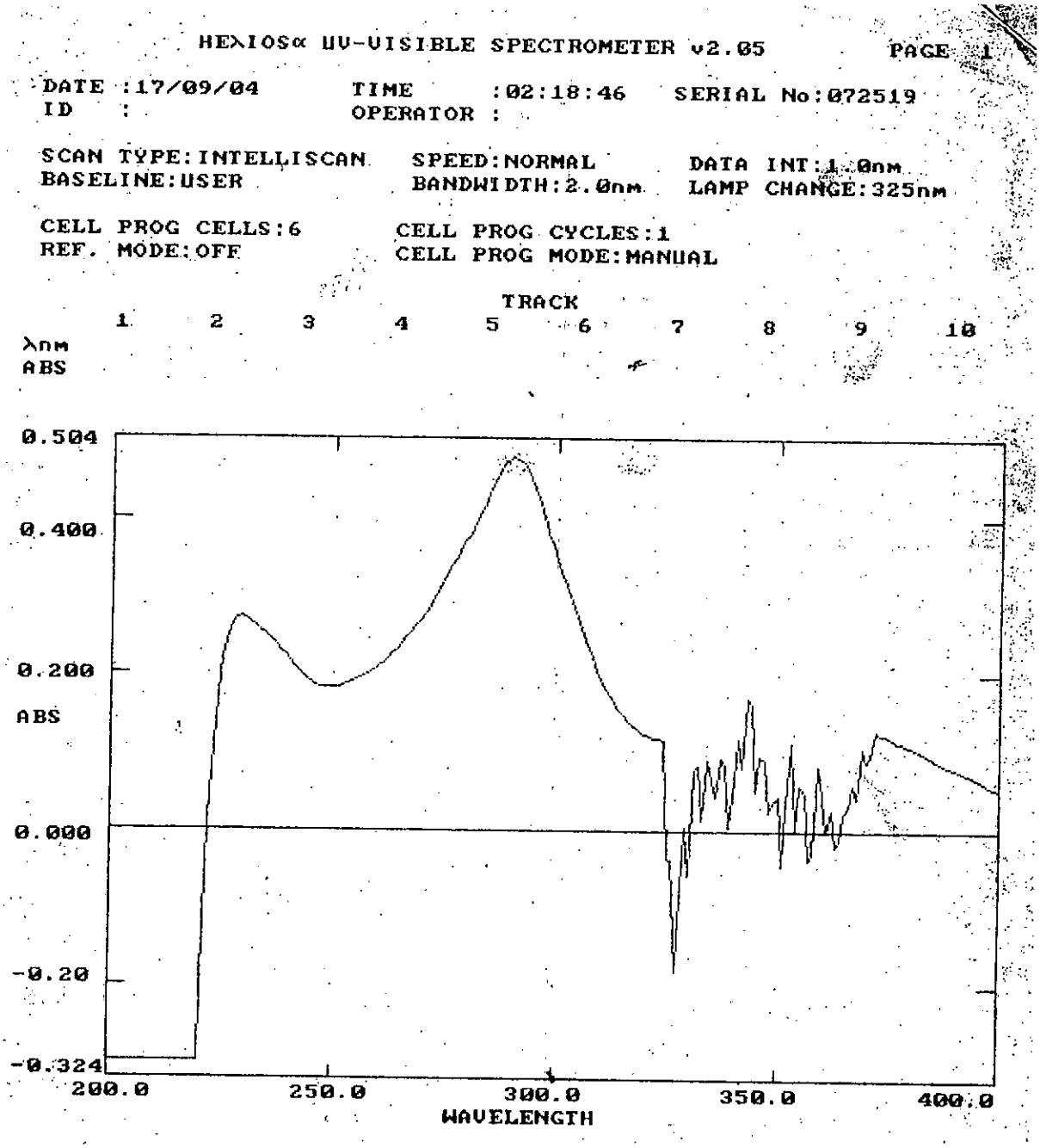


Fig. 4.1b: UV scan of Sparfloxacin at λ_{max} 290nm

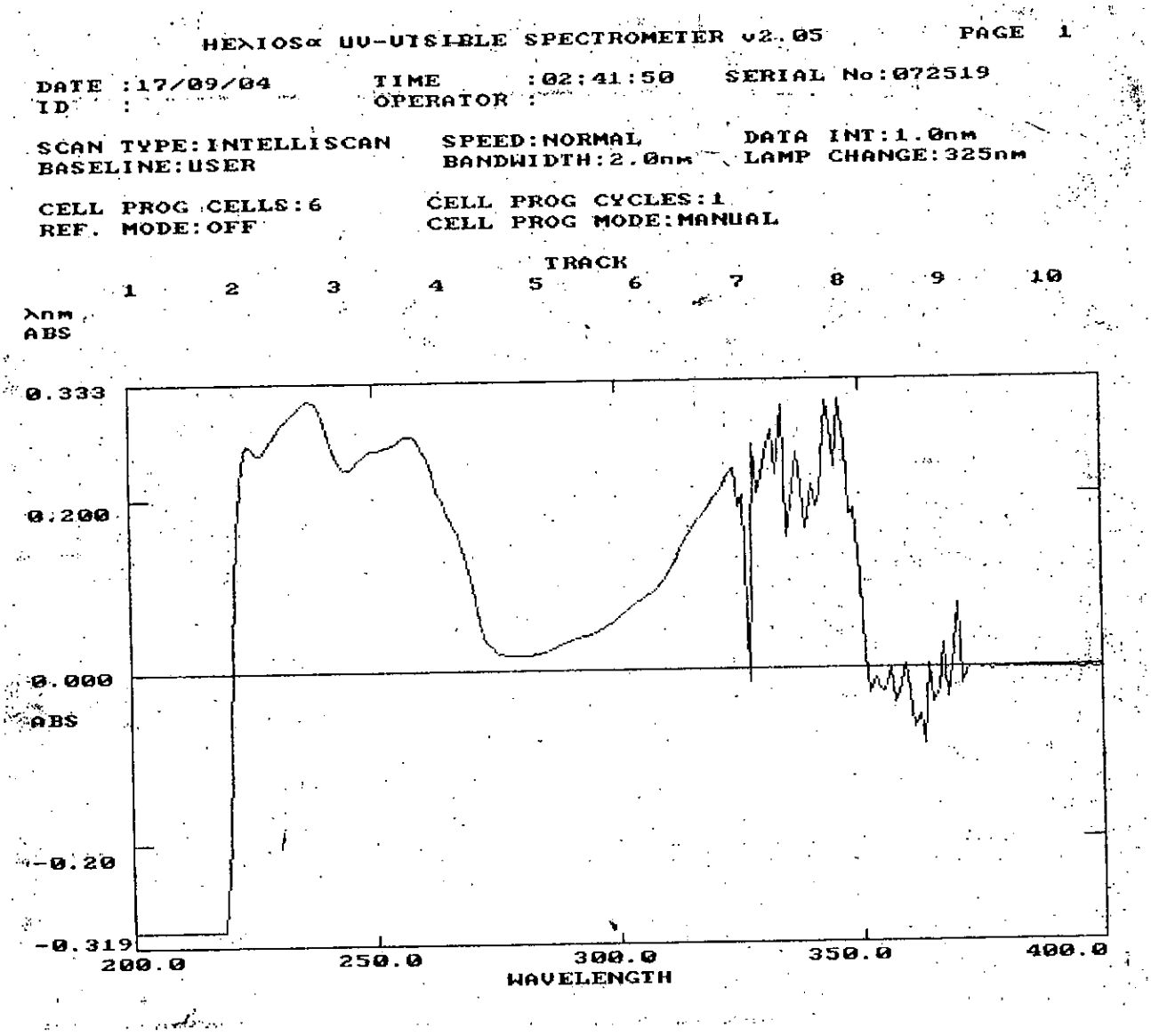


Fig. 4.1c: UV scan of Chloroquine at λ_{max} 220 and 256nm

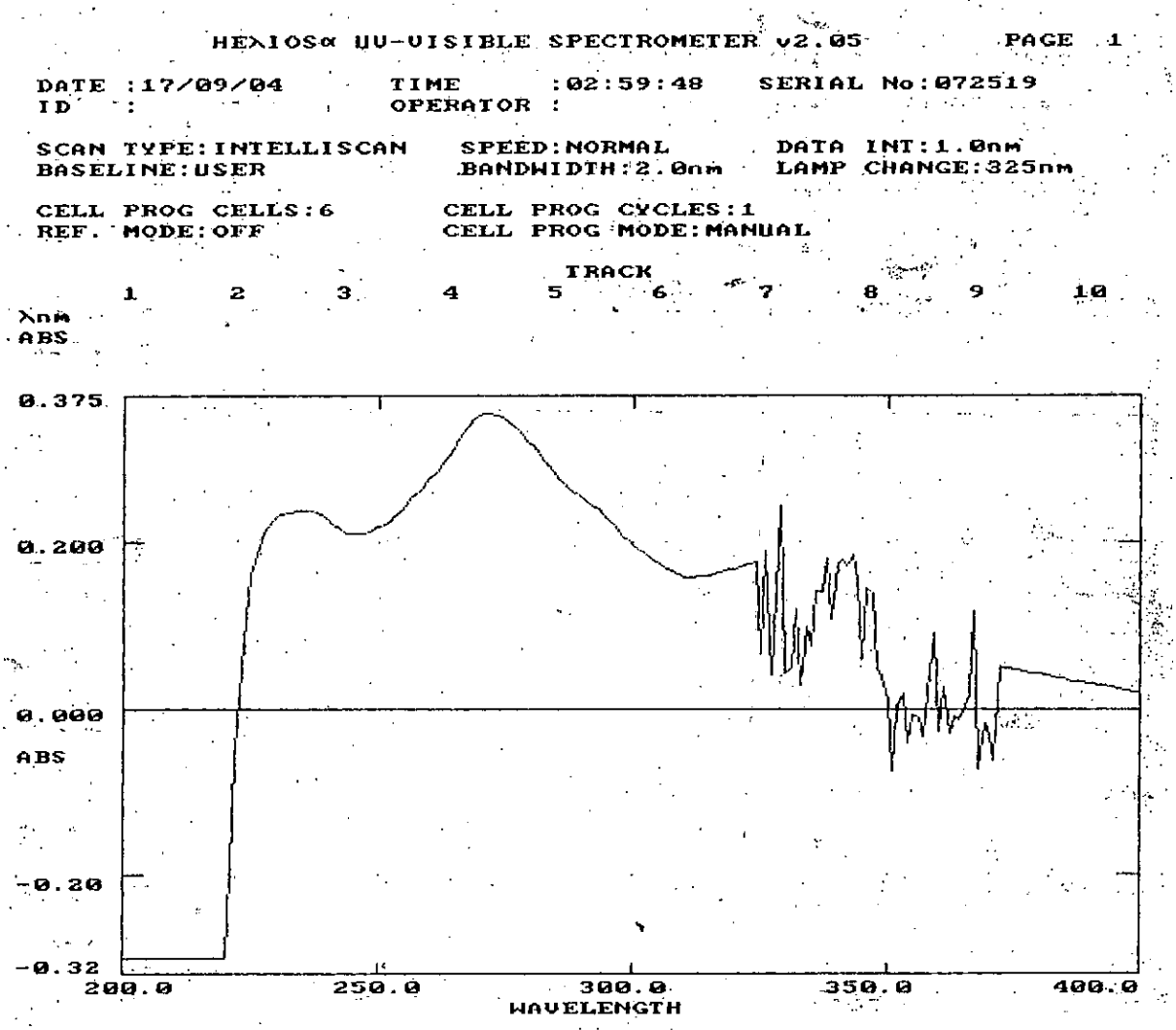


Fig. 4.1d: UV Scan of standards:- CQ/CP/SP mixture at λ_{max} 254nm.

4.2 In-House HPLC developed method

Figure 4.2 shows the scanned spectra for CP, CQ and SP alone and when in combinations (CQ/CP/SP). The wavelength of maximum absorption for CP, CQ and SP alone were 274; (220, 256, 340) and 290nm respectively while the common λ_{max} for CQ/CP/SP was 254nm.

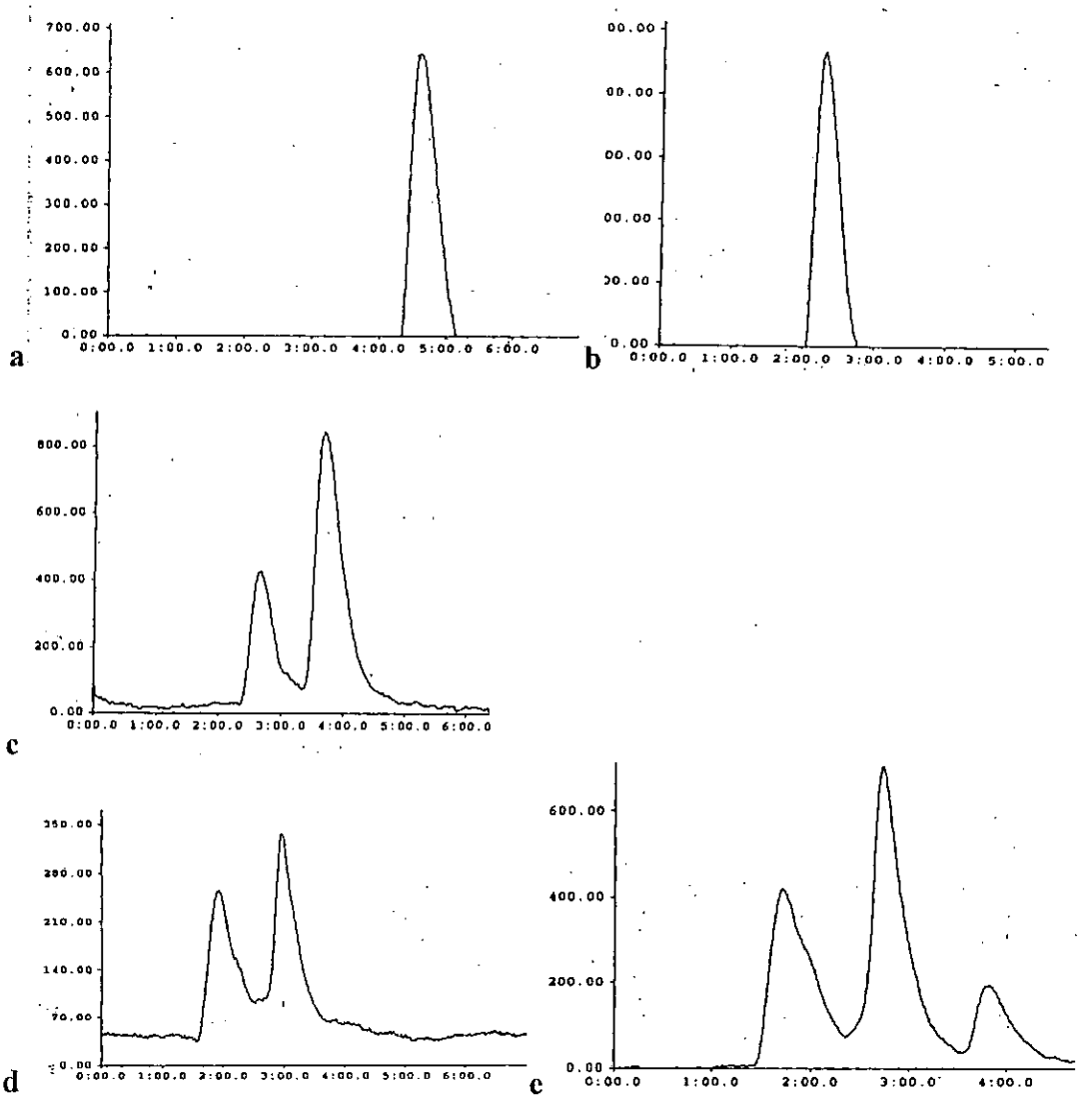


Fig 4.2a: HPLC chromatograms(Cecil® system) of: (a) internal standard (Sparfloxacin); (b) Ciprofloxacin; (c) Ciprofloxacin and Sparfloxacin; (d) Chloroquine and Ciprofloxacin; (e) Chloroquine, Ciprofloxacin and Sparfloxacin.

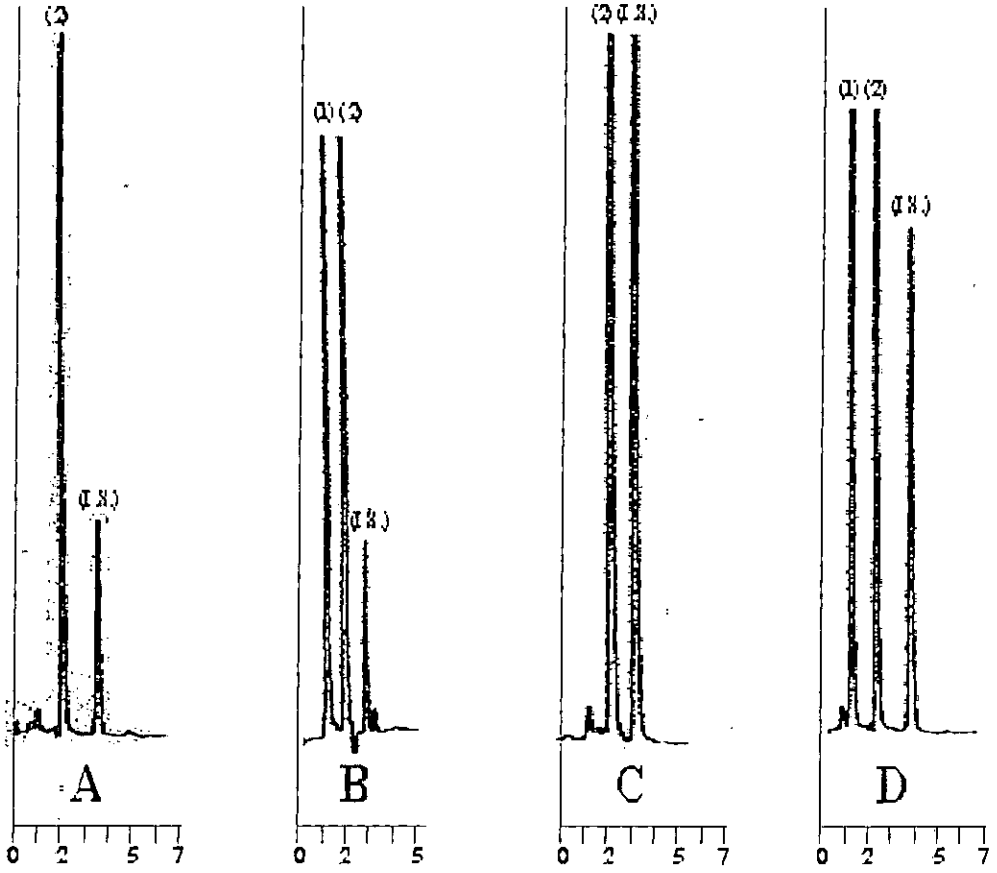


Fig. 4.2b: Representative chromatograms (Waters®) of: (A) Ciprofloxacin (1.2µg/ml)[2] & Sparfloxacin (2.5 µg/ml)[I.S= internal standard]; (B) Chloroquine (1.0 µg/ml)[1], Ciprofloxacin (1.0 µg/ml) and Sparfloxacin (2.5 µg/ml; Dissolution samples of 250mg Ciprofloxacin tablet in (C) 25 µg/ml Sparfloxacin standard at 40minutes and (D) 1.0 µg/ml CQ [1] and 25 µg/ml Sparfloxacin standard at 40minutes.

The chromatographs of the standard confirm average retention times of 1.45; 2.7 and 4.5 minutes for chloroquine; ciprofloxacin and sparfloxacin respectively as shown in **Figures 4.2a and b** which also indicate the response of the method in two different HPLC systems. The limit of detection for chloroquine and ciprofloxacin were 10ng/ml and 50ng/ml respectively.

The linear regression equations $y=0.78545x - 0.24914$ and $y=0.5861x - 0.01161$ where x and y are concentrations and peak area ratios of chloroquine and ciprofloxacin standards respectively. **Table 4.2a and b** show the linearity of the calibrations which was demonstrated by the good correlation coefficient (r) of 0.999 and 0.998 obtained for the regression lines of chloroquine and ciprofloxacin respectively.

Some of the chromatograms are shown in **Appendix I**

Table 4.2a: Linearity data for chloroquine-calibration standard response values (Graph pad®)

Calibration	Set(n=5)	SD	P value
(Day 1)			
Slope	0.007475	0.0001433	0.5172
Intercept	0.000030	0.0009503	
Correlation coefficient (r)	0.9995		
(Day 2)			
Slope	0.00737	0.00005292	0.2863
Intercept	0.00012	0.00035100	
Correlation coefficient (r)	0.9999		

Table 4.2b: Linearity data for ciprofloxacin-calibration standard response value

Calibration	Set (n=5)	SD	P value
(Day 1)			
Slope	0.06544	0.002724	0.4035
Intercept	0.02298	0.016570	
Correlation coefficient (r)	0.997		
(Day 2)			
Slope	0.06249	0.001815	0.2804
Intercept	-0.02135	0.01104	
Correlation coefficient (r)	0.999		

The precision shows intra and inter-day assay coefficient of variation of < 5 and 8 (**Table 4.2c**).

Table 4.2c: Precision of Analytical method

Sample	Concentration µg/ml	CV %	n
Intra-day assay			
Ciprofloxacin	0.2	2.1	6
	1.0	1.1	5
	5.0	0.8	6
	10.0	0.7	6
	100.0	0.7	6
Chloroquine	0.2	4.9	7
	1.0	4.0	8
	5.0	3.7	8
	10.0	3.1	8
	100.0	1.1	8
Inter-day assay			
Ciprofloxacin	0.2	3.2	6
	1.0	2.0	6
	5.0	2.5	6
	10.0	1.1	5
	100.0	0.7	6
Chloroquine	0.2	8.0	6
	1.0	7.1	6
	5.0	6.5	6
	10.0	5.1	6
	100.0	3.8	6

CV = coefficient of variation
n = number of replicates

The absolute % recovery for ciprofloxacin and chloroquine tablets is shown in the range of 98-105% (**Table 4.2d**).

TABLE 4.2d: Absolute recovery for chloroquine and ciprofloxacin ± SD

%Recovery	Concentration µg/ml
Ciprotab® 250mg	50
98±0.6	
	100
101±3.1	
Nivaquine® 200mg	50
100±2.5	
	100
105±2.2	

4.3 Results of interaction potentials of CQ, CP and HF

There was a significant reduction in the maximal quantity of ciprofloxacin (250 mg and 500 mg) % Q_{max} dissolved (deionized water & 0.1N HCl) in the presence of chloroquine (0.69 – 3.47 mole/L concentrations (p= 0.027). An average reduction of 43.6% was recorded as represented in **Table 4.3a and Figure 4.3a-d**

The $\%Q_{30}$ of CP alone (250 mg and 500 mg) met both USP 2004 and FDA recommendations but low $\%Q_{30}$ were recorded when the same strength were in concomitant with chloroquine as shown in **Table 4.3b**.

The total quantity of CP alone (250 mg and 500 mg) and in the presence of varying concentrations of CQ represented by the area under the dissolution (**AUC**) profile curves (**Table 4.3d and Fig. 4.3a, b, c and d**) were found to be highly significantly different (p= 0.0008). Non-significant level was recorded when compared with the Q_{max} of CP500-CQ_{water} versus CP500-CQ_{acid} while others show a significant level of differences **(Table 4.5).**

The % Q_{max} CP was reduced by over 60% in the presence of Halofantrine compared to CP alone (**Table 4.3a and fig. 4.3e and f**). There was significant level of difference between CP alone and CP in varying concentration of HAL ($p<0.05$). The $\%Q_{30}$CP in the presence of HF did not conform to the official specifications [USP2004 & US FDA] (**Table 4.3b**). Likewise the area under

dissolution profile curves (**Fig. 4.3e and f**) showed significant variances compared to the CP alone.

Table 4.3a: $\%Q_{max}$ of CP in dissolution media containing varying concentrations of CQ and HF.

Concentration of CQ (M)	$\%Q_{max}$			
	$CP500mg_{water}$	**$CP500mg_{HCl}$**	**$CP250mg_{water}$**	**$CP250mg_{HCl}$**
0.00	123.4	102.4	120.0	92.7
0.69	105.0	87.3	83.5	78.5
1.39	81.3	70.5	80.4	61.0
2.78	42.0	45.5	56.9	51.0
3.47	29.3	20.4	51.3	40.8
Mean ± SD ($\%Q_{max}$ CP with CQ)	64.4±3.0	55.8±2.5	68.0±1.4	57.8±1.3
% reduction ($\%Q_{max}$CP alone/$\%Q_{max}$CP with CQ)	47.8%	45.5%	43.3%	37.7%
Concentration of HF (M)				
0.0	NA	106.2	NA	91.9
0.56	NA	28.2	NA	49.4
1.11	NA	24.9	NA	45.8
Mean ± SD ($\%Q_{max}$ CP with HF)		26.6±1.65		47.6±1.8
% reduction ($\%Q_{max}$CP alone/$\%Q_{max}$CP with HF)		75.0%		48.2%

NA= Not applicable
Q_{max}= Maximal quantity of dissolved drug.
$\%Q_{max}$= Percentage of drug dissolved compared to Q_{max}
CP_{water}= Ciprofloxacin in distilled water
CP_{HCl} =Ciprofloxacin in 0.1N HCl

Table 4.3b: $\%Q_{30}$ of CP in dissolution media containing varying concentrations of CQ and HF.

Concentration of CQ (M)	$\%Q_{30}$			
	$CP500mg_{water}$	**$CP500mg_{HCl}$**	**$CP250mg_{water}$**	**$CP250mg_{HCl}$**
0.00	74	73.7	88	80
0.69	71	74.4	73	69
1.39	80.5	80.8	68.6	54
2.78	28.1	97.4	58.5	52.3
3.47	68.9	64.3	43.6	46.0
Mean ± SD ($\%Q_{30}$ CP with CQ	62.13±2.0	79.23±1.2	60.93±1.1	55.39±8.5
Concentration of HF (M)				
0.0	NA	91	NA	80
0.56	NA	33.2	NA	53
1.11	NA	28.4	NA	56.8
%Mean ± SD ($\%Q_{30}$ CP with HF)		30.8±2.4		54.9±1.9

$\%Q_{30}$ = Percentage quantity of CP released at 30 minutes.

Table 4.3c: Assessment of pH before and after dissolution of CP 250mg tablets in varying concentrations of Chloroquine.

Concentration of CQ M (Molarity)	pH of CQ alone	pH of CQ solution in the presence of CP 250mg tablet
0.00	6.77	5.95
0.35	6.63	5.79
0.69	6.54	5.72
1.39	6.28	5.65
2.09	6.12	5.62
2.78	6.00	5.60
3.47	5.89	5.59

Table 4.3d: AUC of CP, CP-CQ and CP-HF

Concentration of CQ (M)	*AUC*			
	CP250mg$_{HCl}$	*CP500mg$_{HCl}$*	*CP250mg$_{water}$*	*CP500mg$_{water}$*
0	*22965*	*48540*	*27153*	*48469*
0.56 – 3.47	*16495.5*	*39453*	*19554.3*	*34672.3*
Concentration of HF (M)				
0	*23038*	*55072*	*NA*	*NA*
0.56 – 1.11	*14843*	*16921*	*NA*	*NA*

AUC= Area Under dissolution Curve.

Table 4.4: Mathematical comparison of similarity factor, f_2 of dissolution time points profile of CP/CQ and CP/HF

Sample (M)	CP250mg$_{water}$	CP500mg$_{water}$	CP250mg$_{HCl}$	CP500mg$_{HCl}$
CP/CQ(0.69 – 3.47)	28.5	28.8	35.4	35.3
CP/HF(0.56 – 1.11)	NA	NA	29.2	8.5

Table 4.5: Statistical Results of t-test on $\%Q_{max}$ of CP with CQ and HF in dissolution studies.

Sample	P	Result
$\%Q_{max}$		
CP500mg-CQ_{water} vs. CP500mg-CQ_{HCl}	0.07	NS
CP500mg-CQ_{water} vs. CP250mg-CQ_{water}	0.05	S
CP500mg-CQ_{HCl} vs. CP250mg $_{HCl}$	0.04	S
CP250-CQ_{water} vs. CP250mg $_{HCl}$	0.03	S
CP500mg-HF_{HCl} vs. CP250mg-HF_{HCl}	0.08	NS
CP-alone vs. CP in varying CQ concentration (two tailed)	0.03	S
CP-alone vs. CP in varying HF concentration	0.03	S
AUC		
CP alone vs. CP in varying CQ	0.01	S
CP-alone vs. CP in varying HF	0.2	NS

S= significant; NS= non significant

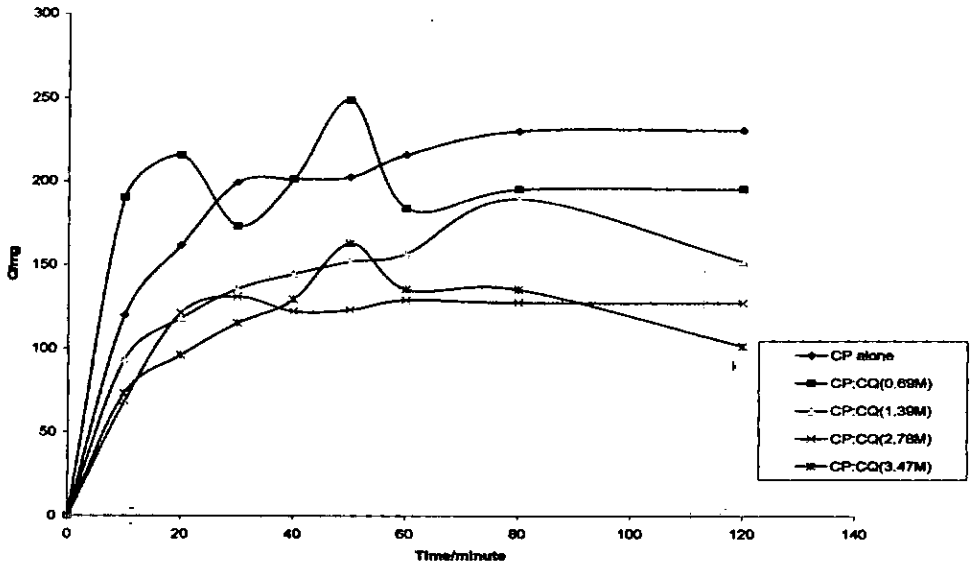


Fig. 4.3a: Dissolution profile of CP (250 mg) Tablets with varying concentrations of CQ in dissolution medium (0.1NHCl)

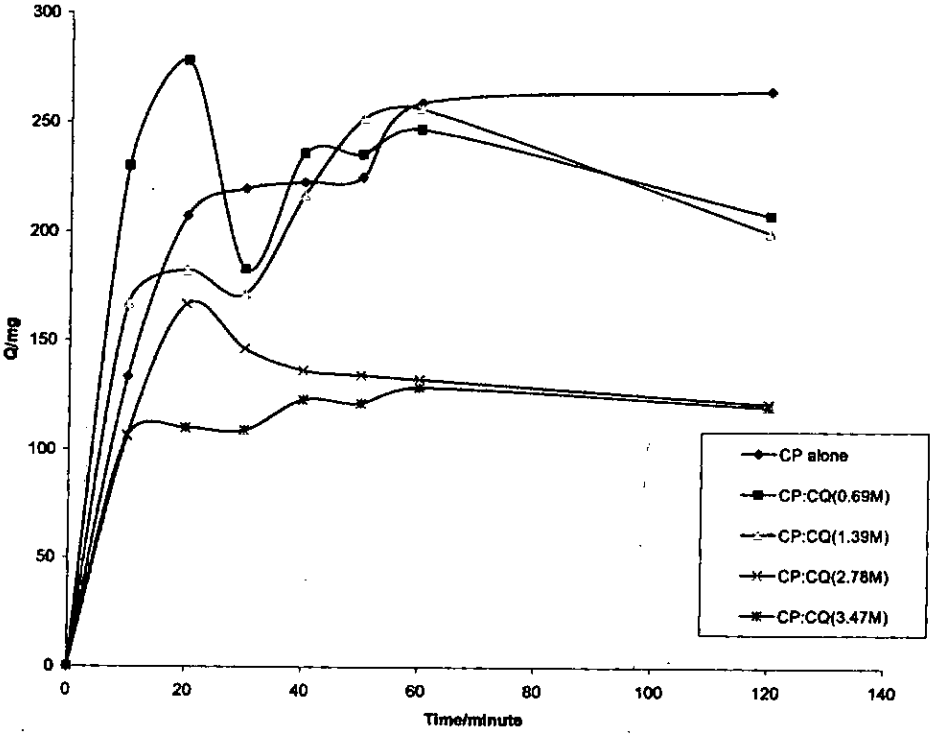


Fig. 4.3b: Dissolution profile of CP (250 mg) tablets with varying concentrations of CQ in deionized water

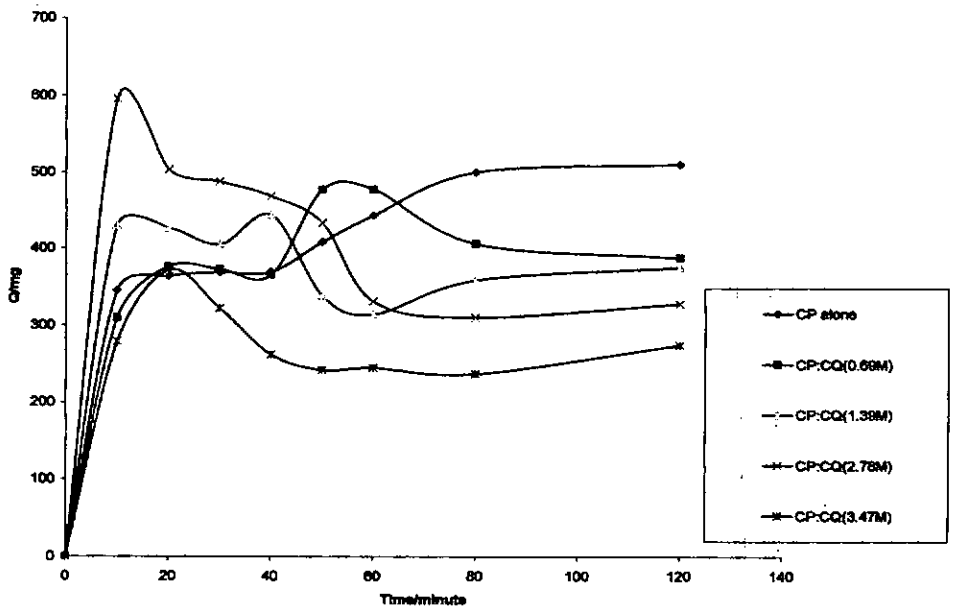


Fig. 4.3c: Dissolution profile of CP (500 mg) tablets with varying concentrations of CQ in 0.1N HCl

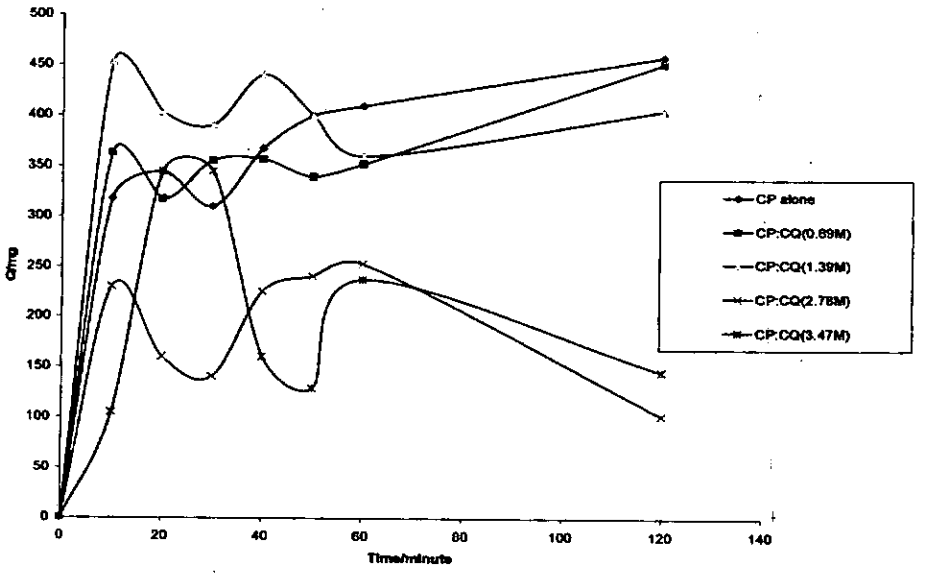


Fig. 4.3d: Dissolution profile of CP (500 mg) tablets with varying concentrations of CQ in deionized water.

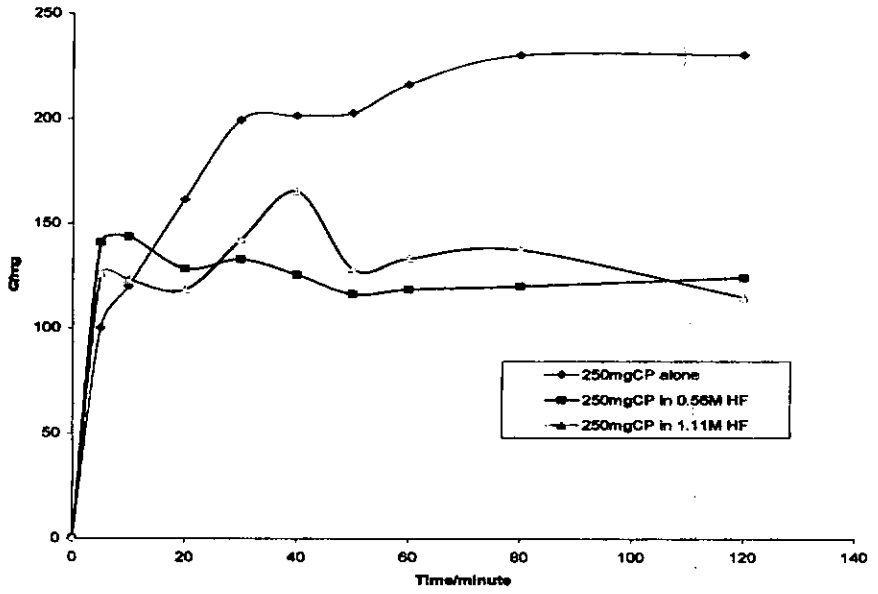


Fig. 4.3e: Dissolution profile of CP (250 mg) tablets with Halofantrine 0.56 and 1.11M respectively in 0.1N HCl.

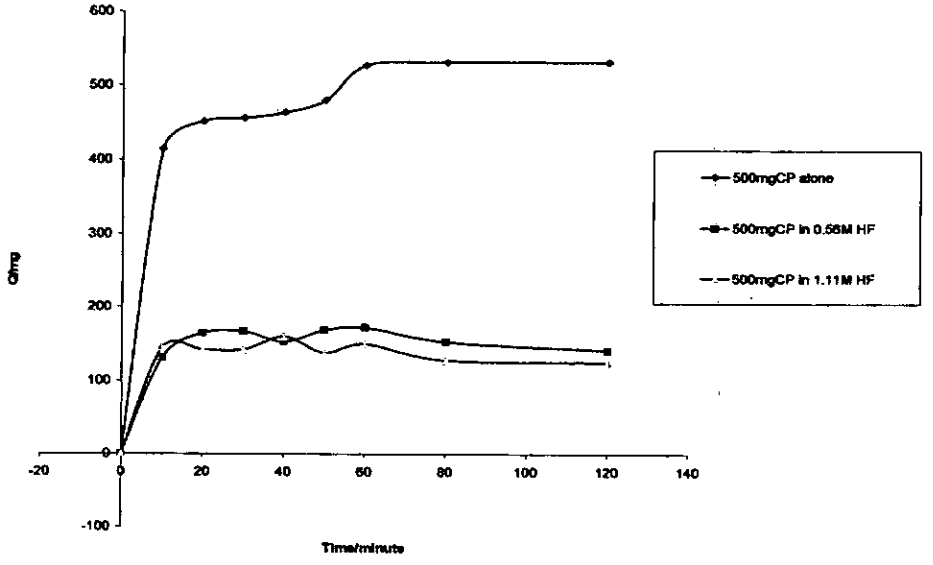


Fig. 4.3f: Dissolution profile of CP (500 mg) tablets with Halofantrine 0.56 and 1.11M respectively in 0.1N HCl.

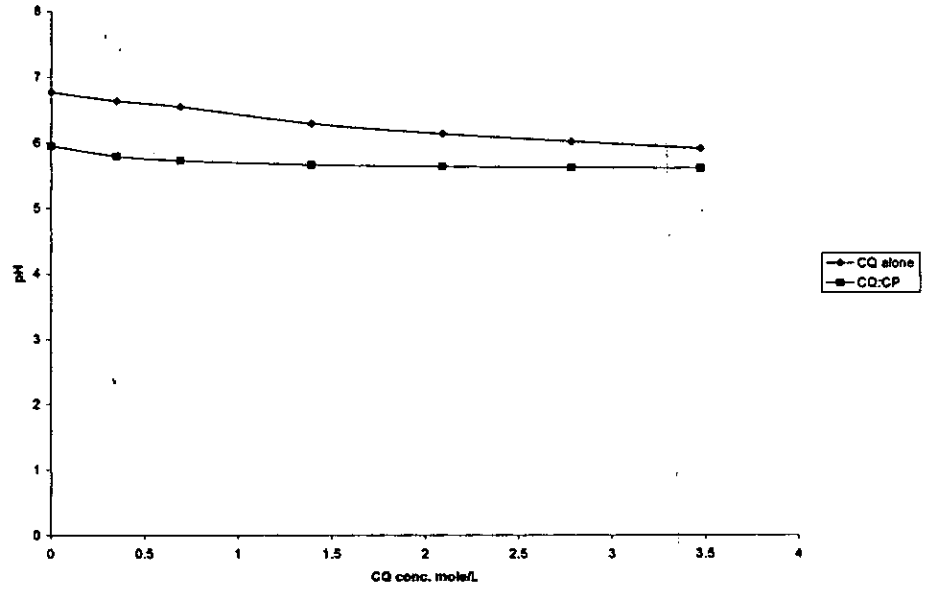


Fig. 4.3g: pH profiles of varying concentration of Chloroquine alone and in the presence of Ciprofloxacin (250 mg) tablets after 120 minutes of dissolution.

Table 4.3c and Figure 4.3g show the change in the pH of varying concentrations of chloroquine in water alone and in the presence of a fixed ciprofloxacin tablets (250mg) at the end of dissolution time of 120minutes. **Figure 4.3h** shows that the pH profile of CQ-HF mixture is below that for ciprofloxacin alone and the decrease in pH shows that complexation occurred almost throughout the neutralization range.

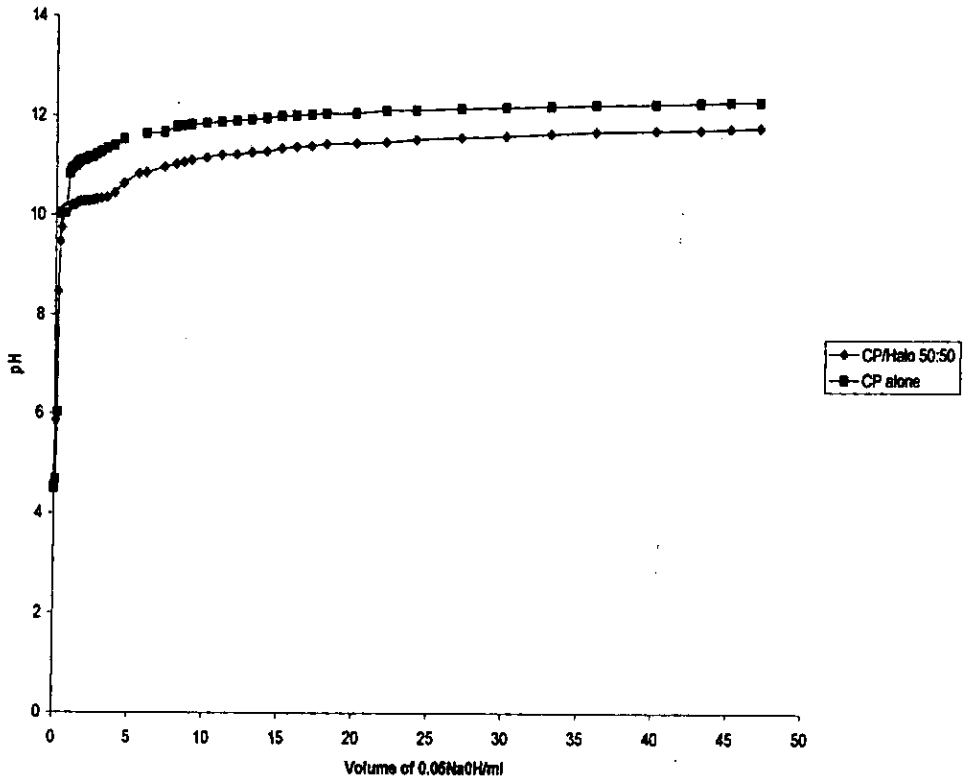


Fig. 4.3h: Titration of Ciprofloxacin alone and in the presence of Halofantrine

4.4 Physicochemical properties of Co-formulated Amodiaquine/Artesunate formulations.

In all the tablets and capsules tested for uniformity of weight, they all conformed to the B.P (1998) specification for uniformity of weight i.e., none of the formulations A, B, C, D, E and F deviated from the average weight by more than ± 5% **(Table 4.6a and b)**.

The co-formulated tablets and capsules disintegrated in the medium within the specification stipulated in official monographs (USP, 2004 & BP, 1998) for disintegration time for tablets and capsules as shown in **Table 4.7**.

Figures 4.4a and b show the calibration curves of Amodiaquine and Artesunate standards in methanol and were found to be linear with excellent determination coefficients (r^2) of 0.999 and 0.999 with average retention times of 2.3 and 10.0 respectively (**Fig. 4.6a and b**). Using the HPLC method, artesunate and amodiaquine components of the formulated products returned percentage purity of 92 – 95.8% and 97 -101% respectively. These values fall within the range percentage purity 90 - 110% and 95 – 105%, specified in the International Pharmacopoeia, (2005) for Artesunate and in USP 2004 for amodiaquine respectively.

Table 4.6a: Results of uniformity of weight of Product A , B Tablets and C capsule.

Nos.	Product A		Product B		Product C	
	Weight(mg)	%Deviation	Weight(mg)	%Deviation	Weight(mg)	%Deviation
1.	604.7	1.77	735.6	1.95	1357.2	0.79
2.	603.8	1.92	733.5	2.25	1359.6	0.13
3.	614.2	0.23	752.4	0.29	1360.1	0.01
4.	624.5	1.44	757.3	0.95	1362.1	0.57
5.	601.4	2.31	731.4	2.51	1359.9	0.04
6.	602.3	2.16	757.4	0.96	1350.5	2.66
7.	600.4	2.47	748.9	0.18	1365.3	1.46
8.	604.5	1.81	754.0	0.51	1369.3	2.57
9.	608.1	1.22	759.0	1.17	1356.5	0.99
10.	610.1	0.90	767.0	2.24	1368.2	2.26
11.	631.2	2.53	752.4	0.29	1354.2	1.63
12.	645.4	4.84	734.0	2.16	1359.2	0.24
13.	631.2	2.53	742.1	1.08	1361.4	0.37
14.	624.1	1.38	761.2	1.47	1362.2	0.59
15.	601.4	2.31	755.6	0.72	1358.9	0.32
16.	632.1	2.68	759.5	1.24	1351.5	2.38
17.	631.3	2.55	735.1	2.01	1365.4	1.48
18.	637.1	3.49	753.2	0.40	1359.3	0.21
19.	600.1	2.52	756.3	0.81	1356.1	1.10
20.	604.3	1.84	758.3	1.09	1364.2	1.15
	Average weight=615.61±14.79mg		Average weight=750.21±10.85mg		Average weight=1360.06±4.99mg	

Table 4.6b: Results of uniformity of weight of Product D, E and F

Nos.	Product D		Product E		Product F	
	Weight(mg)	%Deviation	Weight(mg)	%Deviation	Weight(mg)	%Deviation
1.	847.7	0.99	508.5	1.01	505.3	1.0
2.	830.8	2.97	516.3	0.51	513.7	0.65
3.	831.3	2.90	521.8	1.58	512.1	0.33
4.	834.6	2.52	525.0	2.2	489.9	4.02
5.	856.7	0.06	525.0	2.2	516.6	1.22
6.	820.2	4.20	505.1	1.7	518.0	1.49
7.	874.3	2.11	500.4	2.6	502.6	1.53
8.	856.0	1.24	499.3	2.8	508.0	0.47
9.	897.6	4.80	504.6	1.8	520.4	1.96
10.	856.6	0.05	529.9	3.2	522.3	2.33
11.	845.4	1.26	517.3	0.7	489.9	4.02
12.	852.2	0.47	521.3	1.48	529.3	3.70
13.	864.7	0.99	521.5	1.52	489.8	4.04
14.	855.3	0.10	523.5	0.52	527.0	3.25
15.	864.0	0.91	495.1	3.6	515.4	0.98
16.	855.4	0.09	533.3	3.82	534.0	4.62
17.	824.7	3.68	495.6	3.5	499.8	2.10
18.	843.3	1.51	528.2	2.82	506.4	0.78
19.	856.7	0.06	509.6	0.80	524.5	2.77
20.	850.2	0.70	500.3	2.6	521.7	2.20
	Average weight=856.2 ± 17.83mg		Average weight=513.7 ± 12.24mg		Average weight=510.4 ± 13.19	

Table 4.7: Disintegration time of ACT Products in deionized water

PRODUCTS	DISINTEGRATION TIME/MINUTES
A	4.42 ± 0.25
B	3.90 ± 0.20
C	28.33 ± 2.16
D	5.83 ± 1.47
E	2.80 ± 0.75
F	3.33 ± 1.97

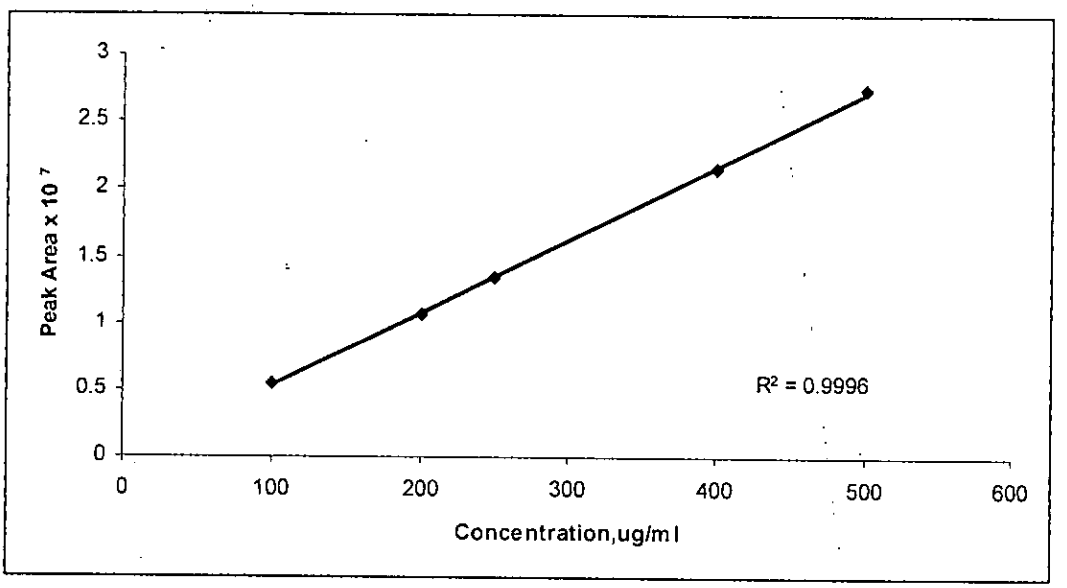


Figure 4.4a: Calibration Plot of Amodiaquine in AMQ/AT standard mixture

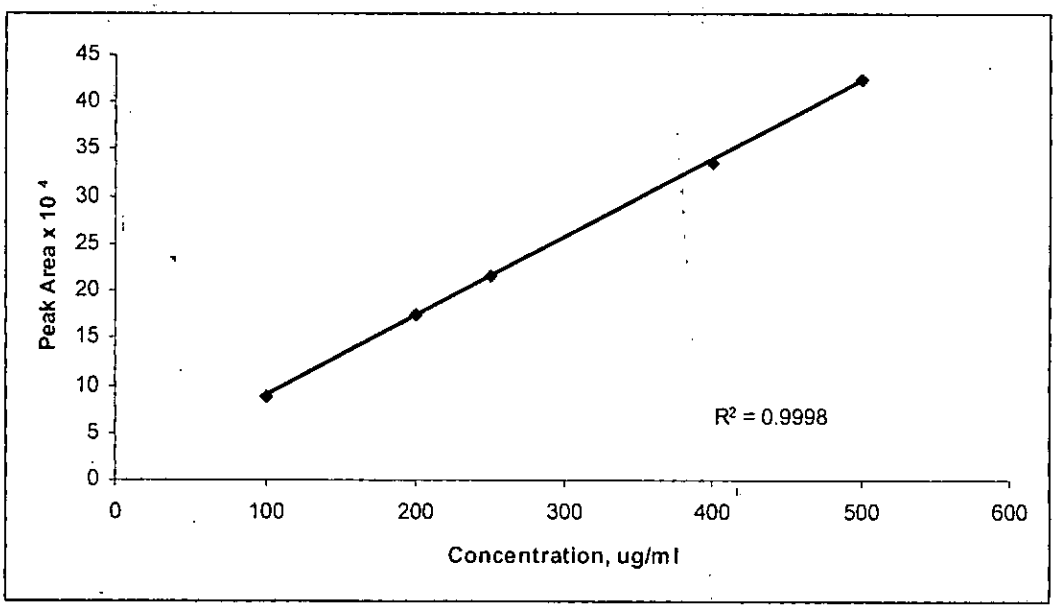


Figure 4.4b: Calibration Plot of Atesunate in AMQ/AT standard mixture

The dissolution profiles of co-formulated tablets and capsules were conducted in 0.05N HCl, 0.1N HCl and in deionized water as dissolution media. In both media, the percentage release of amodiaquine component of the tablets was found to be 94.6-97.5 & 86.9-90% at 25 minutes in deionized water and 0.1N HCl respectively for Product A, B, C and D (**Fig. 4.5a and b**). This is in conformity with official specifications of not less than 80% of formulation is released in 30 minutes (USP, 2004). Product E and F which were in-house formulations showed percentage release of 100% in less than 5 minutes.

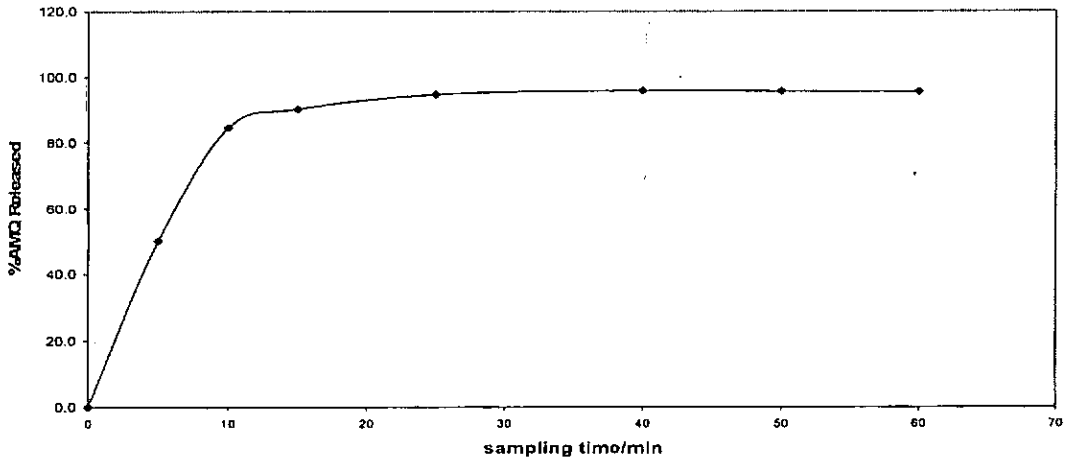


Figure 4.5a: Dissolution Profile of Amodiaquine in AMQ/AT Co-Formulated tablets in deionized water (Product A)

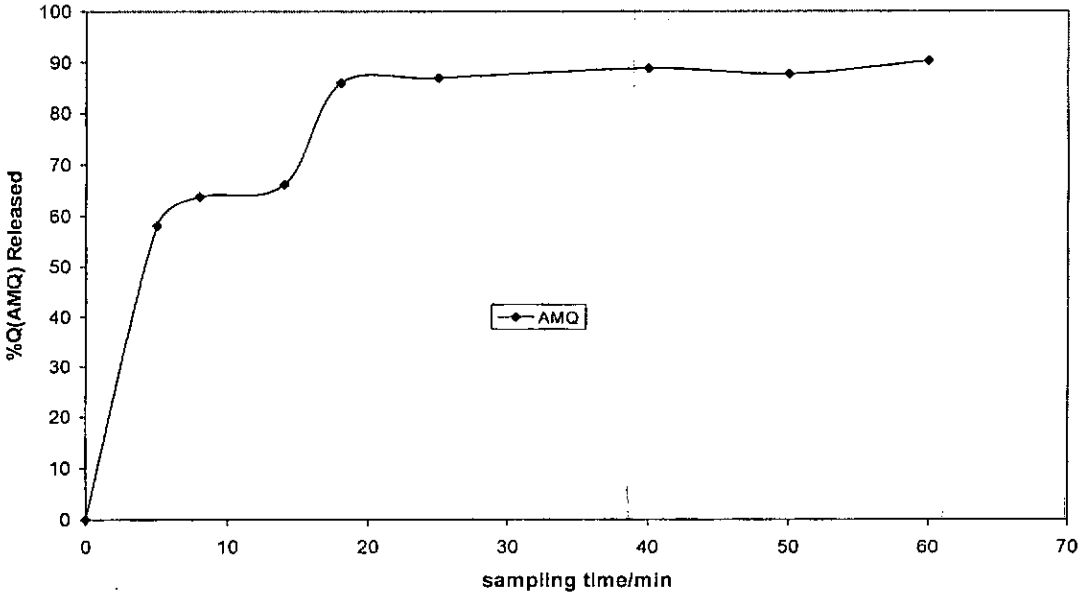


Figure 4.5b: Dissolution Profile of Amodiaquine in AMQ/AT Co-formulated tablets in 0.1N HCl (Product A)

Figure 4.6a confirms the retention time of amodiaquine at 2.1 – 2.3 as shown on the chromatogram compared to the standard. **Figure 4.6b** shows the retention time of artesunate standard at 10minutes while the same retention time is shown in the chromatogram obtained from dissolution of Co-formulated tablets (**Fig.4.6c**) in deionized water.

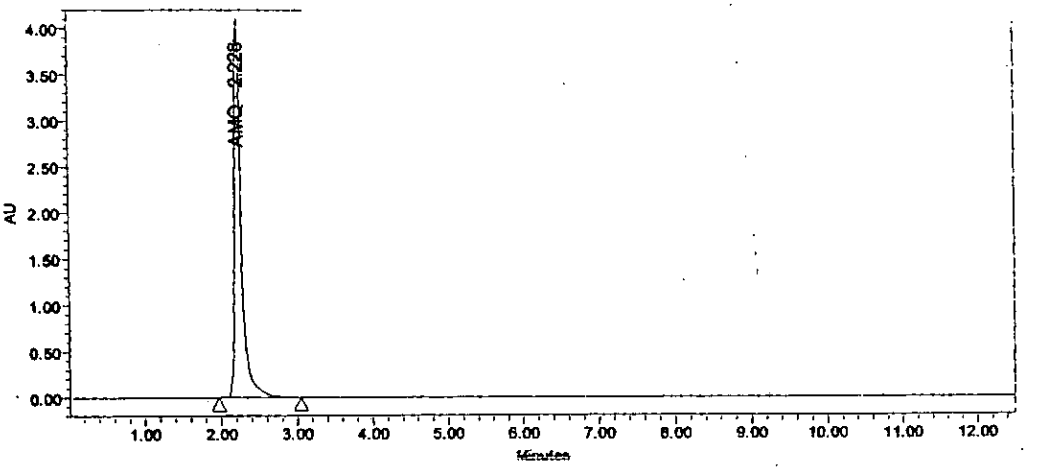


Fig. 4.6a: Chromatogram of amodiaquine in dissolution sample.

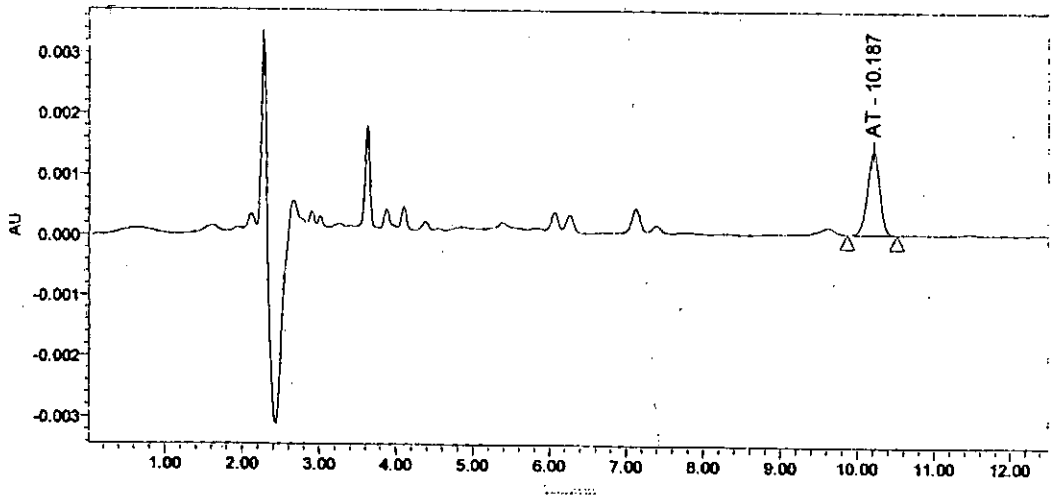


Fig. 4.6b: Chromatogram of Artesunate standard

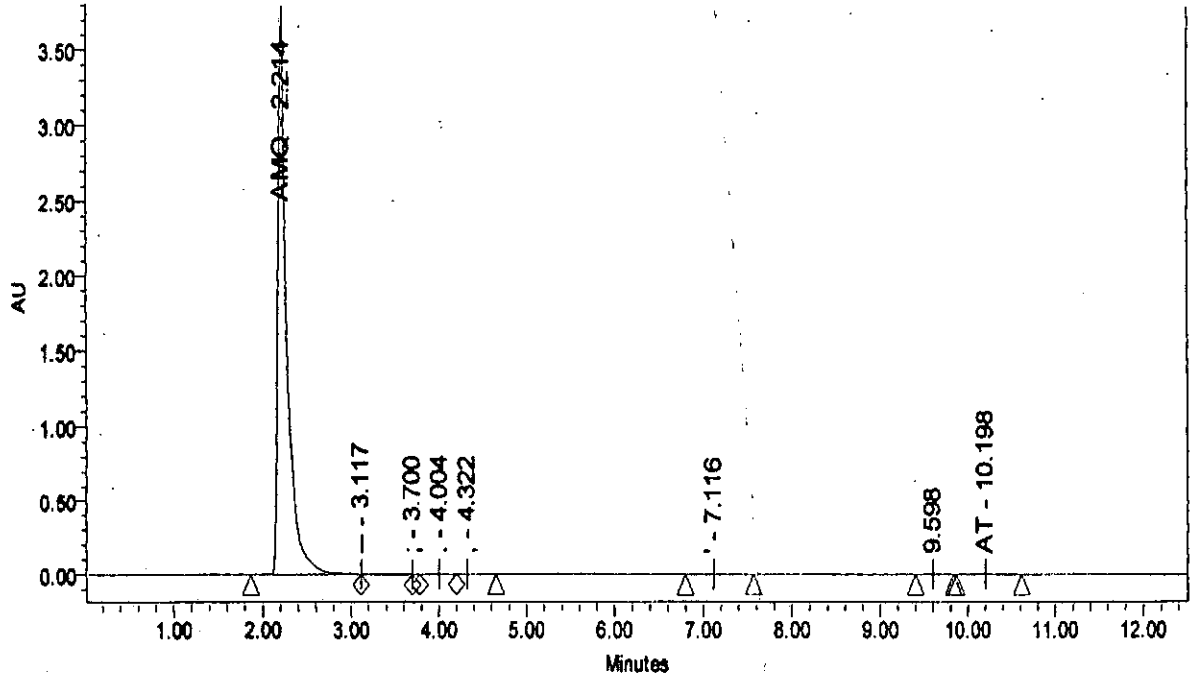


Fig. 4.6c: Chromatogram of dissolution sample of co-formulated amodiaquine and Artesunate in deionized water

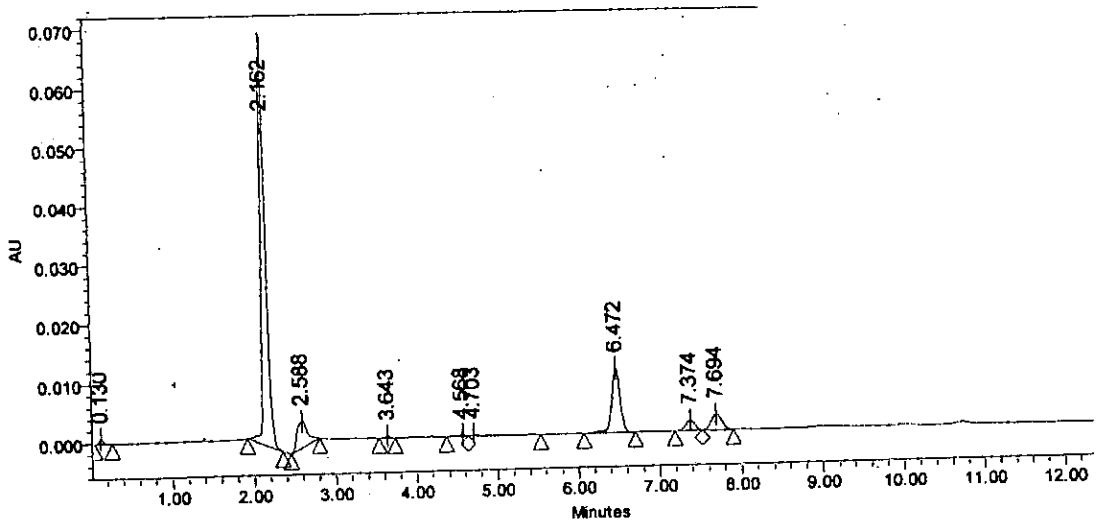


Figure 4.6d: Chromatogram of dissolution sample of co-formulated amodiaquine and Artesunate in 0.05N HCl

However, the artesunate release in the 0.05N HCl dissolution medium could not be confirmed on the chromatogram (**Fig.4.6d**) as nothing was eluted at the established retention time 10.0 min but rather various species came out at 6.4, 7.3 and 7.6 minutes as represented in **Figure 4.7**. Out of these species, only the one at 6.4 minutes showed a progressional increase in peak areas which signified an increase in the release of the specie. Similar results were obtained when Artesunate from other sources were subjected to dissolution testing using same 0.05N.

Appendix II shows more of the chromatograms of amodiaquine and artesunate elution in the formulations during assay and in dissolution studies.

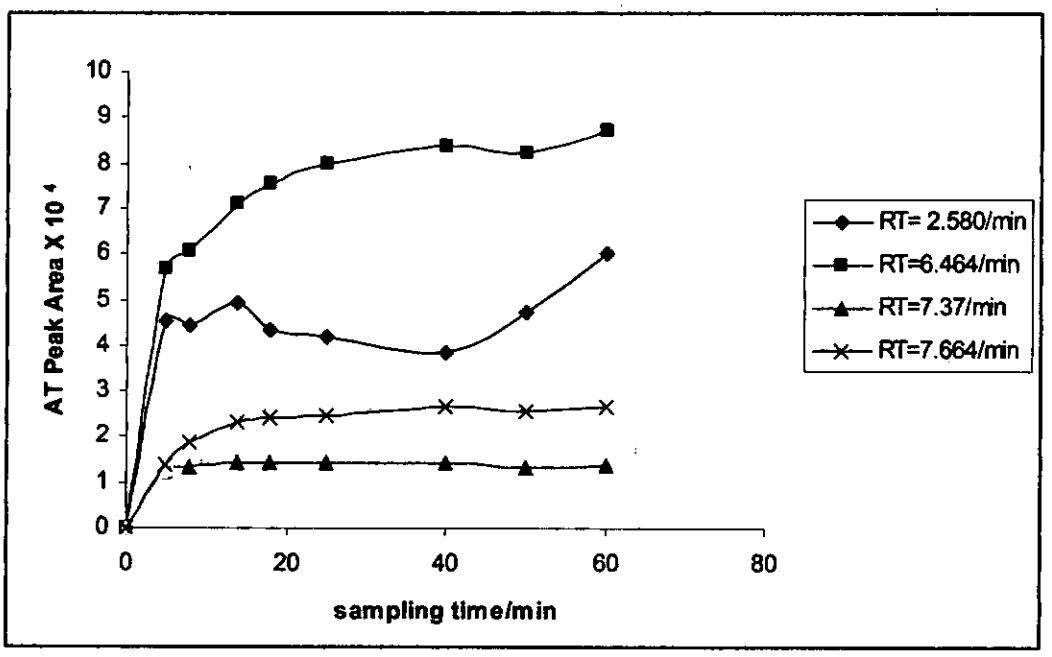


Figure 4.7: Dissolution Profile of Degraded Artesunate in 0.05N HCl (Product A)

4.5 Pharmacokinetic results of Amodiaquine in AMQ/AT formulations.

Figure 4.8 shows the plot obtained from known concentrations of amodiaquine standard spiked in blank plasma. The linearity of the calibration was also demonstrated by the good determination coefficient (r^2) 0.995 obtained from regression line of amodiaquine in plasma.

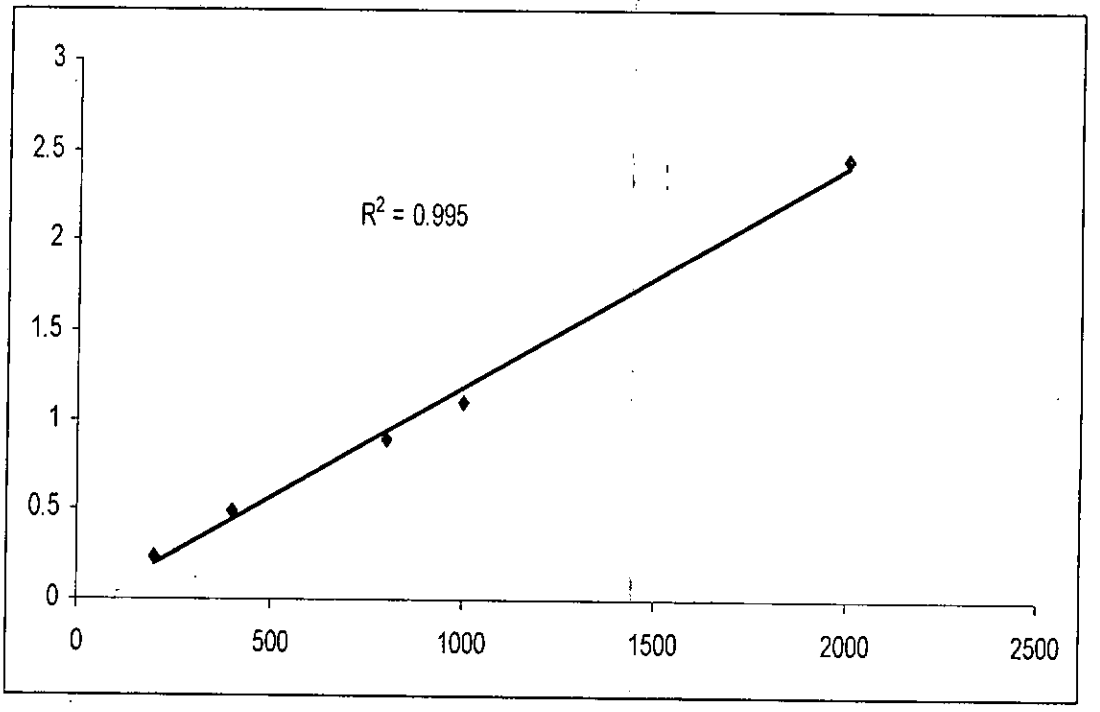


Figure 4.8: Calibration Plot of Amodiaquine standard spiked in blank plasma

Figure 4.9a-d show the chromatograms of blank plasma, amodiaquine, chloroquine spiked in pre-dose plasma and amodiaquine in one of the healthy volunteers who had received 800mg AMQ.HCl. The average retention time of chloroquine (internal standard), administered amodiaquine and extracted monodesethlyamodiaquine are 6.7, 9.7 and 11.2 minutes respectively as also shown in **Appendix II**

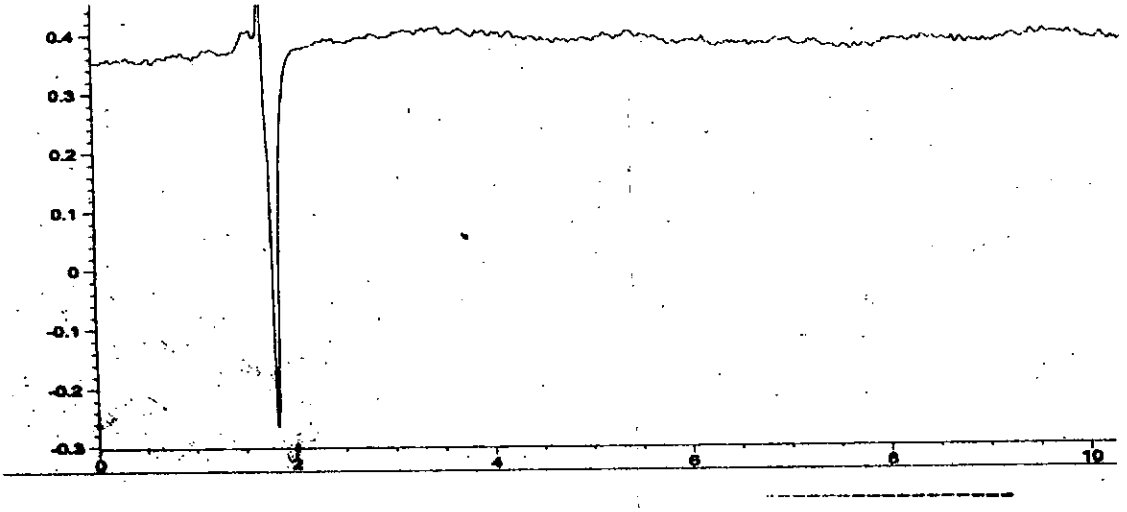


Fig. 4.9a: Chromatogram of blank plasma

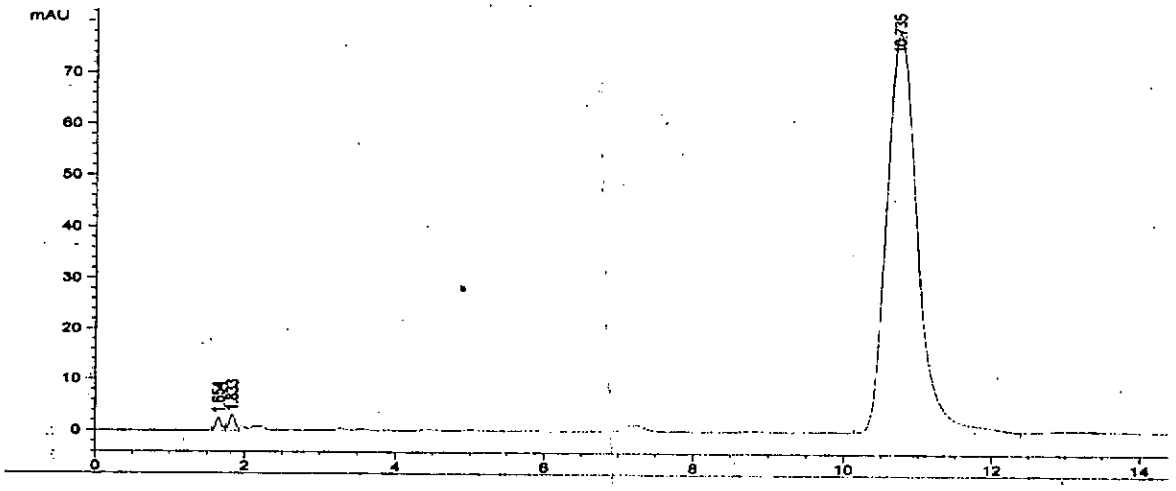


Fig. 4.9b: Chromatogram of extracted pre-dose plasma sample of a healthy volunteer spiked with amodiaquine standard alone

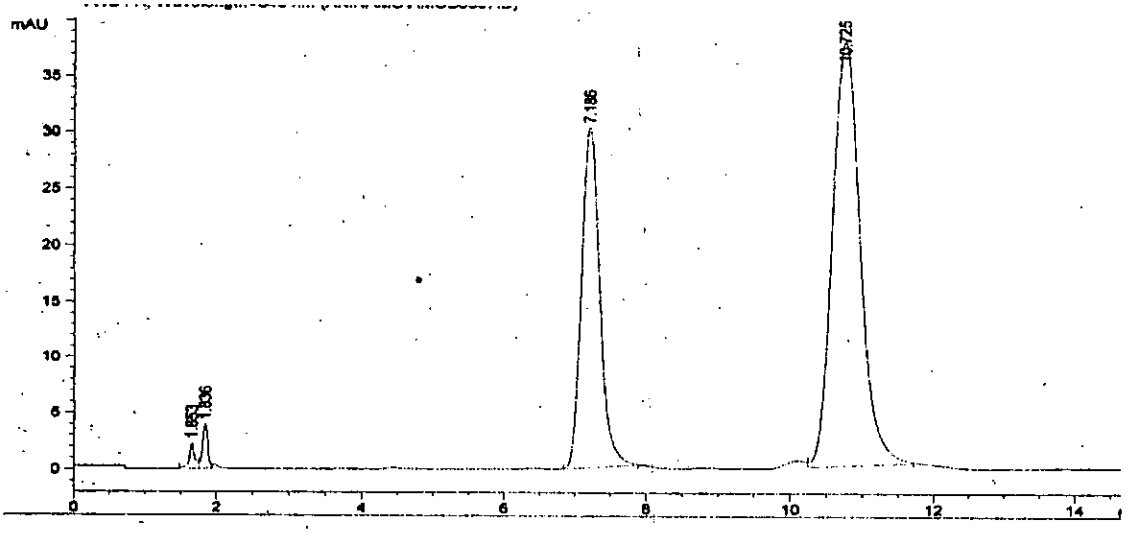


Fig. 4.9c: Chromatogram of extracted pre-dose plasma sample of a healthy volunteer spiked with amodiaquine and internal standard, chloroquine

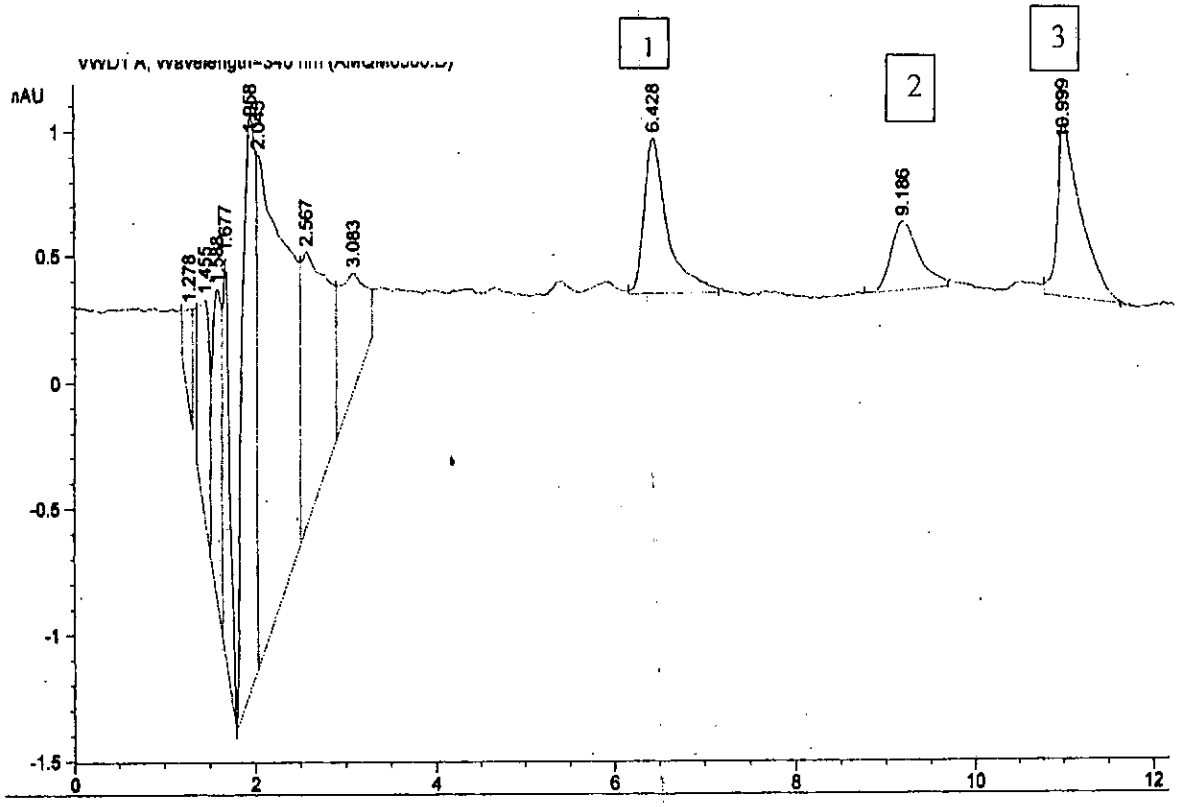


Figure 4.9d: Chromatogram of extracted plasma sample obtained from same subject obtained 2h following a 600mg single oral dose of AMQ base and spiked with 200ng of CQ. Peaks 1, CQ; 2, AMQ and 3, monodesethylAMQ

Table 4.8 shows the percentage recovery of amodiaquine in plasma.

Table 4.8 : Results of recovery studies of amodiaquine in plasma

Concentration (ng/ml)	No. of samples (n)	Percentage recovery
200	3	82.17 ± 2.20
800	3	85.13 ± 2.93

Figure 4.10a shows the plasma concentrations versus time curves of amodiaquine in six of the subjects conducted for 72 h while **figure 4.10b** represents the same study conducted for over 28 days. **Table 9a and b** show the pharmacokinetic parameters derived from the plasma concentration-time plots for 72 h and 28 days respectively.

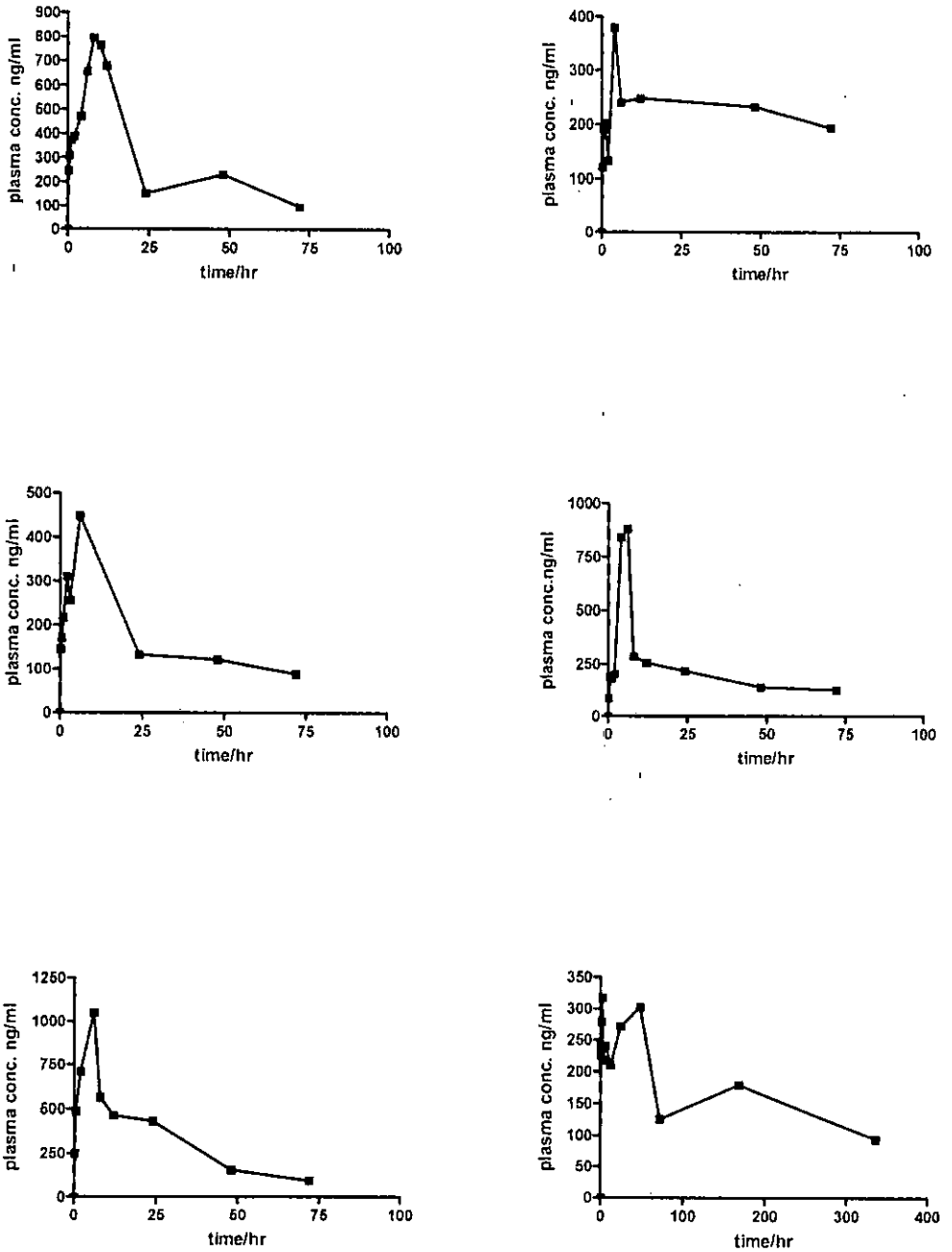


Figure 4.10a: Representative plasma concentration versus time plots of amodiaquine in 6 of the subjects sampled for 72hrs

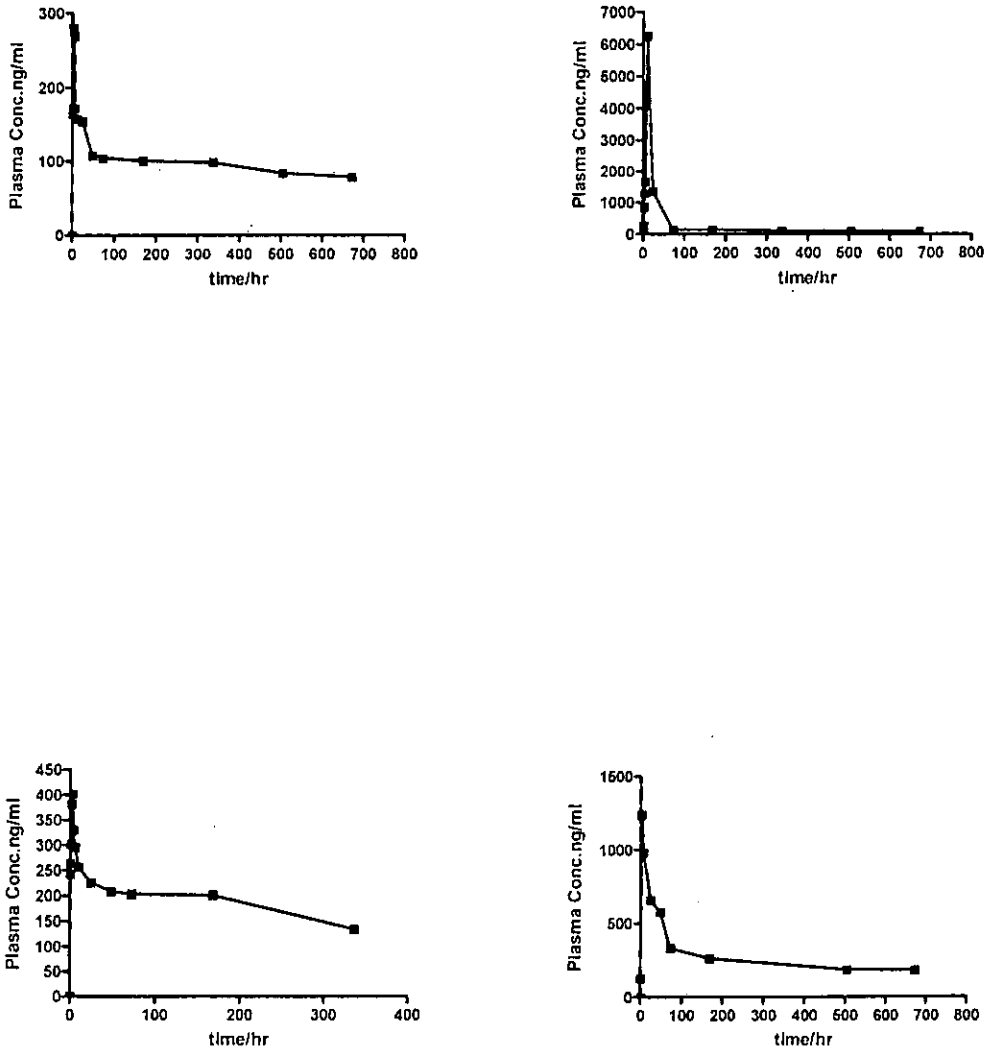


Figure 4.10b: Representative plasma concentration versus time plots amodiaquine in four of the subjects sampled for 28days

Table 4.9a: Summary of some Pharmacokinetic data of AMQ in 6 different ACTs conducted for 72hrs

Brand	Max. Pl. Conc. C_{max} (µg/ml)	Time to C_{max}, T_{max} (hr)	$t_{½ el.}$ (hr)	$AUC_{0-72\ hr}$ (ng.hr/ml)
A	(0.20 – 0.80) 0.62 ±0.21	(1.4 – 5.2) 3.24 ±1.58	(3.35 – 15.1) 11.07±3.26	(132.11 – 189.0) 167.65±19.87
B	(0.26 –1.05) 0.73 ±0.30	(2.0 – 8.0) 4.92 ±2.40	(5.2 – 18.5) 15.57±4.6	(142.4 – 206) 180.17±22.46
C	(0.35 – 0.95) 0.69 ±0.23	(1.5 –6.5) 4.35±1.84	(9.4 – 20.2) 16.38±3.73	(140.4 – 210) 184.20±25.97
D	(0.29 – 1.02) 0.74 ±0.27	(1.1 – 5.6) 3.43 ±1.84	(8.0 – 28.5) 19.51±6.38	(142.5 – 230) 185.01±31.52
E	(0.22 – 0.85) 0.56 ±0.25	(1.5 – 5.5) 3.46 ±1.47	(8.5 – 30.5) 18.41±7.24	(135.2 – 220) 186.35±30.16
F	(0.25 – 0.94) 0.63 ±0.26	(1.6 – 5.0) 3.3 ±1.30	(10 – 30.8) 20.17±8.02	(125 – 205) 146.63±25.18

Table 4.9b: Summary of some Pharmacokinetics of AMQ in 2 different ACTs conducted for 28days

Brand	Max. Pl. Conc. C_{max} (µg/ml)	Time to C_{max}, T_{max} (hr)	$t½$ el. (weeks)	$AUC_{0-28days}$ (µg.hr/ml)	$AUC_{0-\infty}$ (µg.hr/ml)
C	(0.40 – 1.24) 0.97±0.26	(2.0 – 3.0) 2.61±0.28	(2.6 –12.0) 9.02±3.34	(122.15-180.32) 157.24±23.06	(148.73-722.71) 516.01±239.55
D	(0.28 – 6.25) 3.91±1.89	(2.0 – 10.0) 6.87±2.81	(6.8 -11.0) 9.13±1.48	(120.31-175.25) 153.52±20.92	(194.81-413.11) 317.72±99.79

4.6 DENDRIMER-DNA POLYCATION FORMULATIONS (DENDRIPLEXES)

Figure 4.11a and b show evidence that an **N:P ratio** in excess of 8 and / or a DNA concentration in excess of 250μg/ml leads to non-colloidal complexes of large particle size in micrograms. The area encircled in **figure 24a** seems to follow a pattern and the particle size within the region was found to be below 500 nm. Other parts of the curves where the N:P was higher than 8 with increase in DNA gave quite particle sizes in the range of micron-size [**Appendix IIIa**].

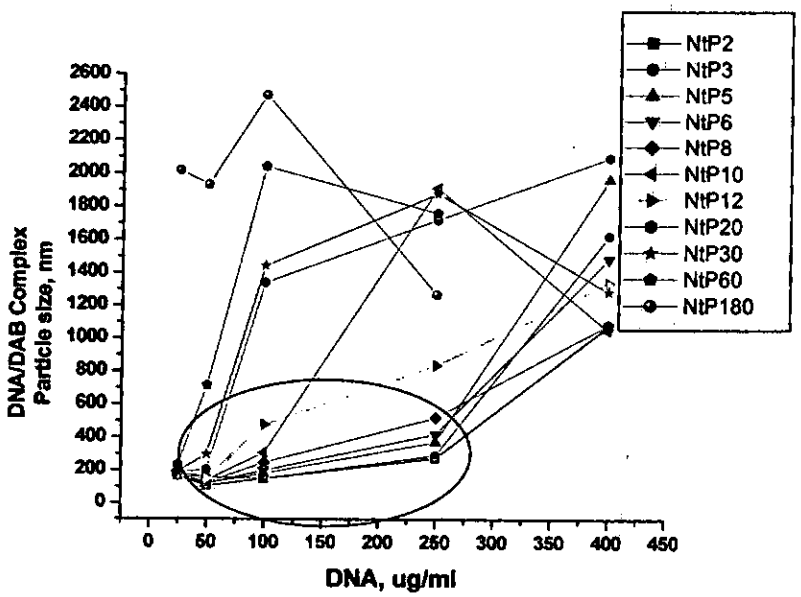


Fig. 4.11a: Effect of DNA concentration on dendriplex after an incubation time of 1 h.

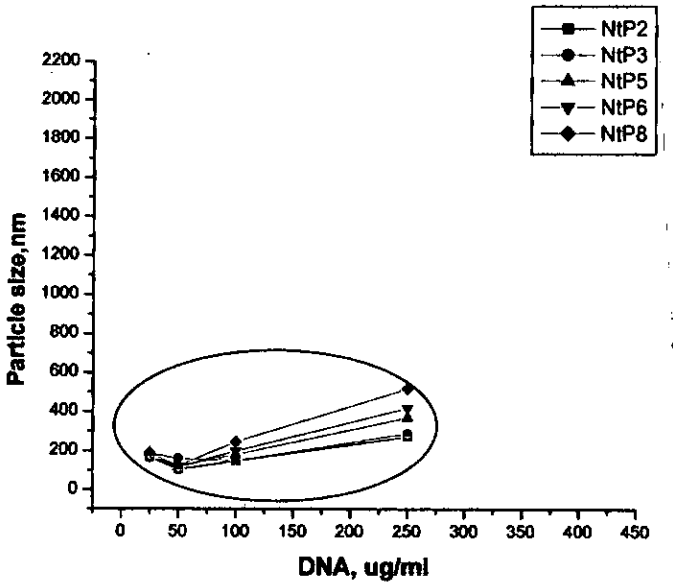


Fig. 24b: Dendriplex colloid dormain

Figure 4.12a and b show that higher levels of dendrimer to dendriplex lead to a diminished dendriplex surface charge.

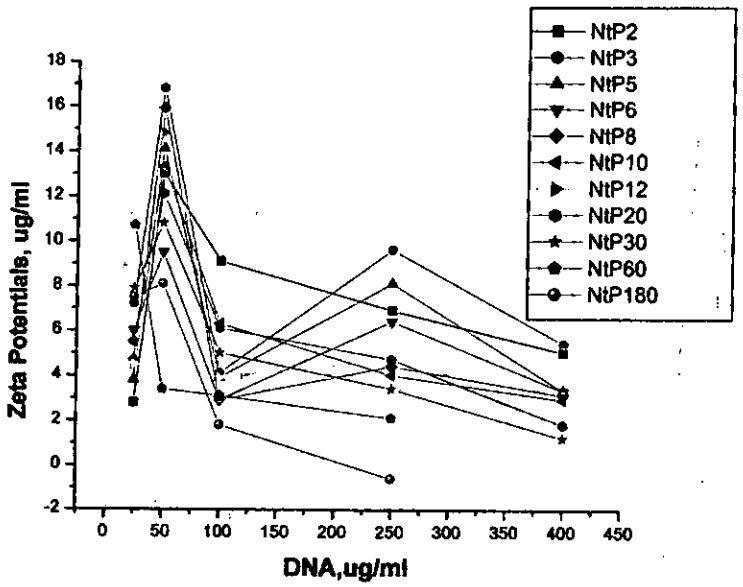


Fig. 4.12a: Effect of DNA on dendriplex zeta potential after a 1 h incubation time.

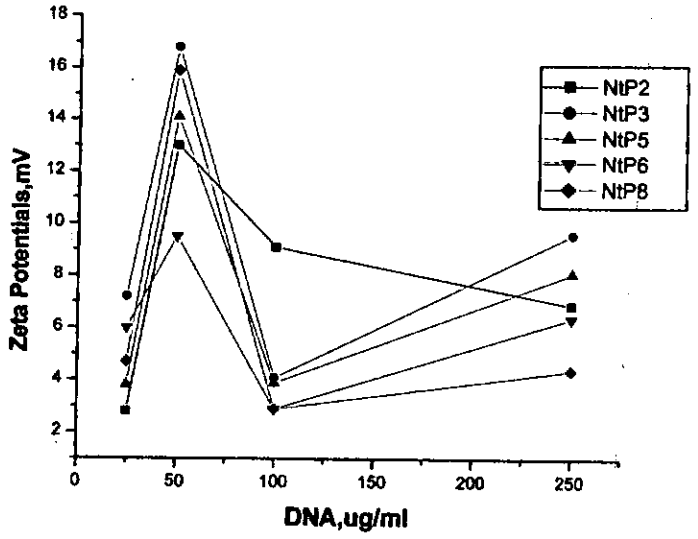


Fig. 4.12b: Dendriplex zeta potential at low N:P ratio.

a)

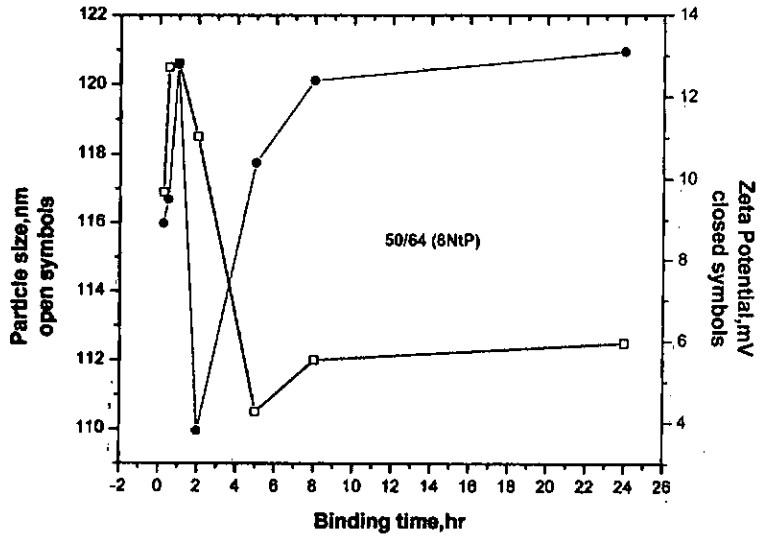


Figure 4.13a: Effect of Binding time on the particle size and zeta potential of DNA/DAB complexes using samples prepared at N:P ratio of 8 with DNA concentration of 50μg/ml

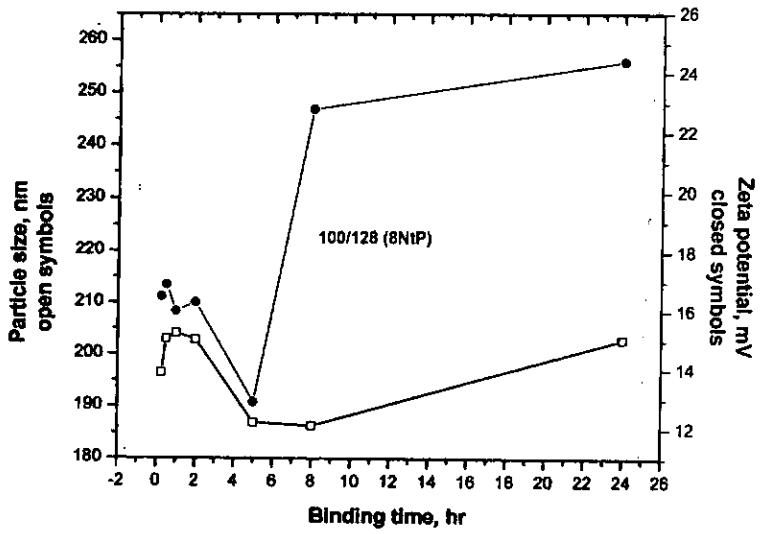


Figure 4.13b: Effect of Binding time on the particle size and zeta potential of DNA/DAB complexes using samples prepared at N:P ratio of 8 with DNA concentration of 100μg/ml

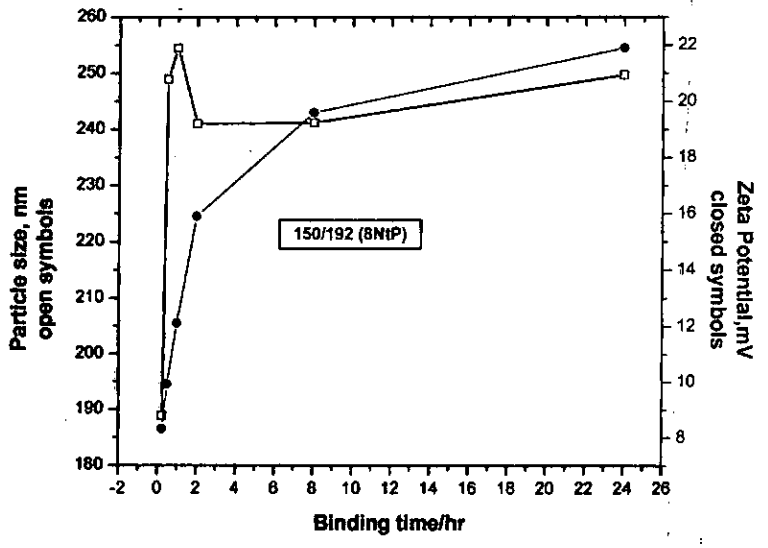


Figure 4.13c: Effect of Binding time on the particle size and zeta potential of DNA/DAB complexes using samples prepared at N:P ratio of 8 with DNA concentration of 150μg/ml

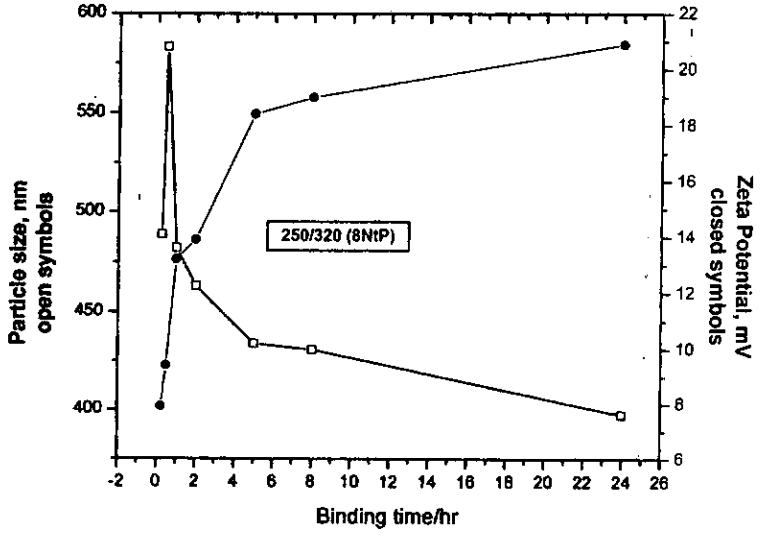


Figure 4.13d: Effect of Binding time on the particle size and zeta potential of DNA/DAB complexes using samples prepared at N:P ratio of 8 with DNA concentration of 250μg/ml

Figures 4.13a, b and d show that as the binding time of dendriplexes increases, the particle size of the complexes decreases while the zeta potential increases. However **Figure 4.13c** shows no appreciable variations in particle size and zeta potential.

The resulting particle size and shape of the DAB/DAB complexes examined using transmission electron microscope (**TEM**) **(Fig.4.14a, b and c)** were found to be below 200nm nanoscale for formulations containing 50-250ug/ml at N:P8 while **Figure 4.14d** which is sample of higher N:P 60 above the selected field gave larger size in the range of microscale.

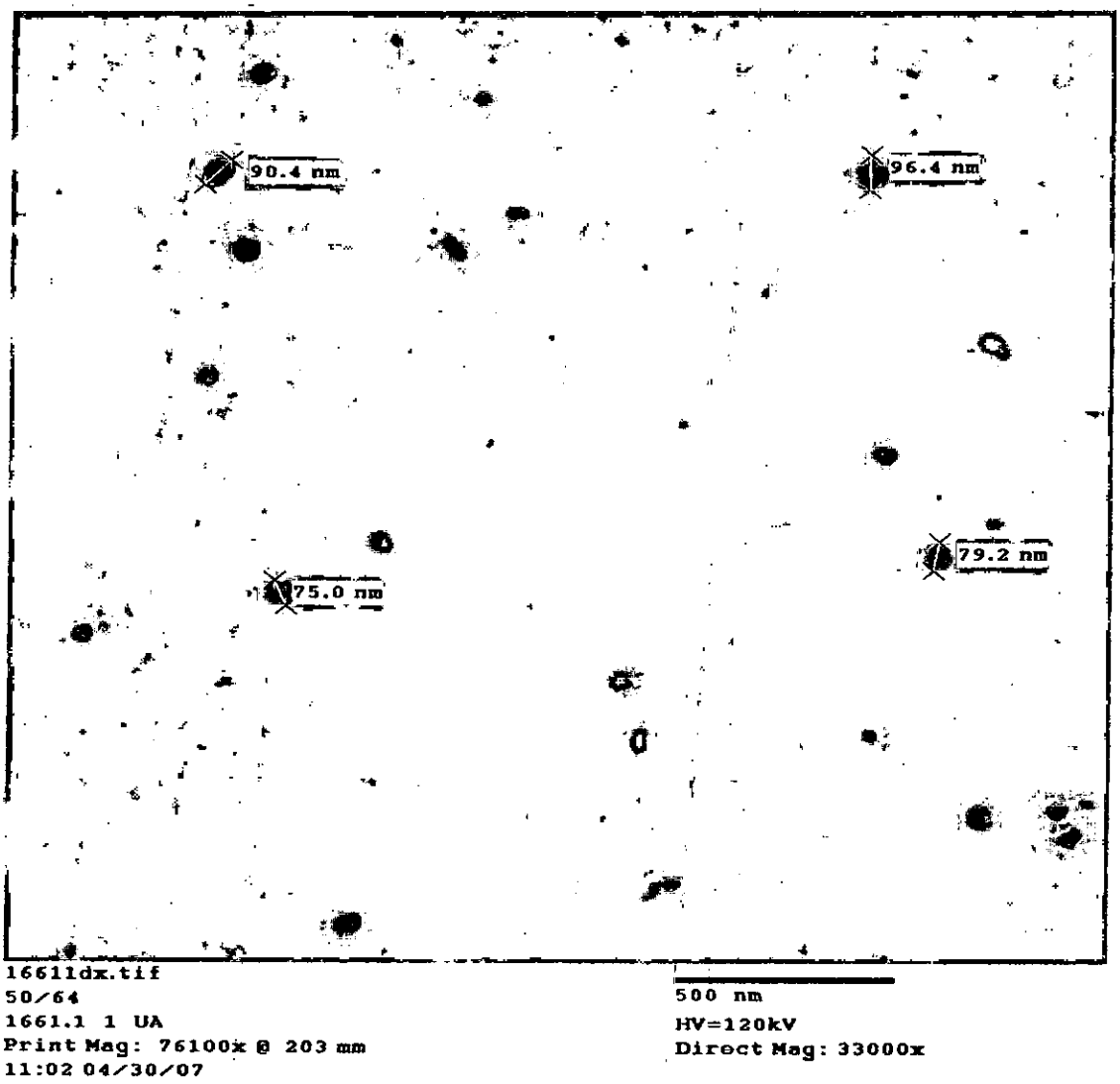


Figure 4.14a: Negative stained Transmission Electron Micrograph of a DNA/DAB16 formulation (DNA concentration = 50µg/ml, DAB16 concentration = 64µg/ml, N:P ratio = 8). Sample was imaged 5h after preparation and presented as a clear liquid.

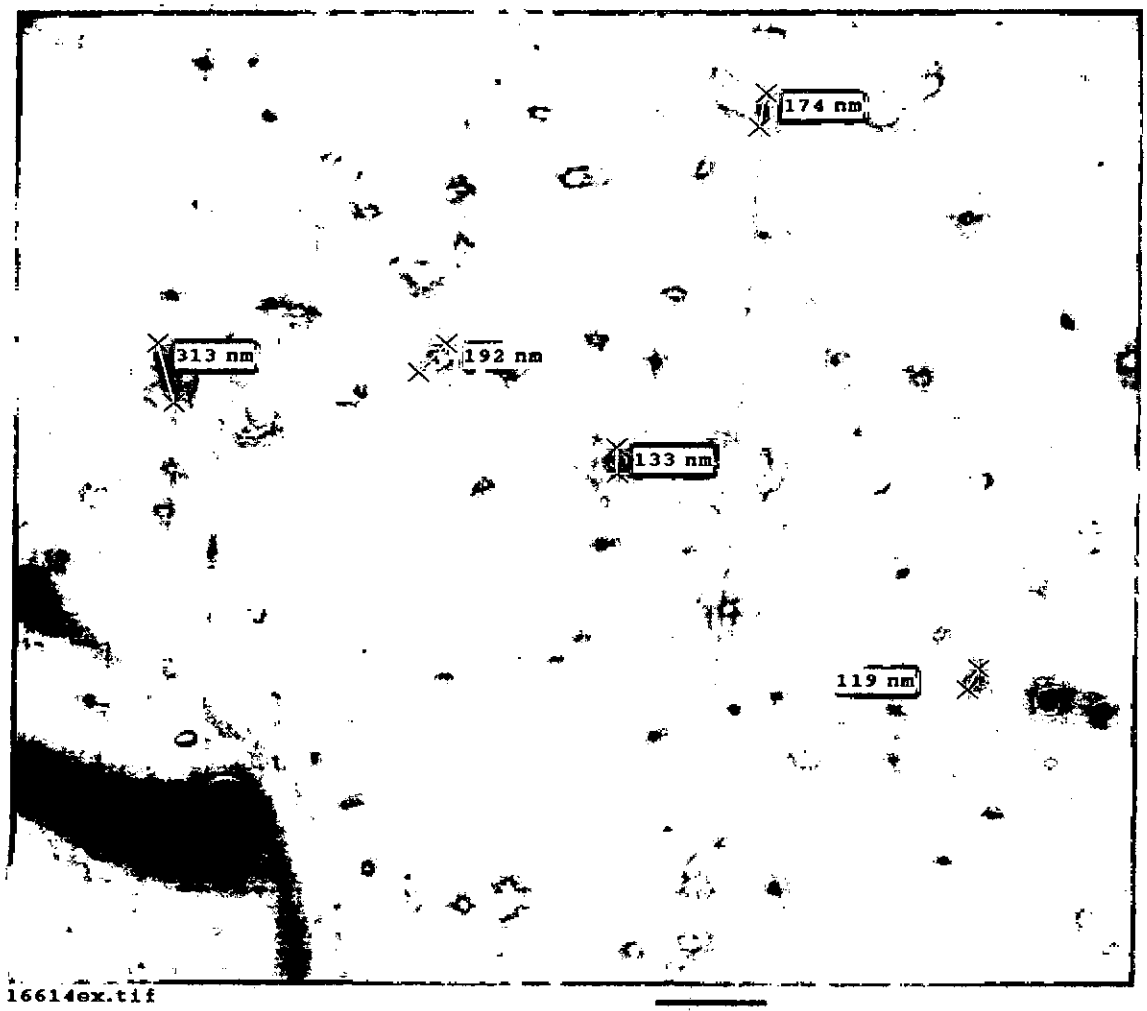


Figure 4.14b: Negative stained Transmission Electron Micrograph of a DNA/DAB16 formulation (DNA concentration = 100μg/ml, DAB16 concentration = 128μg/ml, N:P ratio = 8). Sample was imaged 5h after preparation and presented as a clear liquid.

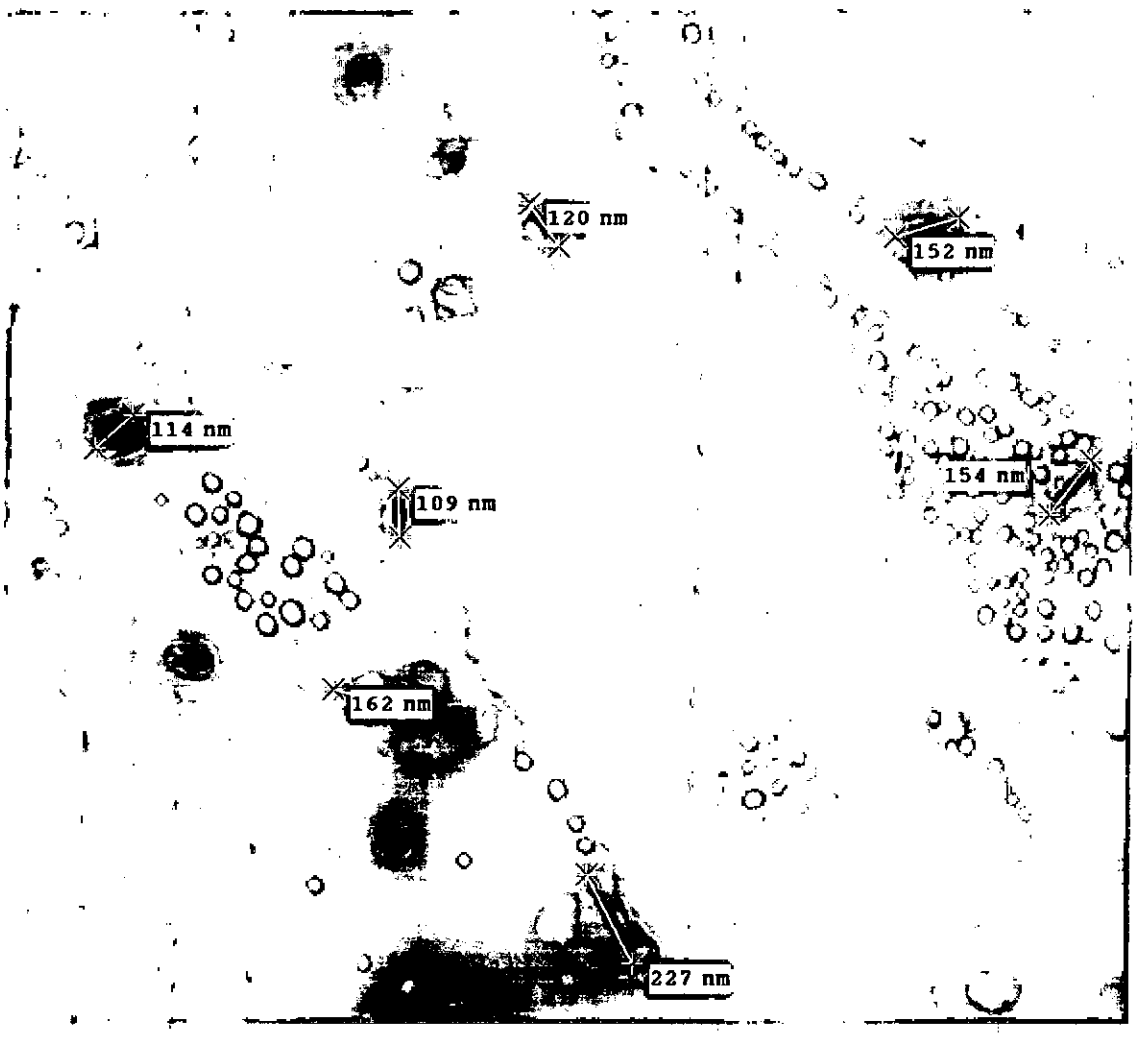


Figure 4.14c: Negative stained Transmission Electron Micrograph of a DNA/DAB16 formulation (DNA concentration = 250µg/ml, DAB16 concentration = 320µg/ml, N:P ratio = 8). Sample was imaged 5h after preparation and presented as a clear liquid.

Figure 4.14d:Negative stained Transmission Electron Micrograph of a DNA/DAB16 formulation (DNA concentration = 250µg/ml, DAB16 concentration = 2400µg/ml, N:P ratio = 60). Sample was imaged 5h after preparation and presented as a slightly cloudy solution.

Figure 4.15 shows the opacity of some of the formulations.

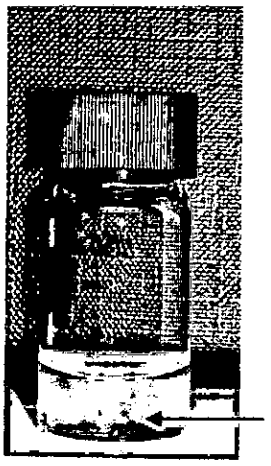


Figure 4.15a: Photograph of a dendriplex dispersion (DNA concentration = 500μg/ml, DAB 16 concentration = 160μg/ml, N:P ratio = 2). Formulation was imaged 24 h after preparation. DAB 16 was added to DNA solution. A precipitate is clearly visible in the dispersion (arrowed).

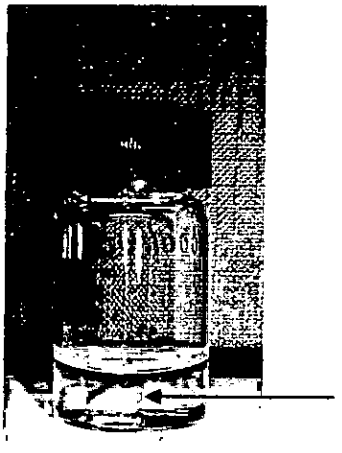


Figure 4.15b: Photograph of a dendriplex dispersion (DNA concentration = 1000μg/ml, DAB 16 concentration = 320μg/ml, N:P ratio = 2). Formulation was imaged 24 h after preparation. DNA was added to DAB 16 solutions. A precipitate is clearly visible in the dispersion (arrowed).

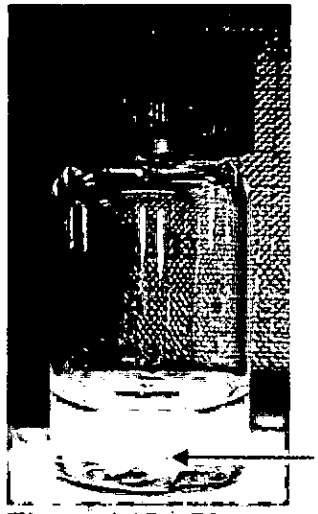


Figure 4.15c: Photograph of a dendriplex dispersion (DNA concentration = 1000μg/ml, DAB 16 concentration = 320μg/ml, N:P ratio = 2). Formulation was imaged 24 h after preparation. DAB 16 was added to DNA solution. A precipitate is clearly visible in the dispersion (arrowed).

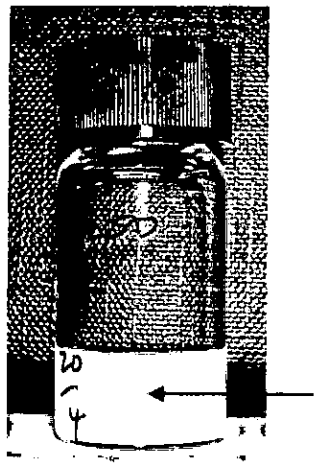


Figure 4.15d: Photograph of a dendriplex dispersion (DNA concentration = 250μg/ml, DAB 16 concentration = 7200μg/ml, N:P ratio = 180). Formulation was imaged 24 h after preparation. DAB 16 was added to DNA solution. A cloudy solution is clearly visible in the dispersion (arrowed).

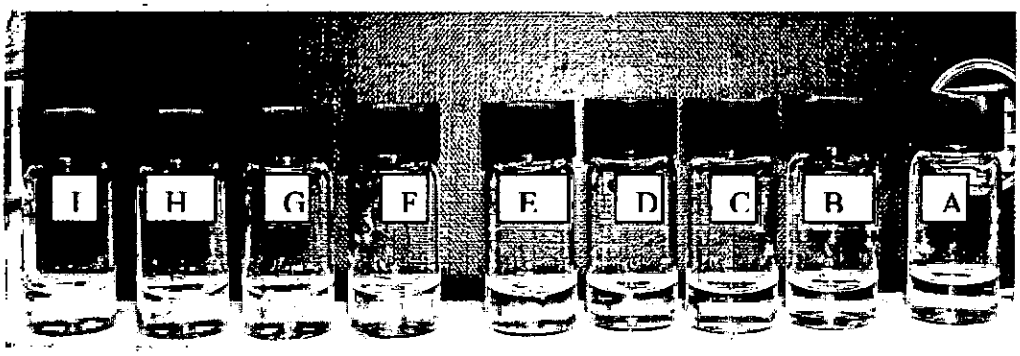


Figure 4.15e: Photograph of dendriplex dispersion (DNA concentration = 400μg/ml, DAB 16 concentration A = 128μg/ml, B=192 μg/ml, C=320 μg/ml, D=384 μg/ml, E=512 μg/ml, F=640 μg/ml, G=768 μg/ml, H=1280 μg/ml and I=1920 μg/ml at 2,3,5,6,8,10,12,20 and 30 N:P ratio respectively. Formulation A to E formed clear dispersion while cloudy solutions are visualised on F to I

CHAPTER FIVE

5.0 DISCUSSION

5.1 Validation of UV Spectrophotometry and HPLC methods.

The non-specificity and selectivity of UV spectrophotometric method to separate and resolve the drugs (CQ, CP, HF & SP) preclude the use of the analytical method for this study. Therefore, the need to develop a better analytical method that will be more specific, selective and with a good precision becomes very expedient.

The HPLC method reported here involved a less tedious procedure. Initially solvent systems such as methanol : water (50:50); methanol: phosphate buffer (1:4) and Acetonitrile : phosphate buffer (1 : 4) were used. There was no clear resolution as some of the peaks overlapped. When ion-pairing agent, tetrabutylammonium bromide was included with acetonitrile solvent and the pH of the solvent mixture was adjusted to 2.0, peak resolution was greatly improved. Chloroquine was well resolved from the ciprofloxacin and the internal standard. Initially, the total elution time for the drugs at trial stage was 20 minutes (CQ 1.25;CP 7.96 and SP 19.16min) [**Appendix 1**] when mobile phase ratio, acetonitrile: tetrabutylammonium bromide (TBAB) (1:9) was used. This run time was not economical and time wasting considering the volume of mobile phase consumed and time factor per sample analysis. Therefore, optimum separation in a shorter elution time less than 5 minute was achieved by adjusting the acetonitrile:

TBAB (1:4) [**Appendix 1**]. Wavelengths such as 274, 290 and 340nm were also tried for the elution of the three components but wavelength 254nm seems to be the best in terms of response and sensitivity. Scanning of mixture of the three standards also showed that all components have intense UV absorption at 254nm as shown in **figure 4.1d.** The method has good linearity, precision and accuracy across the concentration range used. There was no interference from impurities and degradative materials of the two drugs.

The method was applied to the assay of samples generated from *in-vitro* interaction study of effect of chloroquine/halofantrine on the dissolution profile of ciprofloxacin tablets.

5.2 The effect of Chloroquine and Halofantrine on the dissolution profile of Ciprofloxacin.

The reduction of ciprofloxacin release in the presence of varying concentrations of chloroquine phosphate was found to be significant. Generally, the release profile appears to be more stable in acid than in water, while the quantity released tends to be more in water than in acid. It is not clear if the observed reduction in the amount of CP released in the presence of the substrates is entirely dependent in the changes in pH associated with the presence of the substrates in solution. It is possible that availability of the substrates in solution as cations may play a role in possible complexation mechanisms, as has been reported (Mizuki *et al.*, 1996). It has also been shown that the presence of cations decreases the Q_{max} of CP

(Rodriguez *et al.,* 1999). Therefore, there is possibility that the substrate cation might interact with the CP anion and/or complex with the zwitterion through some sort of hydrogen-bridge.

The use of potentiometric titration to investigate complexation reaction has also been reported (*Martin et al.,* 1993). The results of similar study conducted on CP-HF mixture whereby the difference in pH for a given quantity of base added in potentiometric titration indicates the occurrence of a complex. Similar results have been obtained with other zwitterions (e.g. copper-glycine and NN diacetylethylenediamine diacetic acid) which have been studied for its complexing action with copper and calcium ions. It is not impossible that the reduction of ciprofloxacin release by chloroquine and halofantrine may have followed the same mechanism since they can also generate cationic species in solutions.

5.3 Dissolution profiles of Amodiaquine and Artesunate in ACTs.

Instability of artesunate in 0.05N HCl which led to degredation of artesunate confirms the work done and presented in WHO workshop (Pogany, 2005) on the decomposition of artesunate in aqueous solution where 0.1N HCl resulted to 74% decomposition of artesunate. The specie eluted at 6.4 min (**Fig.4.7**) is presumed to be one of the degradative products of decomposition of artesunate by the acid.

The present results indicate rather that artesunate though released in both acidic and neutral media is structurally more stable and intact in deionized water. The artesunic acid may further lower the pH of the medium which in turn may augment auto-induced degradation of the endoperoxide linkage in the sesquiterpene lactone ring moiety.

5.4 Pharmacokinetics of amodiaquine in amodiaquine-artesunate formulations

Although the use of amodiaquine for treatment has during the last 17 years been limited after the reports of fatal adverse drug reactions in the mid-1980s (Blessborn et al., 2006). Lately, several clinical trials have demonstrated the safety and efficacy of AMQ when used alone and in combination (Winstanley *et al.,* 1987; Menard *et al.,* 2005; Agnamey *et al.,* 2006). After oral administration of co-formulated amodiaquine/artesunate, the loading dose of AMQ for each brand was 600mg AMQ base (equivalent to 800mg AMQ. HCl) to 10 healthy subjects, amodiaquine underwent rapid change to desethylamodiaquine (**Fig. 4.9d and Appendix II**). Evaluation of pharmacokinetic data of amodiaquine in the presence of artesunate gave data in **Table 4.9a and b**. These values were compared with pharmacokinetic parameters of amodiaquine monotherapy obtained by other scientists (Winstanley *et al.,* 1987; Menard *et al.,* 2005; Agnamey *et al.,* 2006).

The peak plasma concentrations got from the first study conducted over 72hrs varied from 0.22 to 1.05μg/ml at T_{max} 1.1 to 8.0 h. The quantum $t_{1/2}$ of elimination ranges from 3.35 to 30.8 h while the quantum AUC $_{0\text{-}72hr}$ deviates from 125 to 230μg.hr/ml **(fig. 4.10a and b)**. Comparing these data with that of Winstanley *et al,* (1987), the $AUC_{0\text{-}72hr}$ obtained from this study agreed with $AUC_{0\text{-}96hr}$ 154 ± 38ng.hr/ml, while the apparent terminal $t_{i/2}$ of elimination 5.2 ± 1.7 h was in contrast with our results.

Krishna and White, (1996) in their work which was conducted for 28 days obtained the terminal half life of between 1-3weeks while an average of 9 weeks (2.6-12 weeks) was obtained in our study conducted within the same 28 days **(Table 4.8b and fig. 4.10b)**. Assessment of subject chromatograms showed that the concentration of amodiaquine declined rapidly while desethylamodiaquine metabolite increased appreciably within 6 hours. This confirms the fast metabolizing feature of amodiaquine (a pro-drug) in plasma. Recent comparative *in-vitro* studies with isolates of *P. falciparum* showed a significant correlation between the activities of amodiaquine and monodesethylamodiaquine (DesAMQ) that indicates similarities in the way they act against the parasite (Gerstner *et al.,* 2003). It has been posited that the mean plasma concentration of DesAMQ is about 6 to 7 fold higher than the parent drug. The desethylamodiaquine (amodiaquine metabolite) could not be evaluated in this study because of the

unavailability of its reference standard. The HPCLC method gave good recovery for amodiaquine (>80%). The HPLC chromatograms show that with products A, B & C therapeutic concentrations of amodiaquine were achieved within 1 hr of drug administration.

5.5 DNA-Dendrimer16 Complex (Dendriplexes) formulations.

Considerable research efforts have been devoted to establishing the causes of poor *in-vivo* gene transfection and agglomeration of DNA-polycation complexes. It has been posited that aggregation of plasmid/lipid complexes is a function of cationic lipid species, DNA/cationic lipid ratio, DNA and lipid concentrations, shearing force, temperature, solution viscosity, time and mixing procedures (Mahato,*et al.*, 1997). Manipulation of these factors might give a lead way to what could be responsible for DNA/DAB aggregate formation. It is a noted fact that electrically neutral DNA/Polycation complex solution is not only difficult to prepare but it also prone to particle aggregation. It is also a fact that suspension of complex particles only tend to be colloidally stable if the particles are charged, that is, the cationic carrier will be present in excess to create particles which repel one another (Mislick and Baldeschwieler, 1996). Hence, the positive charge on the complex is

also important because it facilitates cell adsorption and mediate efficient endosomal uptake into cells. It then becomes imperative that the formulations or the dendriplexes must carry excess positive charge.

The results of this study then demonstrated that the particle size of the product of electrostatic interaction of DNA with polycation-dendrimer (DAB) varies according to ratio of nitrogen to phosphate of DAB and DNA respectively. An N:P higher than 10 was found not to be suitable for formulation of DNA-DAB complexes (dendriplexes) of nanosize.

The resulting particle size and shape of the dendriplex formulations examined using transmission electron microscope (TEM) (**Fig. 4.14a, b, c and Appendix IIIb**) was found to be below 200 nm nanoscale. **Figure 4.14d** which shows the micrograph of higher N:P (60) above the selected field resulted in a larger particle size in micro-range. The bigger size is an indication of aggregation of particles as shown in the TEM image.

It can be inferred that as the zeta potential (+ve) of the dendriplexes increases, the particle size decreases **(Figure 4.13)**. This translates into formation of more compact particles that could permeate cell membranes better than the bigger particle size of other formulations above 400 μg/ml DNA at N:P higher than 10.

Various incubation or binding time has been postulated by several authors ranging from 5- 30 minutes [Anna, *et al.,* 1997; Zinselmeyer, *et al*, 2002; Andrei, *et al.,*

2003 and Chad, *et al.*,2004]. With this kinetic study of complexes over 24hrs, it becomes clearer that an optimum time for complex formation needs to be selected. It is therefore suggested based on this results that a period between 5 and 8 hour could be appropriate for efficient dendriplexes binding.

Order of adding DAB to DNA and the mixing method arrived at; in this work should be maintained (**section 3.2.6.2**) in order to avoid instant precipitation/aggregation of DNA.

CHAPTER SIX

6.0 CONCLUSION

The maximum quantity (Q_{max}) of ciprofloxacin released in dissolution media (deionized and 0.1N HCl) was inhibited by the presence of chloroquine and halofantrine.

This suggests that there is a potential for interaction of CP and CQ at the absorption site, which may lead to reduced availability of CP in the presence of any of the substrates. This has an implication in therapeutic outcomes particularly in a malaria endemic environment, such as ours, where both drugs are frequently co-administered. It is advised that when there is a need to use these drugs together, the doses have to be staggered.

The result of instability of artesunate in acid (0.05 and 0.1N) [**Appendix II**] suggests that the fragile dioxygen bridge or endoperoxide linkage may not be stable under acidic conditions and/or the sodium salt of artesunic acid may itself be unstable under harsh acidic conditions. Also the HPLC modified methods for *in-vitro* assay of artesunate and amodiaquine including biological assay of amodiaquine have been found to be satisfactory.

Finally, it was also discovered that agglomeration of DNA-polycation complexes was as a result of inappropriate selection of nitrogen to phosphate ratio of dendritic polymers/DNA which might likely explain the mechanism for poor gene/drug *in-vivo* transfection/delivery. Therefore, stable colloids of consistent particle size and shape are formed if the binding time, mixing process and zeta potentials are skillfully controlled and monitored.

6.1 CONTRIBUTIONS TO KNOWLEDGE

This study demonstrates the following:

1. The HPLC method developed in aqueous solution for concomitant elution of chloroquine, ciprofloxacin, halofantrine and sparfloxacin is being reported for the first time.
2. Co-administration of ciprofloxacin with chloroquine and halofantrine leads to inhibition of ciprofloxacin release in-vitro.
3. The instability effect of acid on artesunate formulated alone is also found to be similar to artesunate co-formulated with amodiaquine.

4. It has also been established that the release, absorption and disposition of co-formulated ACTs (Diasunate®, Macsunate®, Erimal®, Dart®) in Nigerian markets including the in-house formulation compared favourably well to the results obtained by other authors.

5. The equilibration of dendrimer/DNA complexes (Dendriplexes) is established between 5 and 8 h; and stable formulation of consistent particle size and zeta potential are achieved between 2 to 8 N:P with DNA concentration of 50 - 250μg/ml.

6. It is also the first time to establish that particle size and charge potential of dendriplexes are time dependent.

6.2 PROPOSAL FOR FURTHER STUDIES

- A study of the ultra-structure of plasmodium parasite membrane.
- Determination of dendrimer incorporating ACTs capable of penetrating such parasite membrane.

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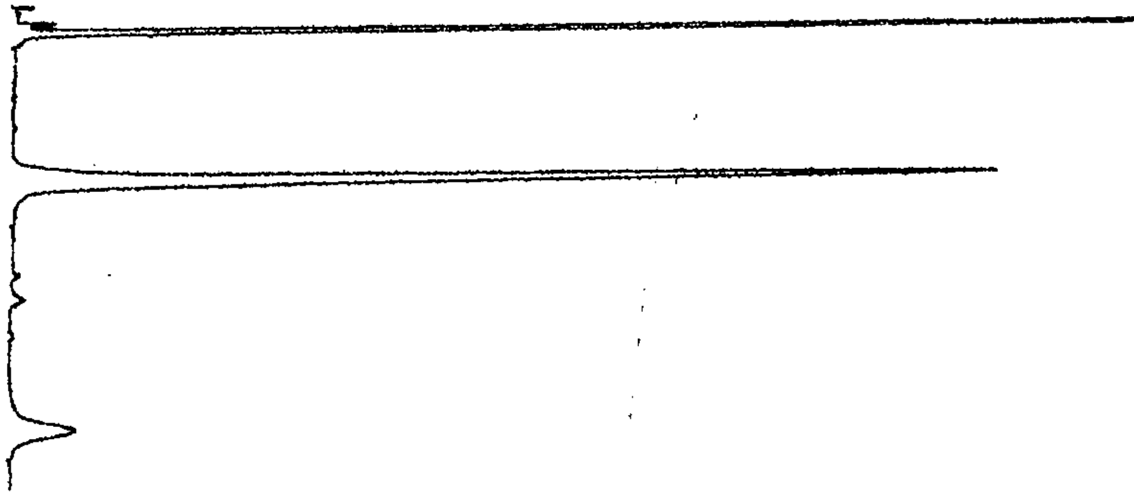
APPENDIX I: Shows HPLC chromatograms of Chloroquine, Ciprofloxacin and Sparfloxacin (internal standard) generated at the trial, validation and application stages of method development.

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A INJECT 31-01-03 13:56:42 STORED TO BIN # 11



DATA SAVED TO BIN # 11

CP-CQ INTERACTION STUDIES 31-01-03 13:57:42 CH "A" PS 1.

FILE 1. METHOD 0. RUN 7 INDEX 7 BIN 11

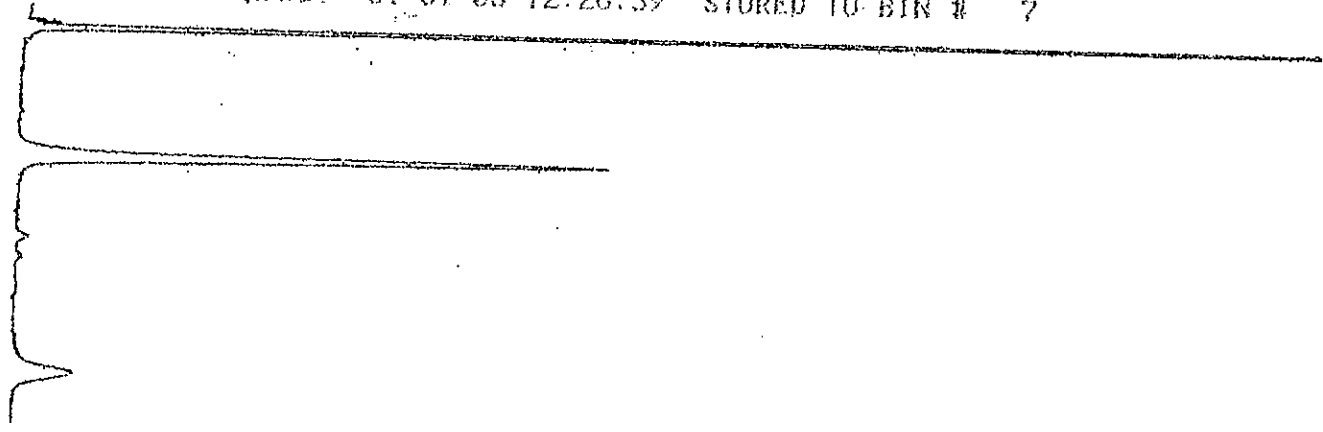
PEAK#	AREA%	RT	AREA BC
1	52.494	1.25	1617565 03
2	40.773	7.96	1256718 02
3	6.533	13.16	200668 03
TOTAL	100		[illegible]

TRIAL

1	81.476	7.89	514589 @3
2	18.524	19.04	116984 @8
TOTAL	100.		631523

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A - INJECT 31-01-03 12:26:59 STORED TO BIN # 7



DATA SAVED TO BIN # 7

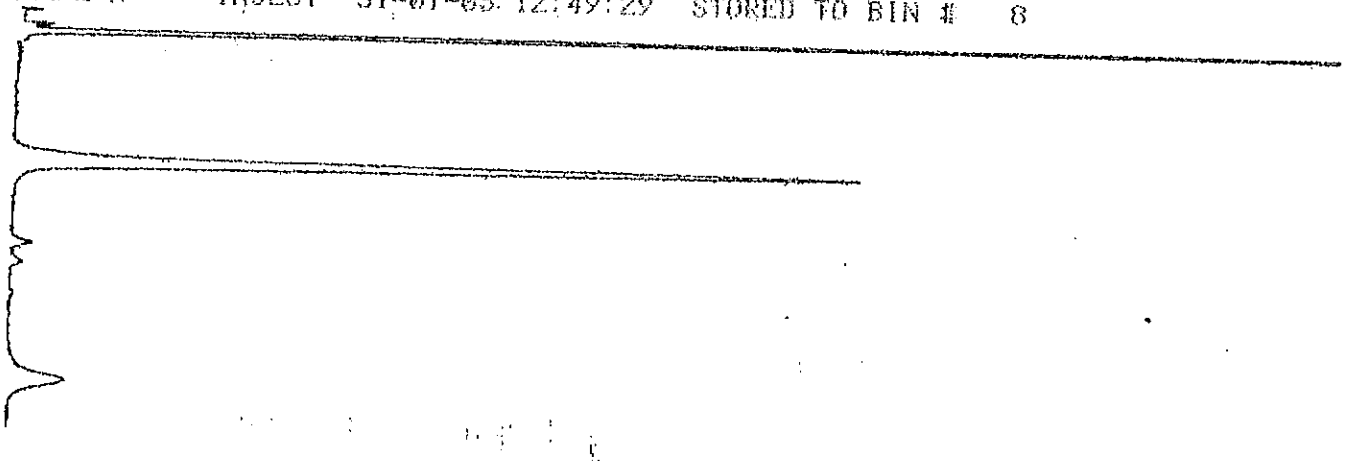
CP-CO INTERACTION STUDIES 31-01-03 12:27:59 CH= "A" PG= 1.

FILE 1. METHOD @. RUN 3 INDEX 3 BIN 7

PEAK#	AREA%	RT	AREA @:
1	62.894	1.24	1337938 @9
2	30.323	7.97	645072 @8
3	6.783	12.29	144296 @7
TOTAL	100.		3127306

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A INJECT 31-01-03 12:49:29 STORED TO BIN # 8



DATA SAVED TO BIN # 8

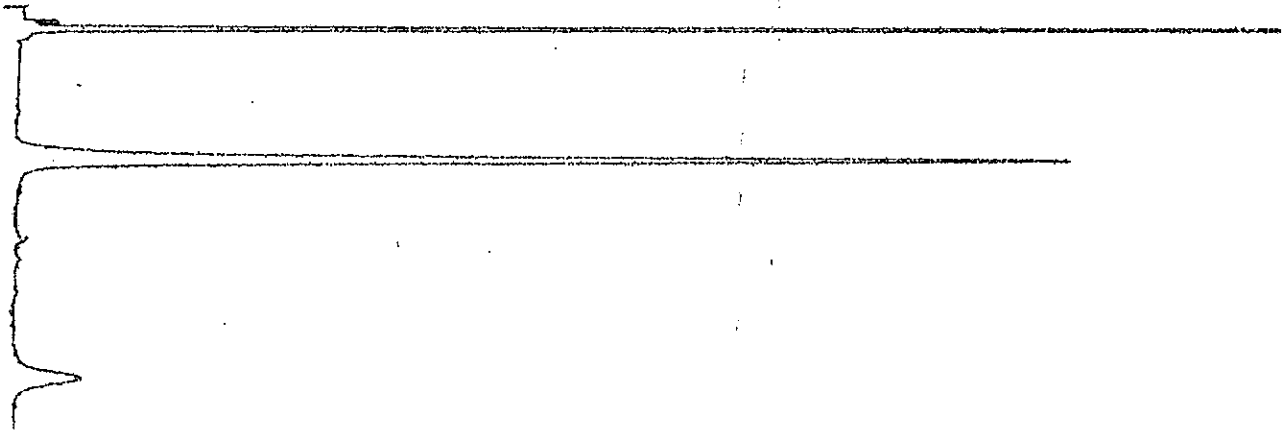
CP-CO INTERACTION STUDIES 31-01-03 12:49:22 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 4 INDEX 4 BIN 8

PEAK#	AREA%	RT	AREA BC
1	55.341	1.24	1297570 09
2	39.029	8.04	915095 08
8	5.63	19.48	132084 08
TOTAL	100.		2344677

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A INJECT 31-01-03 13:11:54 STORED TO BIN # 9



DATA SAVED TO BIN # 9

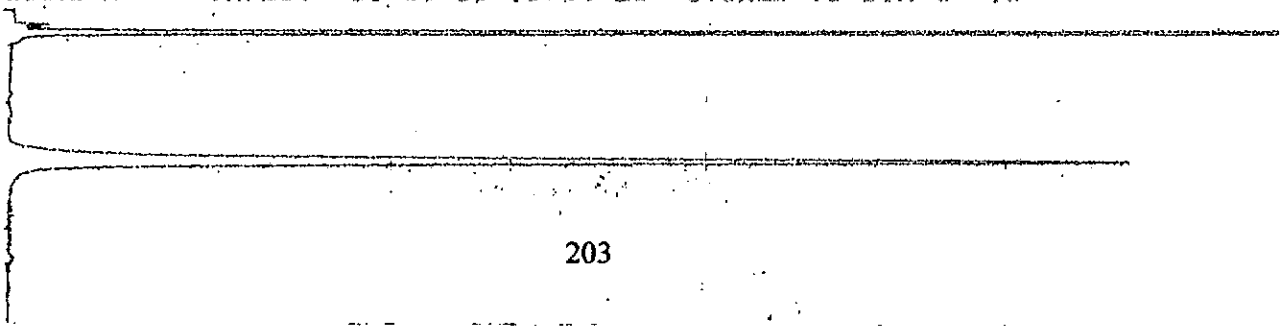
CP-CO INTERACTION STUDIES 31-01-03 13:11:54 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 5 INDEX 5 BIN 9

PEAK#	AREA%	RT	AREA BC
1	53.879	1.24	1514497 09
2	40.528	8.05	1139224 09
3	5.593	19.47	157211 08
TOTAL	100.		2810932

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A INJECT 31-01-03 13:34:20 STORED TO BIN # 10



DATA SAVED TO BIN # 10

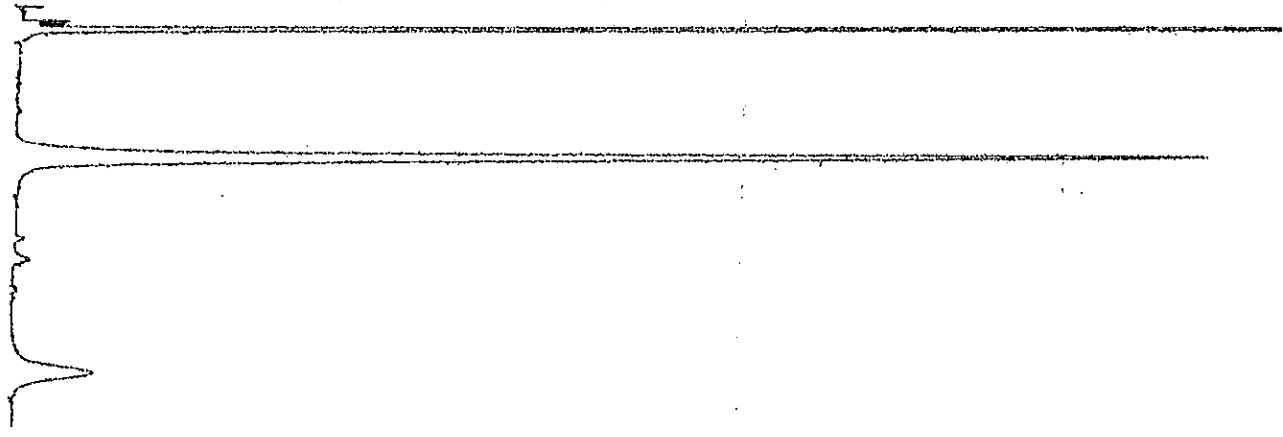
CP-CO INTERACTION STUDIES 31-01-03 13:34:20 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 6 INDEX 6 BIN 10

PEAK#	AREA%	RT	AREA PC
1	53.206	1.24	1563035 09
2	40.994	8.07	1204265 08
3	5.8	19.43	170308 08
TOTAL	100.		2937608

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A INJECT 31-01-03 13:56:42 STORED TO BIN # 11



DATA SAVED TO BIN # 11

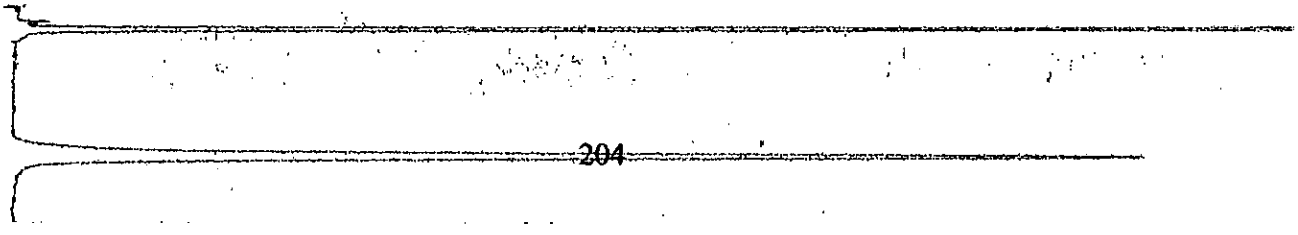
CP-CO INTERACTION STUDIES 31-01-03 13:57:42 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 7 INDEX 7 BIN 11

PEAK#	AREA%	RT	AREA PC
1	52.494	1.25	1612563 08
2	40.973	7.96	1258718 08
3	6.532	19.16	200680 08
TOTAL	100.		3072961

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A INJECT 31-01-03 14:22:53 STORED TO BIN # 12



DATA SAVED TO BIN # 12

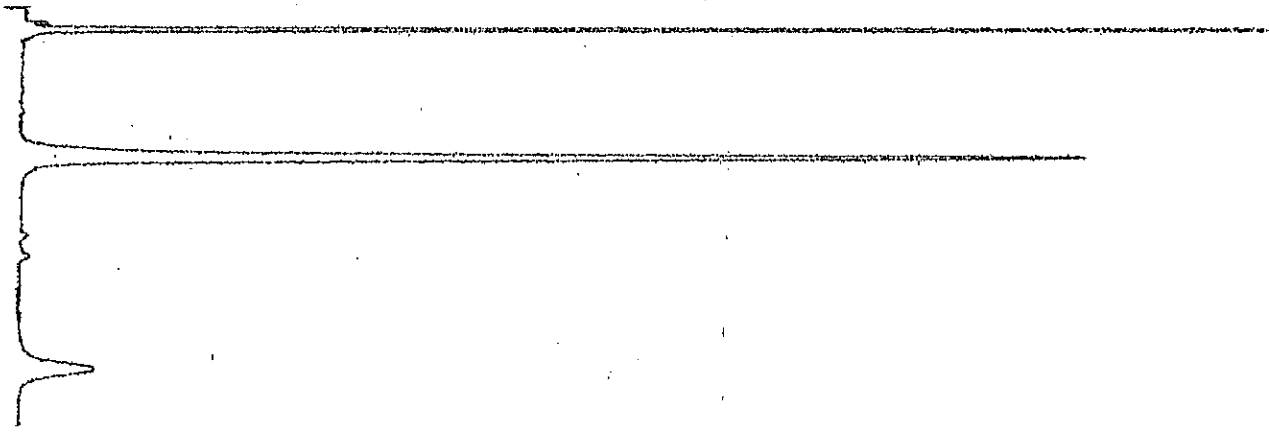
CP-CC INTERACTION STUDIES 31-01-03 14:22:53 CH= "A" RS= 1.

FILE 1. METHOD 0. RUN 8 INDEX 8 BIN 12

PEAK#	AREA%	RT	AREA BC
1	53.307	1.23	1560849 09
2	40.98	7.99	1199383 09
3	5.713	19.26	167194 08
TOTAL	100.		2926546

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A INJECT 31-01-03 14:45:18 STORED TO BIN # 13



DATA SAVED TO BIN # 13

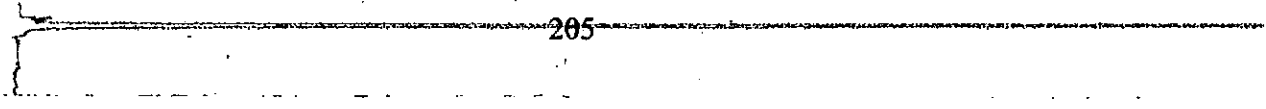
CP-CC INTERACTION STUDIES 31-01-03 14:45:18 CH= "A" RS= 1.

FILE 1. METHOD 0. RUN 9 INDEX 9 BIN 13

PEAK#	AREA%	RT	AREA BC
1	54.064	1.23	1511821 09
2	39.777	7.87	1112359 09
3	6.159	18.99	172282 08
TOTAL	100.		2796491

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A INJECT 31-01-03 15:07:44 STORED TO BIN # 14



DATA SAVED TO BIN # 14

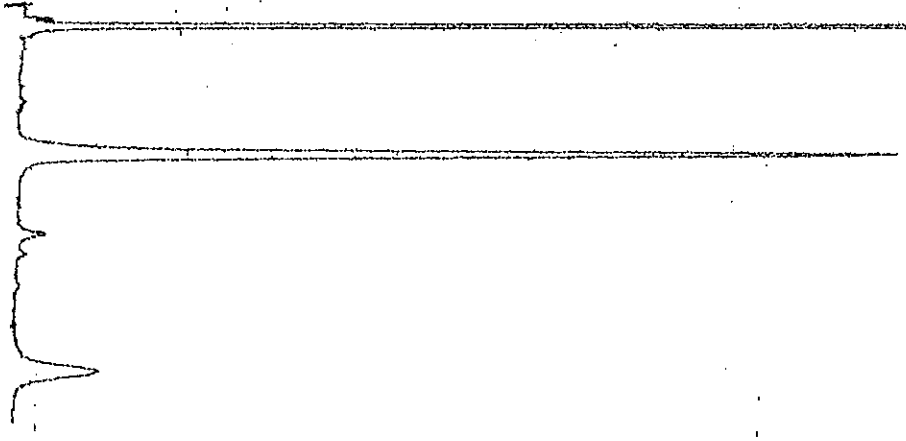
CP-CO INTERACTION STUDIES 31-01-03 15:07:44 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 10 INDEX 10 BIN 14

PEAK#	AREA%	RT	AREA BC
1	47.382	1.23	1502297 09
2	46.643	7.94	1481353 09
3	6.055	19.2	192491 08
TOTAL	100.		3176941

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A INJECT 31-01-03 15:30:19 STORED TO BIN # 15



DATA SAVED TO BIN # 15

CP-CO INTERACTION STUDIES 31-01-03 15:50:19 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 11 INDEX 11 BIN 15

PEAK#	AREA%	RT	AREA BC
1	72.416	1.25	2958452 09
2	22.91	8.01	936390 08
3	4.674	19.33	190615 08
TOTAL	100.		4085457

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

DATA SAVED TO BIN # 30

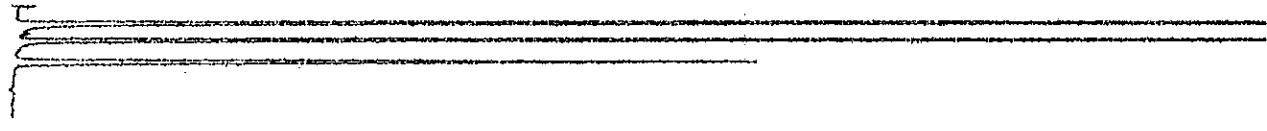
CP/CQ INTERACTIONS. 27-06-03 14:37:14 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 26 INDEX 26 BIN 30

PEAK#	AREA%	RT	AREA BC
1	66.635	1.09	2373775 09
2	22.379	2.1	797202 05
3	10.986	3.41	391368 07
TOTAL	100.		3562345

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A INJECT 27-06-03 14:43:36 STORED TO BIN # 31



DATA SAVED TO BIN # 31

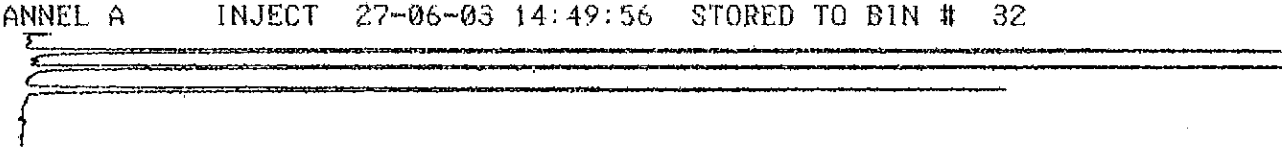
CP/CQ INTERACTIONS. 27-06-03 14:43:36 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 27 INDEX 27 BIN 31

PEAK#	AREA%	RT	AREA BC
1	64.804	0.96	1781794 09
2	24.654	1.85	677853 05
3	10.542	3.01	289849 07
TOTAL	100.		2749496

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A INJECT 27-06-03 14:49:56 STORED TO BIN # 32



DATA SAVED TO BIN # 32

CP/CQ INTERACTIONS. 27-06-03 14:49:56 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 28 INDEX 28 BIN 32

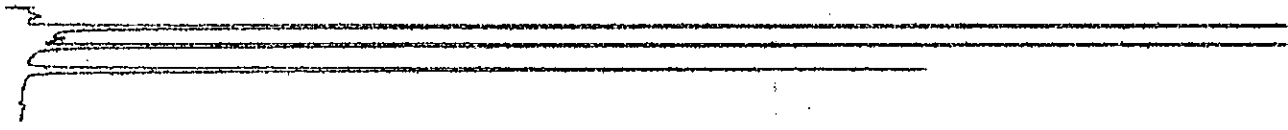
PEAK# AREA% RT AREA BC

2	25.45	1.85	923850 07
3	10.58	3.01	384080 07

TOTAL 100. 3630096

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A INJECT 27-06-03 14:56:17 STORED TO BIN # 33



DATA SAVED TO BIN # 33

CP/CQ INTERACTIONS. 27-06-03 14:56:17 CH= "A" PS= 1.

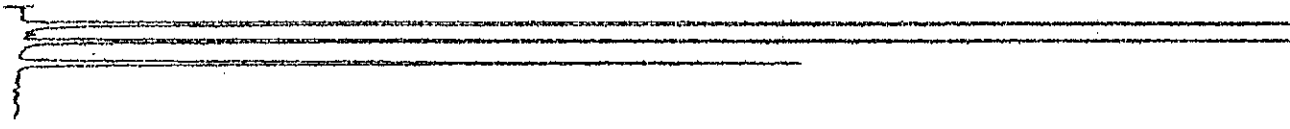
FILE 1. METHOD 0. RUN 29 INDEX 29 BIN 33

PEAK#	AREA%	RT	AREA BC
1	65.106	1.07	2346397 09
2	24.475	2.06	882072 07
3	10.419	3.36	375479 01

TOTAL 100. 3603948

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A INJECT 27-06-03 15:02:37 STORED TO BIN # 34



DATA SAVED TO BIN # 34

CP/CQ INTERACTIONS. 27-06-03 15:02:37 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 30 INDEX 30 BIN 34

PEAK#	AREA%	RT	AREA BC
1	64.112	0.97	1939754 09
2	25.749	1.85	779048 05
3	10.139	3.01	306770 09

TOTAL 100. 3025570

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A INJECT 27-06-03 15:10:37 STORED TO BIN # 35

DATA SAVED TO BIN # 35

CP/CQ INTERACTIONS. 27-06-03 15:10:37 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 31 INDEX 31 BIN 35

PEAK#	AREA%	RT	AREA BC
1	63.284	0.96	2558285 08
2	25.577	1.85	1033967 07
3	11.138	3.	450270 01
TOTAL	100.		4042522

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A INJECT 27-06-03 15:16:59 STORED TO BIN # 36

DATA SAVED TO BIN # 36

CP/CQ INTERACTIONS. 27-06-03 15:16:59 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 32 INDEX 32 BIN 36

PEAK#	AREA%	RT	AREA BC
1	65.334	1.04	2556776 09
2	25.217	2.	986840 06
3	9.449	3.26	369790 07
TOTAL	100.		3913406

INK LOW - CHANGE PRINT CARTRIDGE AND PRESS [CTRL-SHIFT-C]

CHANNEL A INJECT 27-06-03 15:23:32 STORED TO BIN # 37

DATA SAVED TO BIN # 37

CP/CQ INTERACTIONS. 27-06-03 15:23:32 CH= "A" PS= 1.

APPENDIX IIa: Shows HPLC Chromatograms of Amodiaquine-Artesunate in ACT formulations (dissolution sample in distill water).

Empower software

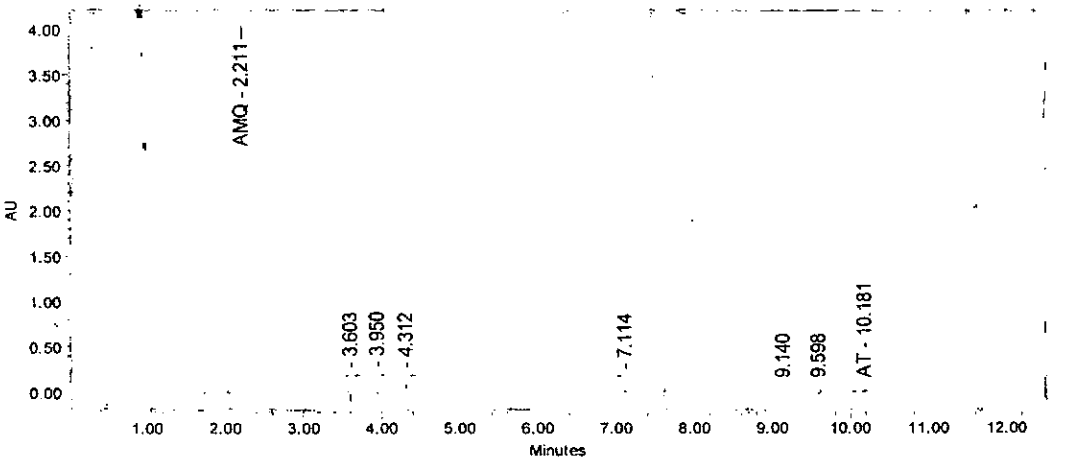
Untitled

Reported by User: System Project Name: ACT

SAMPLE INFORMATION

Sample Name:	AMQ /AT 60 min	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	6/7/2006 3:33:39 PM
Vial:	29	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	6/8/2006 10:35:27 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	AMQ	2.211	30116802	4172757		
2		3.117				
3		3.603	14756	2185		
4		3.950	63970	9435		
5		4.312	8179	1122		
6		6.591				
7		7.114	17890	2060		
8		9.140	3154	139		
9		9.598	3846	326		
10	AT	10.181	46517	4289		

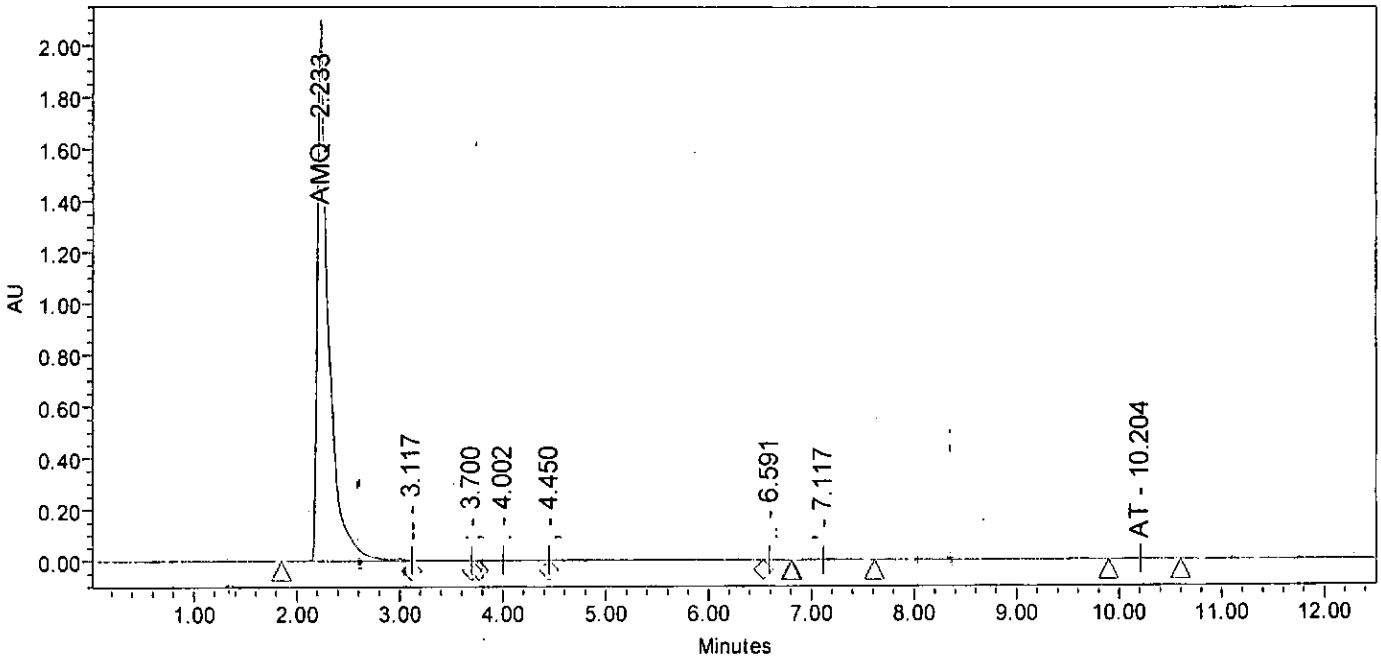
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	AMQ /AT 5 min	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	6/7/2006 2:13:01 PM
Vial:	23	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	6/8/2006 10:35:27 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	AMQ	2.233	15735445	2039832		
2	--	3.117	43280	2410		
3	;	3.700	1848	502		
4	.	4.002	31060	2769		
5	,	4.450	13949	185		
6	..	6.591	109	11		
7	'	7.117	6714	777		
8	AT	10.204	22325	2056		

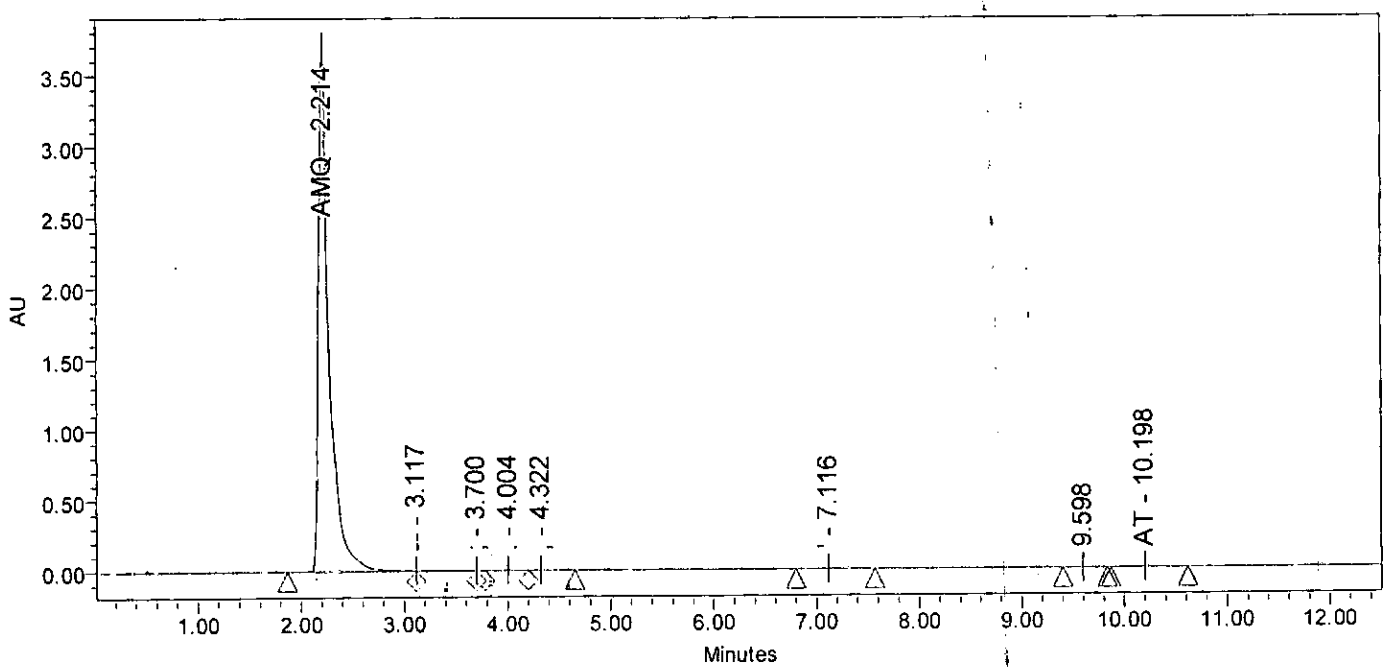
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	AMQ /AT 10 min	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	6/7/2006 2:26:25 PM
Vial:	24	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	6/8/2006 10:35:27 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	AMQ	2.214	26660421	3693543		
2	--	3.117	47035	2510		
3	;	3.700	2407	537		
4	.	4.004	27525	3455		
5	,	4.322	7725	1067		
6	..	6.591				
7	'	7.116	14308	1755		
8		9.598	2230	257		
9	AT	10.198	47362	4365		

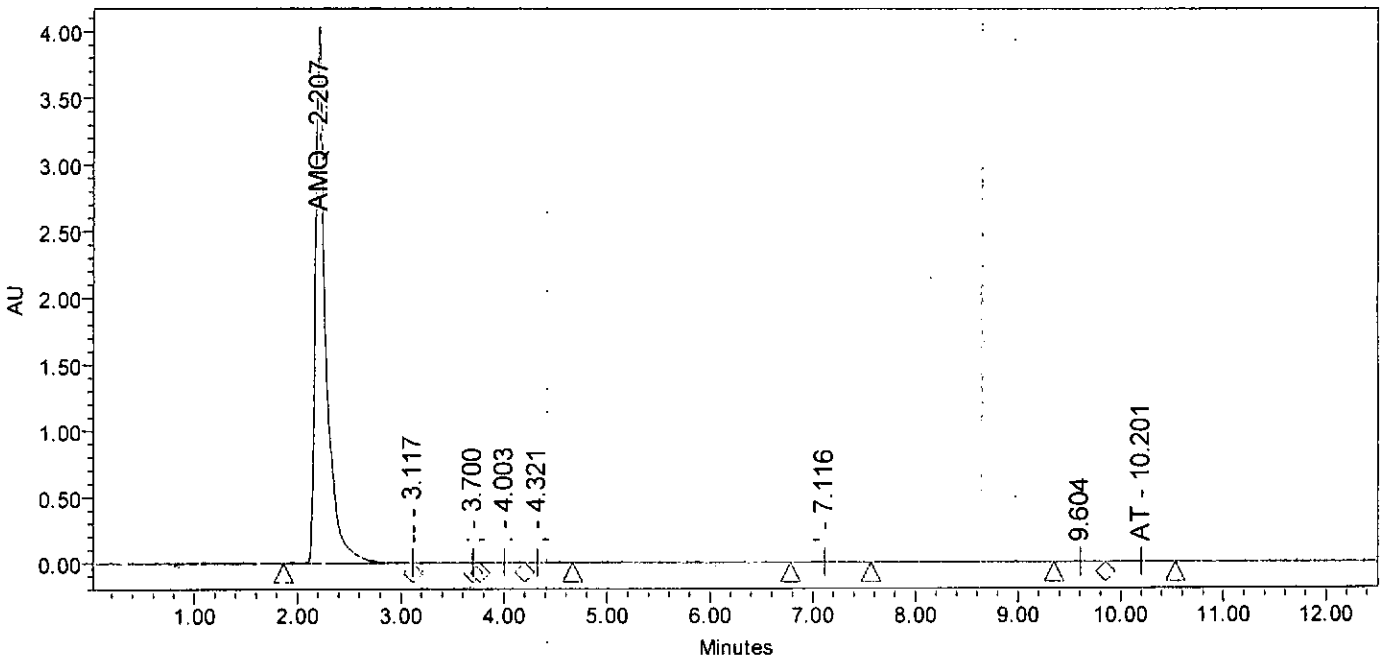
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	AMQ /AT 15 min	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	6/7/2006 2:40:03 PM
Vial:	25	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	6/8/2006 10:35:27 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	AMQ	2.207	28466694	4004310		
2	--	3.117	45240	2312		
3	;	3.700	1910	524		
4	.	4.003	27790	3508		
5	,	4.321	7749	1100		
6	..	6.591				
7	'	7.116	13364	1625		
8		9.604	2261	226		
9	AT	10.201	43192	3978		

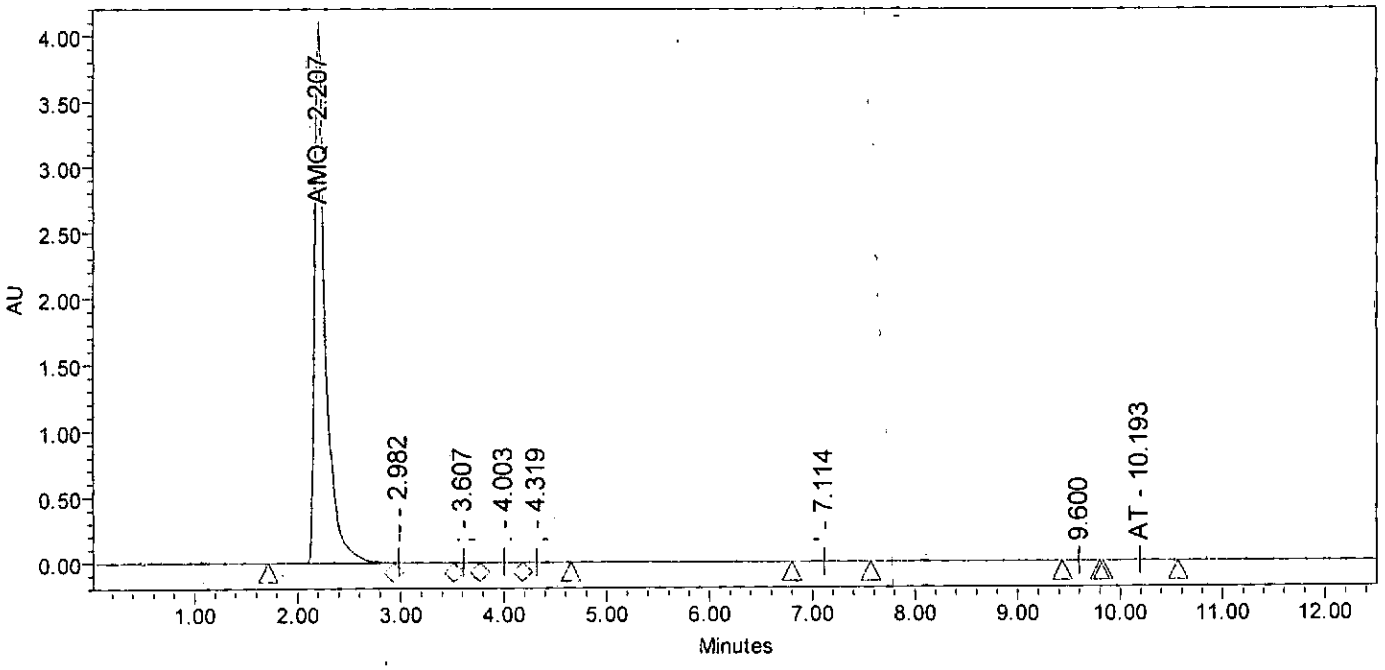
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	AMQ /AT 25 min	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	6/7/2006 2:53:27 PM
Vial:	26	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	6/8/2006 10:35:27 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	AMQ	2.207	29880245	4166151		
2	–	2.982	75586	6572		
3	;	3.607	14918	2182		
4	.	4.003	25169	3507		
5	,	4.319	8009	1120		
6	..	6.591				
7	'	7.114	14602	1794		
8		9.600	2022	223		
9	AT	10.193	46066	4255		

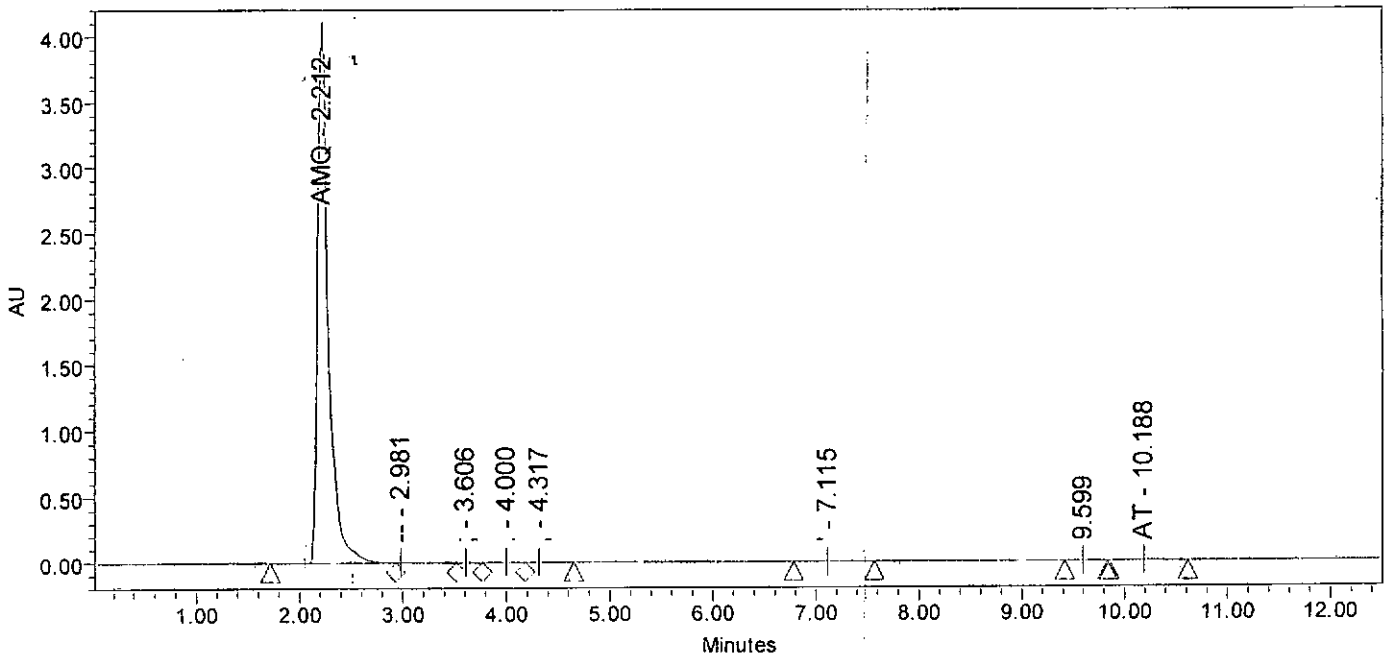
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	AMQ /AT 40 min	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	6/7/2006 3:06:51 PM
Vial:	27	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	6/8/2006 10:35:27 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	AMQ	2.212	30245015	4184115		
2	--	2.981	71163	5785		
3	;	3.606	15321	2212		
4	.	4.000	30999	3680		
5	,	4.317	8259	1144		
6	..	6.591				
7	'	7.115	15403	1897		
8		9.599	2395	252		
9	AT	10.188	47522	4396		

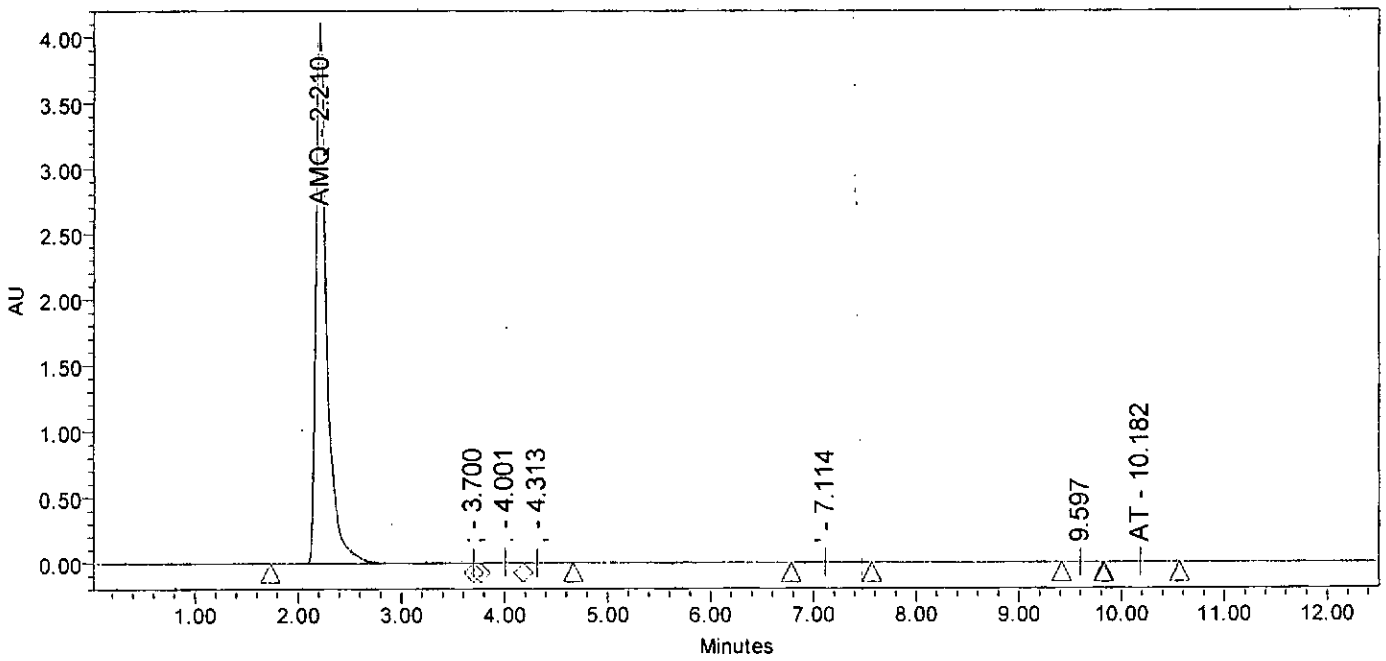
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	AMQ /AT 50 min	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	6/7/2006 3:20:15 PM
Vial:	28	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	6/8/2006 10:35:27 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	AMQ	2.210	30178543	4188952		
2	--	3.117				
3	;	3.700	1843	495		
4	,	4.001	27198	3504		
5	,	4.313	8183	1130		
6	..	6.591				
7	'	7.114	15875	1951		
8		9.597	2302	251		
9	AT	10.182	47383	4379		

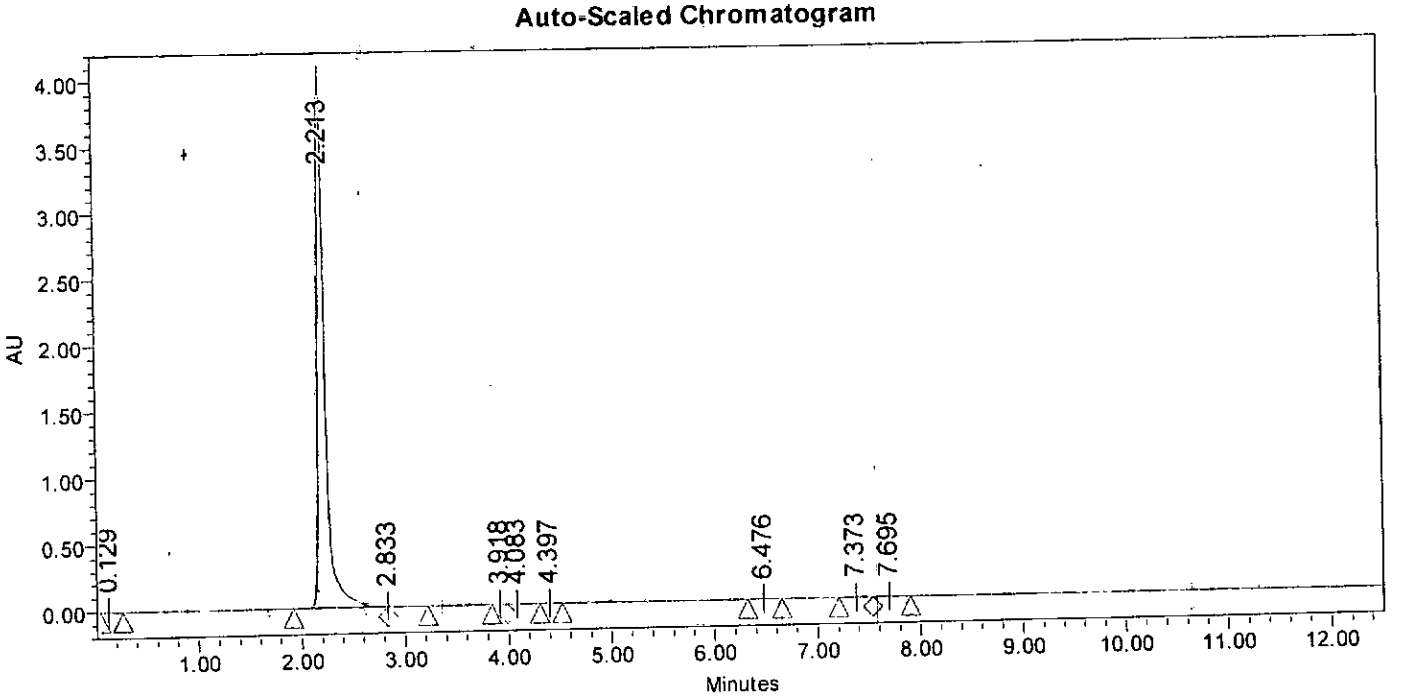
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	ACT S2	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	5/11/2006 10:55:48 AM
Vial:	3	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	5/12/2006 2:21:48 PM
Injection Volume:	20.00 ul	Processing Method:	Default
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT Assay	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

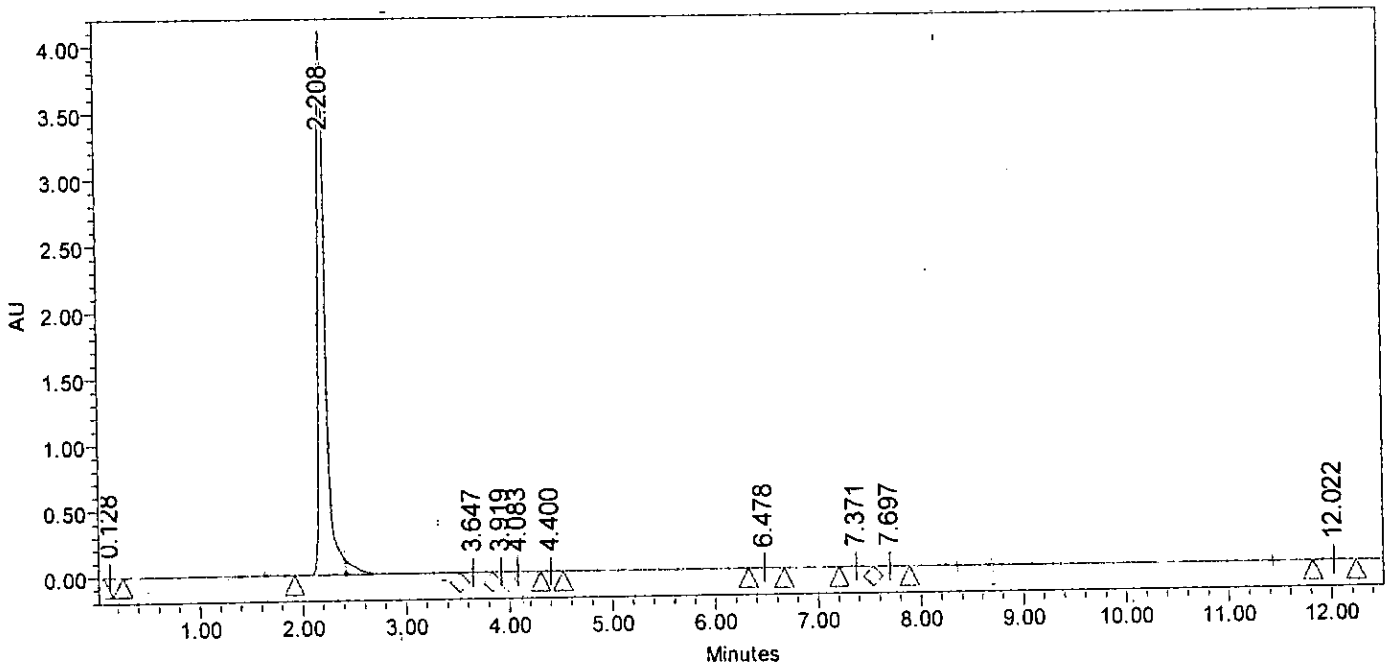
	Name	RT	Area	Height	Amount	Units
1		0.129	3256	934		
2		2.213	20057951	4117353		
3		2.833	22916	3528		
4		3.918	2468	570		
5		4.083	14568	2937		
6		4.397	4243	683		
7		6.476	38228	5690		
8		7.373	9075	1201		
9		7.695	11194	1322		

8 mins

SAMPLE INFORMATION

Sample Name:	ACT S 3	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	5/11/2006 11:09:11 AM
Vial:	4	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	5/12/2006 2:21:48 PM
Injection Volume:	20.00 ul	Processing Method:	Default
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT Assay	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1		0.128	3196	930		
2		2.208	20814923	4113076		
3		3.647	3647	431		
4		3.919	3694	810		
5		4.083	18445	3508		
6		4.400	4247	725		
7		6.478	44650	6639		
8		7.371	11253	1474		
9		7.697	12585	1518		
10		12.022	6515	575		

14 min

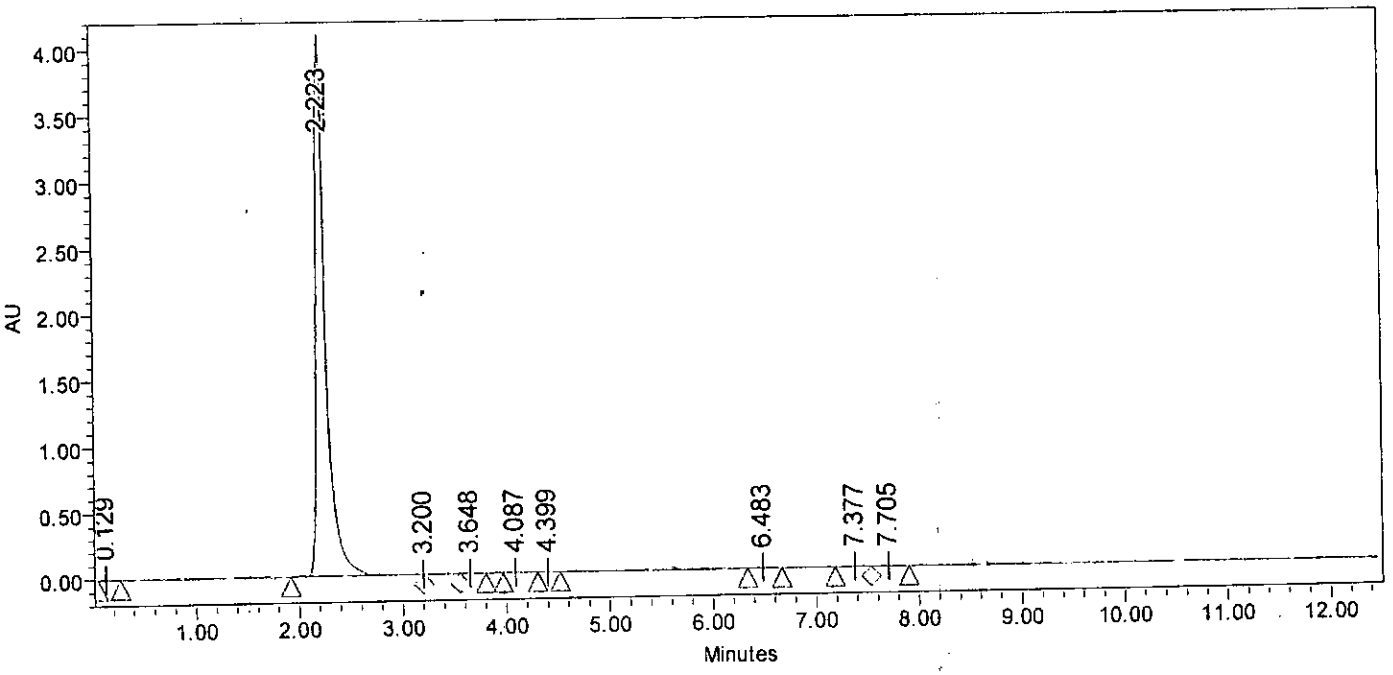
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	ACT S 4	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	5/11/2006 11:22:35 AM
Vial:	5	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	5/12/2006 2:21:48 PM
Injection Volume:	20.00 ul	Processing Method:	Default
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT Assay	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1		0.129	3244	929		
2		2.223	27099548	4227615		
3		3.200	7101	601		
4		3.648	2509	403		
5		4.087	16580	3335		
6		4.399	4304	760		
7		6.483	57192	8522		
8		7.377	12144	1587		
9		7.705	15723	1874		

18 mins

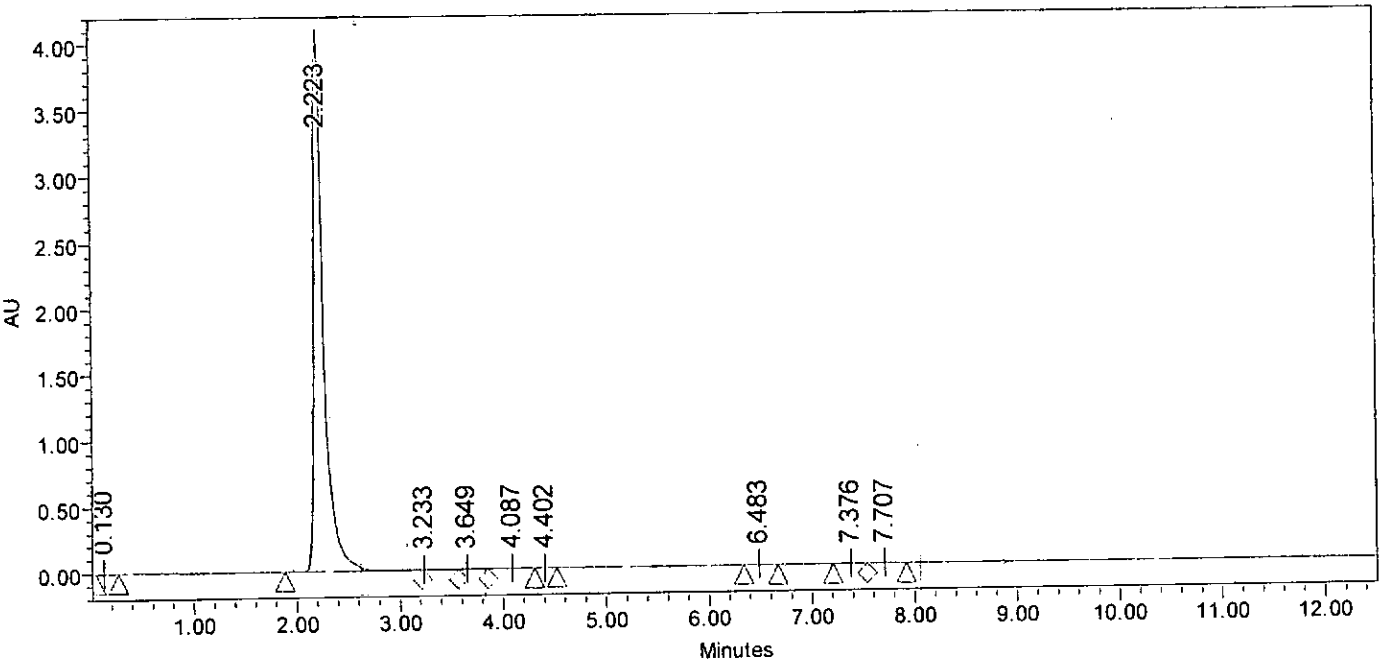
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	ACT S 5	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	5/11/2006 11:35:59 AM
Vial:	6	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	5/12/2006 2:21:48 PM
Injection Volume:	20.00 ul	Processing Method:	Default
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT Assay	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1		0.130	3234	933		
2		2.223	27417721	4232315		
3		3.233	7557	603		
4		3.649	3612	461		
5		4.087	18841	3428		
6		4.402	4236	773		
7		6.483	53775	8016		
8		7.376	10765	1408		
9		7.707	15368	1787		

25min

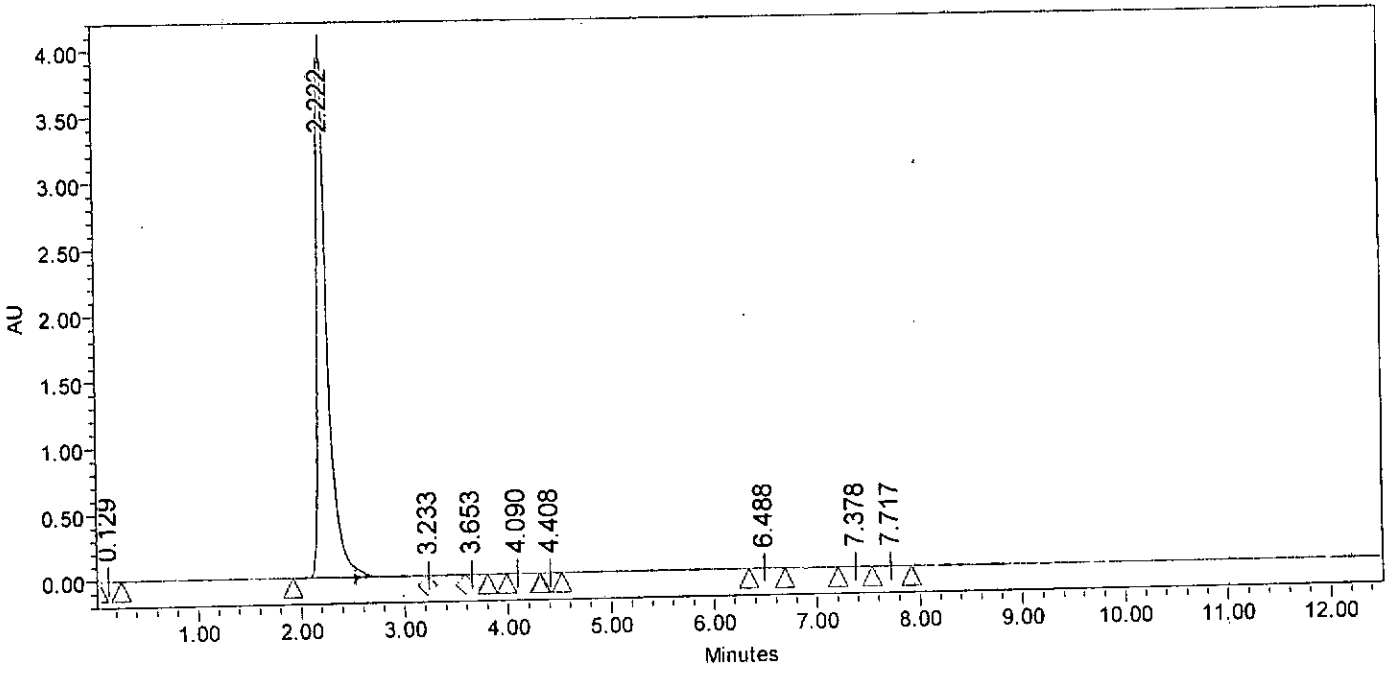
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	ACT S 6	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	5/11/2006 11:49:23 AM
Vial:	7	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	5/12/2006 2:21:48 PM
Injection Volume:	20.00 ul	Processing Method:	Default
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT Assay	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1		0.129	3077	886		
2		2.222	28031823	4225937		
3		3.233	6107	531		
4		3.653	2062	389		
5		4.090	16272	3322		
6		4.408	4068	748		
7		6.488	57675	8554		
8		7.378	10619	1422		
9		7.717	16281	1895		

40 min

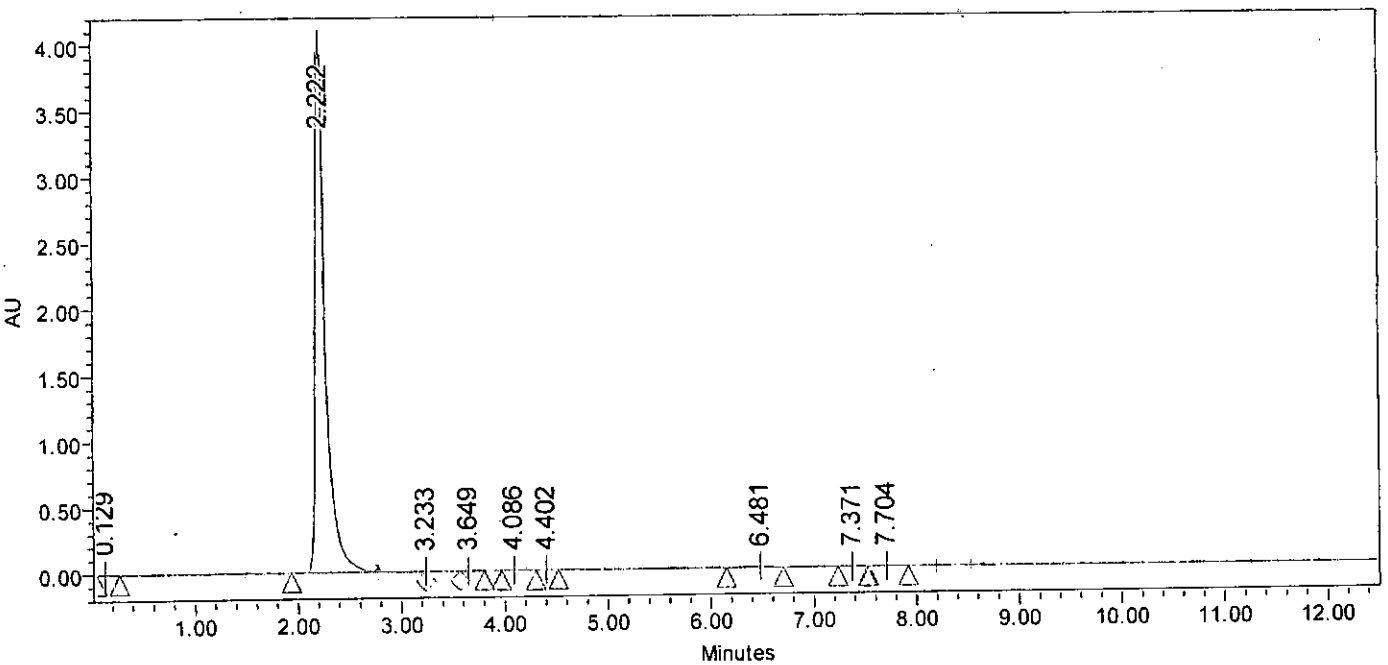
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	ACT S 7	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	5/11/2006 12:02:47 PM
Vial:	8	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	5/12/2006 2:21:48 PM
Injection Volume:	20.00 ul	Processing Method:	Default
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT Assay	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1		0.129	3106	886		
2		2.222	27685732	4224077		
3		3.233	5498	537		
4		3.649	2249	388		
5		4.086	16389	3360		
6		4.402	4333	785		
7		6.481	65026	9187		
8		7.371	10072	1394		
9		7.704	16982	1997		

50 mm

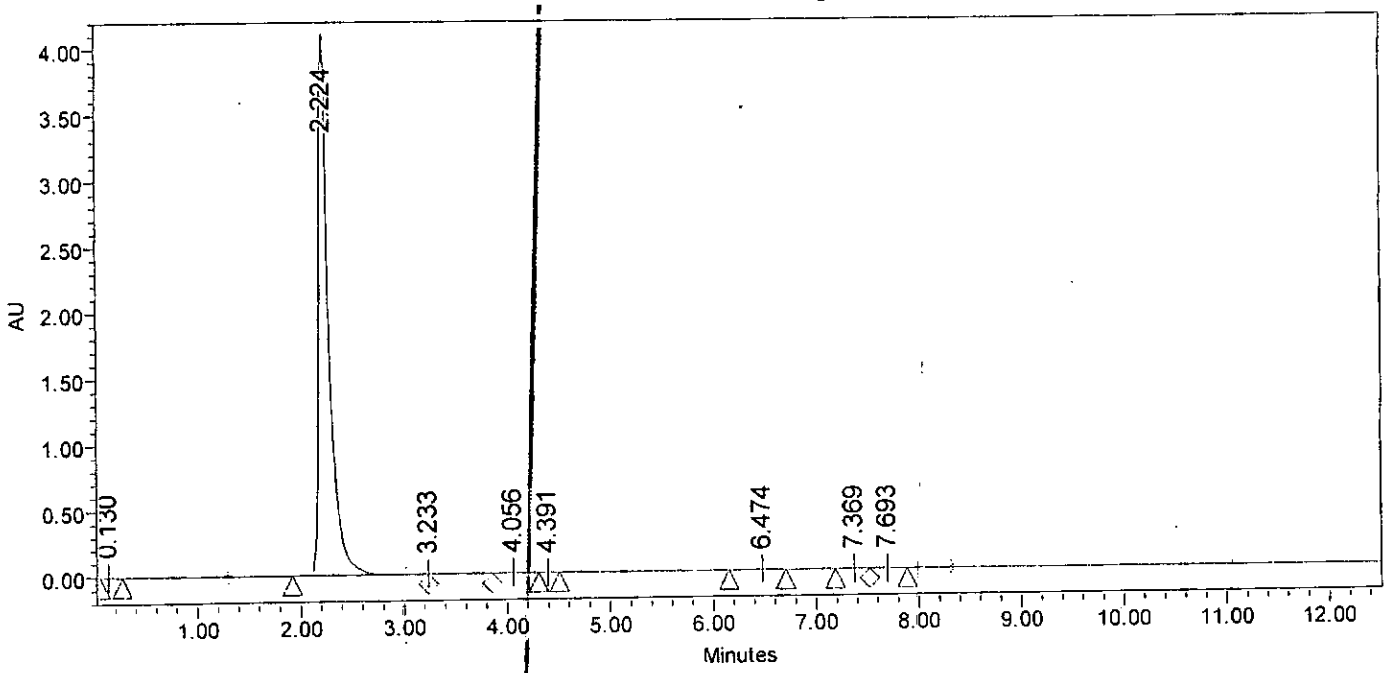
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	ACT S 8	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	5/11/2006 12:16:11 PM
Vial:	9	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	5/12/2006 2:21:48 PM
Injection Volume:	20.00 ul	Processing Method:	Default
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT Assay	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1		0.130	3124	896		
2		2.224	28485673	4224562		
3		3.233	10182	625		
4		4.056	44810	7364		
5		4.391	4124	766		
6		6.474	61769	8744		
7		7.369	9827	1317		
8		7.693	16323	1912		

60min

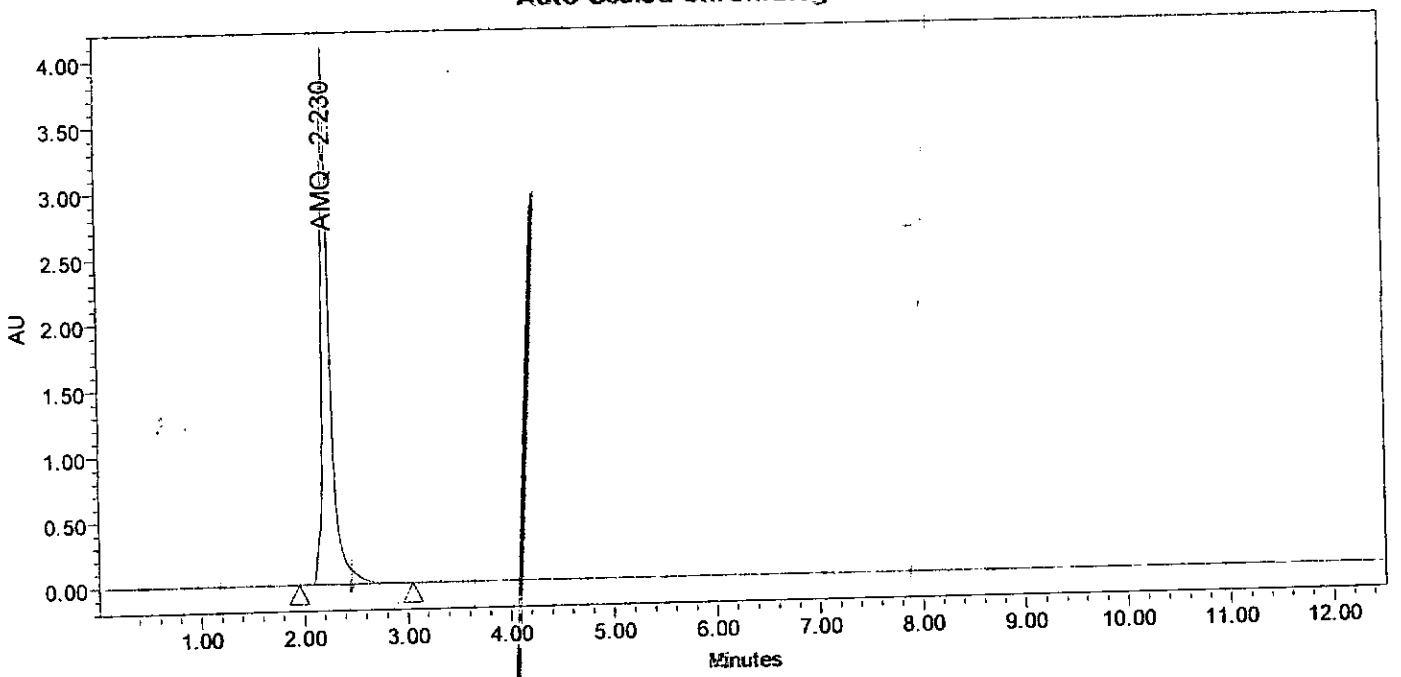
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	AMQ 5 min	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	6/7/2006 12:39:20 PM
Vial:	16	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	6/8/2006 10:15:48 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



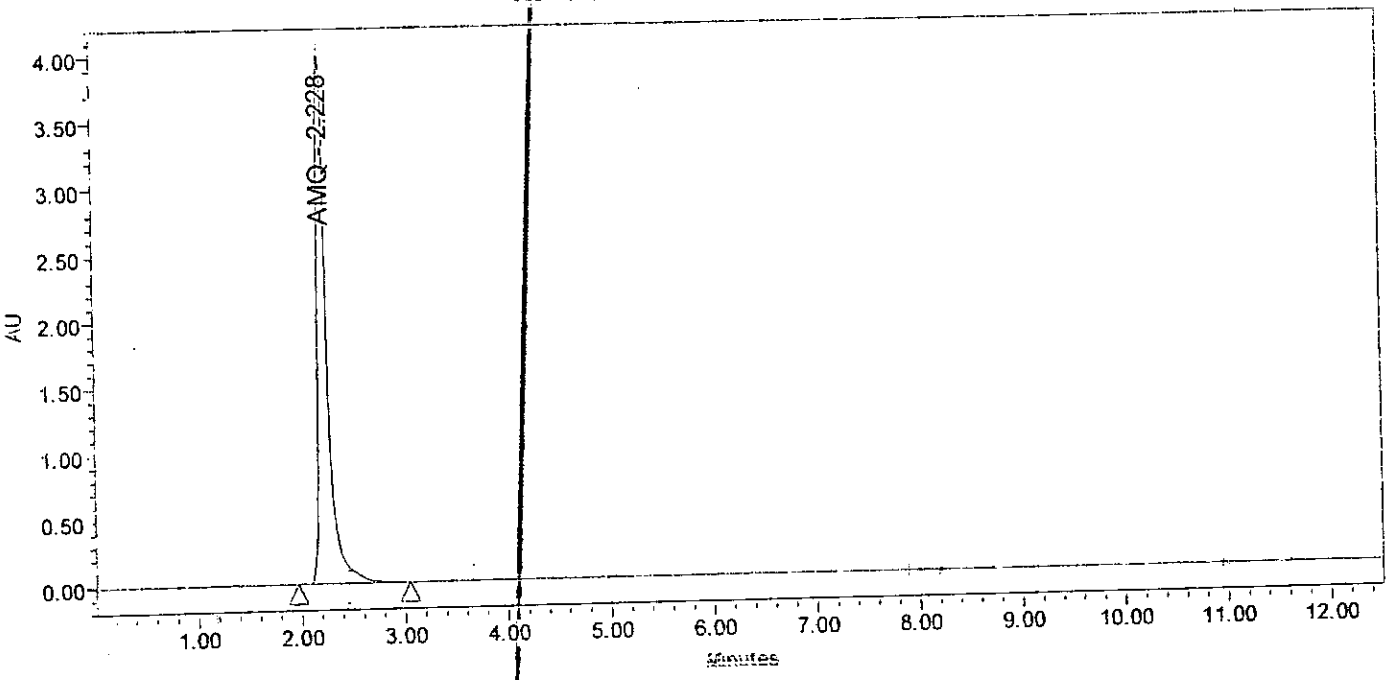
Peak Results

	Name	RT	Area	Height	Amount	Units
1	AMQ	2.230	22850769	4021486		

SAMPLE INFORMATION

Sample Name:	AMQ 10 min	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	6/7/2006 12:52:41 PM
Vial:	17	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	6/8/2006 10:15:48 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



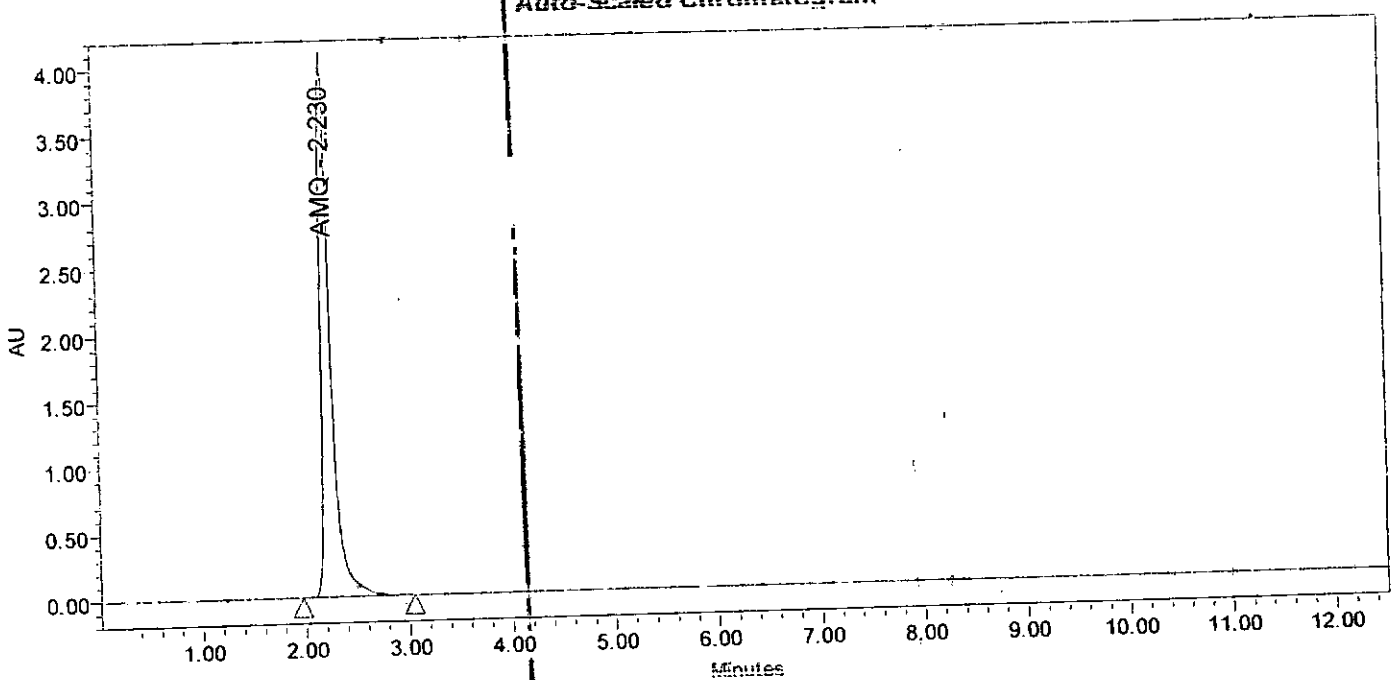
Peak Results

	Name	RT	Area	Height	Amount	Units
1	AMQ	2.228	25913091	4259074		

SAMPLE INFORMATION

Sample Name:	AMQ 15 min	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	6/7/2006 1:06:05 PM
Vial:	18	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	6/8/2006 10:15:48 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram

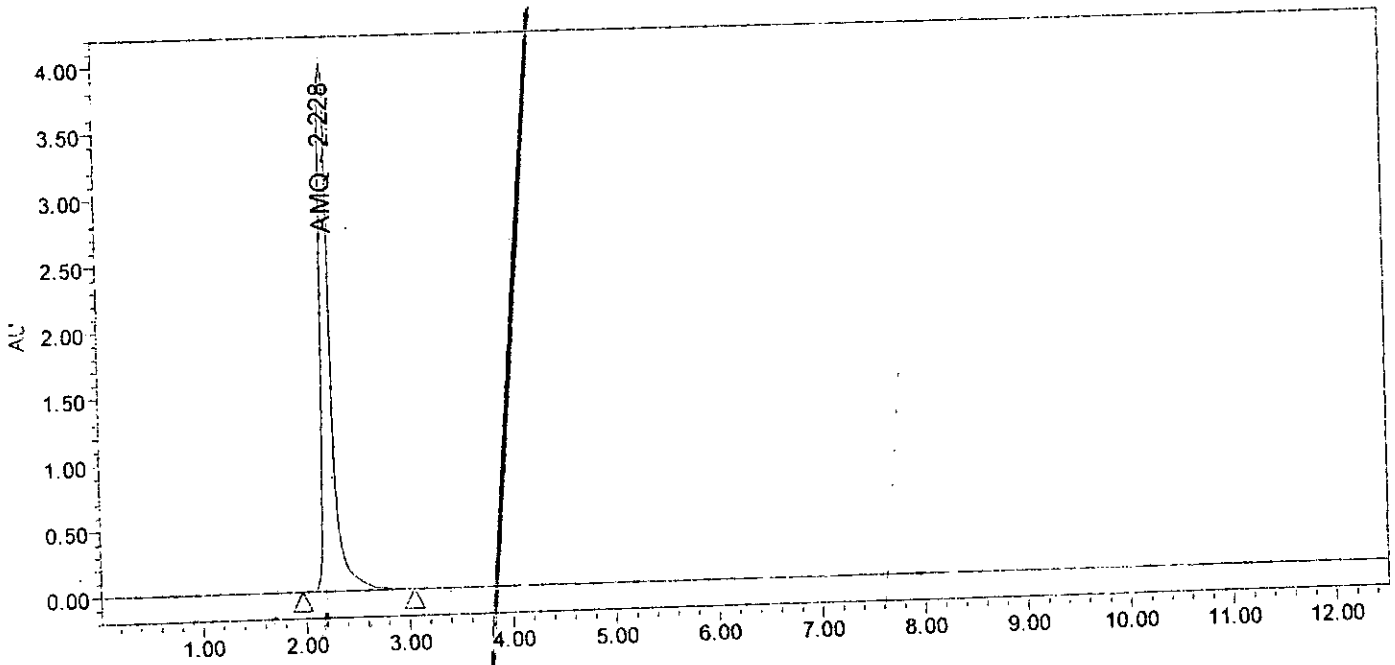


Peak Results

	Name	RT	Area	Height	Amount	Units
1	AMQ	2.230	26856051	4284806		

SAMPLE INFORMATION

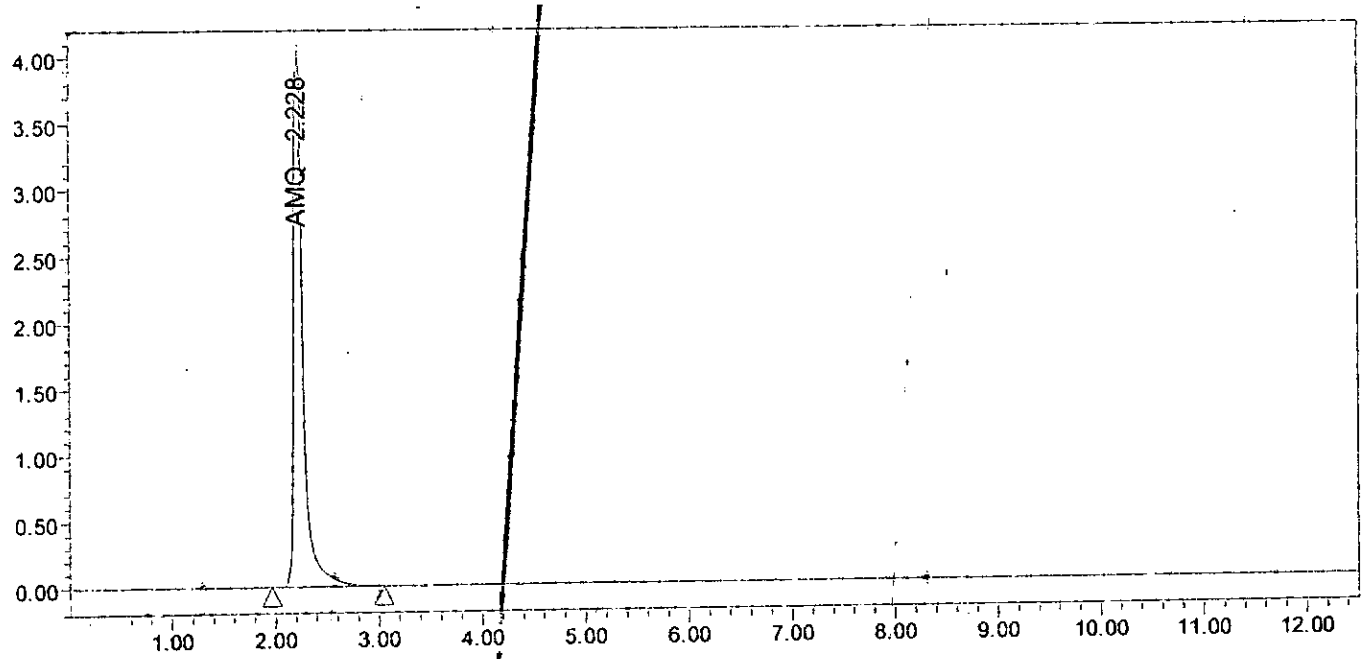
Sample Name:	AMQ 25 min	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	6/7/2006 1:19:28 PM
Vial:	19	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	6/8/2006 10:15:48 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	



	Name	RT	Area	Height	Amount	Units
1	AMQ	2.228	26150797	4293382		

SAMPLE INFORMATION

Sample Name:	AMQ 40 min	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	6/7/2006 1:32:50 PM
Vial:	20	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	6/8/2006 10:15:48 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	



	Name	RT	Area	Height	Amount	Units
1	AMQ	2.228	26013607	4291579		

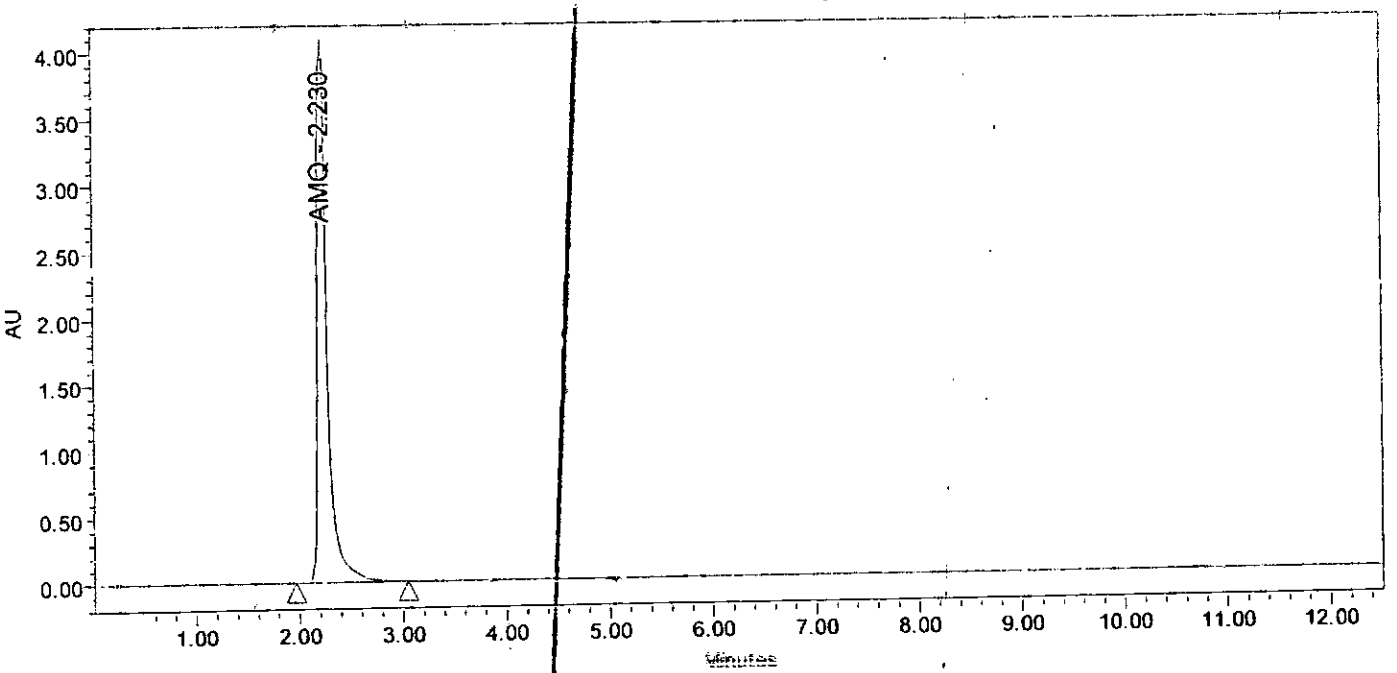
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	AMQ 50 min	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	6/7/2006 1:46:14 PM
Vial:	21	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	6/8/2006 10:15:48 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	AMQ	2.230	27261141	4305447		

Empower software

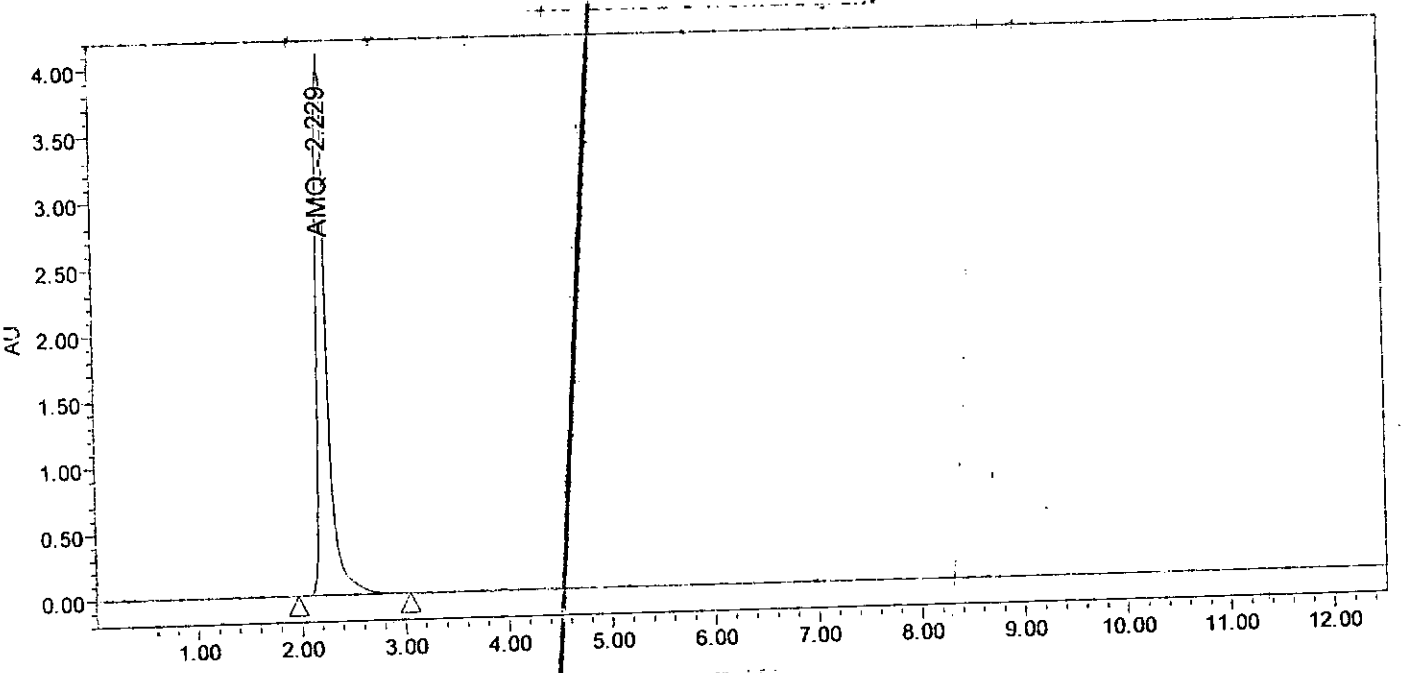
Untitled

Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	AMQ 60 min	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	6/7/2006 1:59:37 PM
Vial:	22	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	6/8/2006 10:15:48 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	



	Name	RT	Area	Height	Amount	Units
1	AMQ	2.229	27179919	4305506		

Untitled

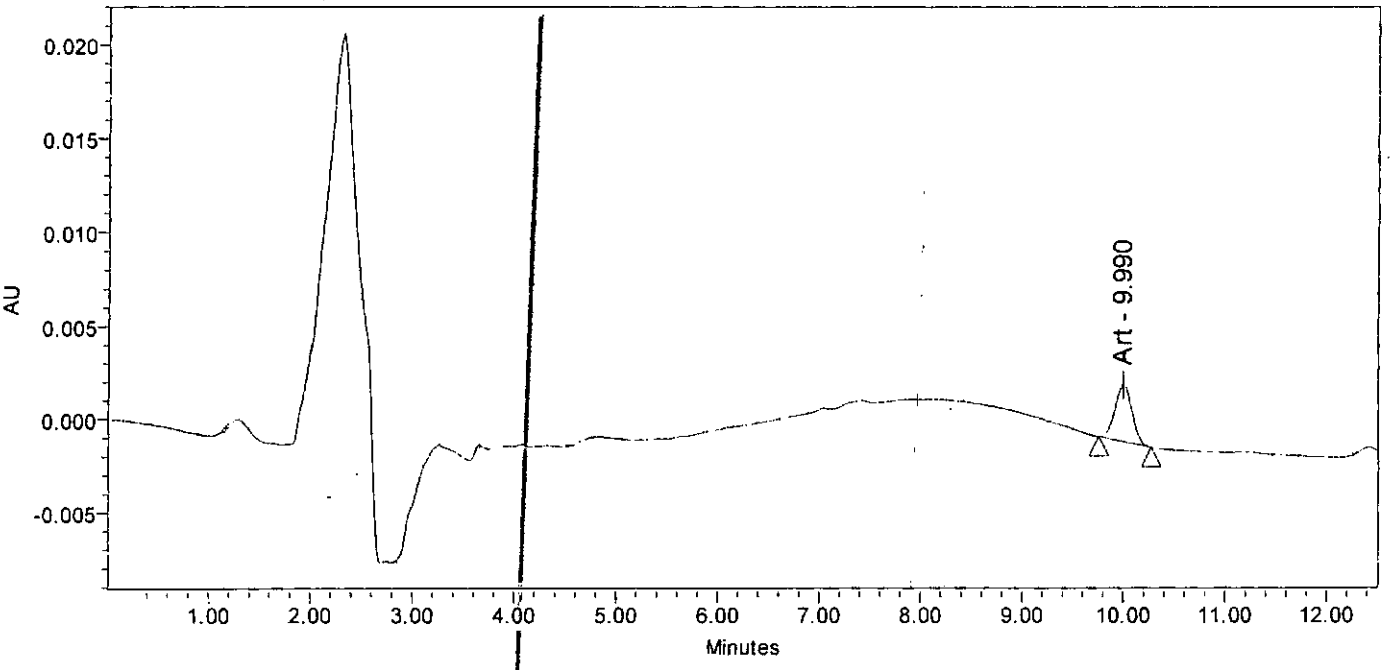
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	Std 003 (50µg/ml)	Acquired By:	System
Sample Type:	Standard	Date Acquired:	5/23/2006 10:08:43 AM
Vial:	3	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	5/24/2006 9:08:16 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Art	9.990	35189	3085		

Std 50µg/ml

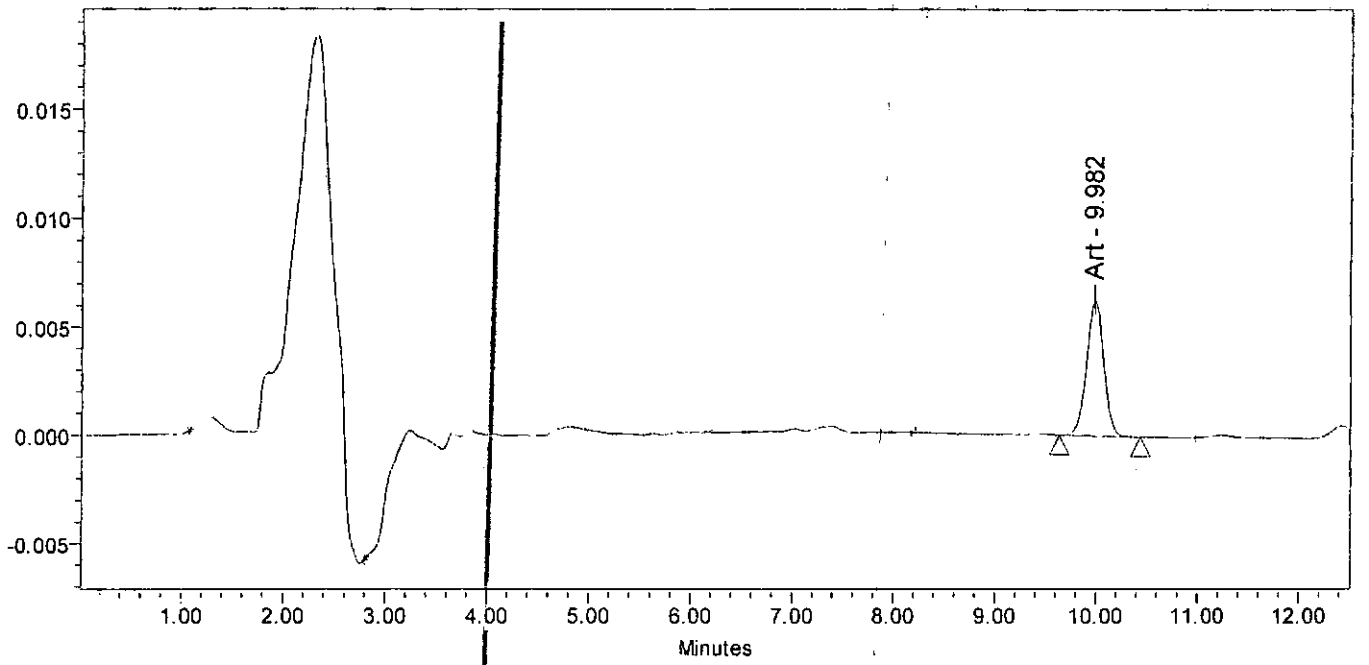
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	Std 004	Acquired By:	System
Sample Type:	Standard	Date Acquired:	5/23/2006 10:22:07 AM
Vial:	4	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	5/24/2006 9:08:16 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Art	9.982	73988	6319		

Std 100 pg/mL

Empower software

Untitled

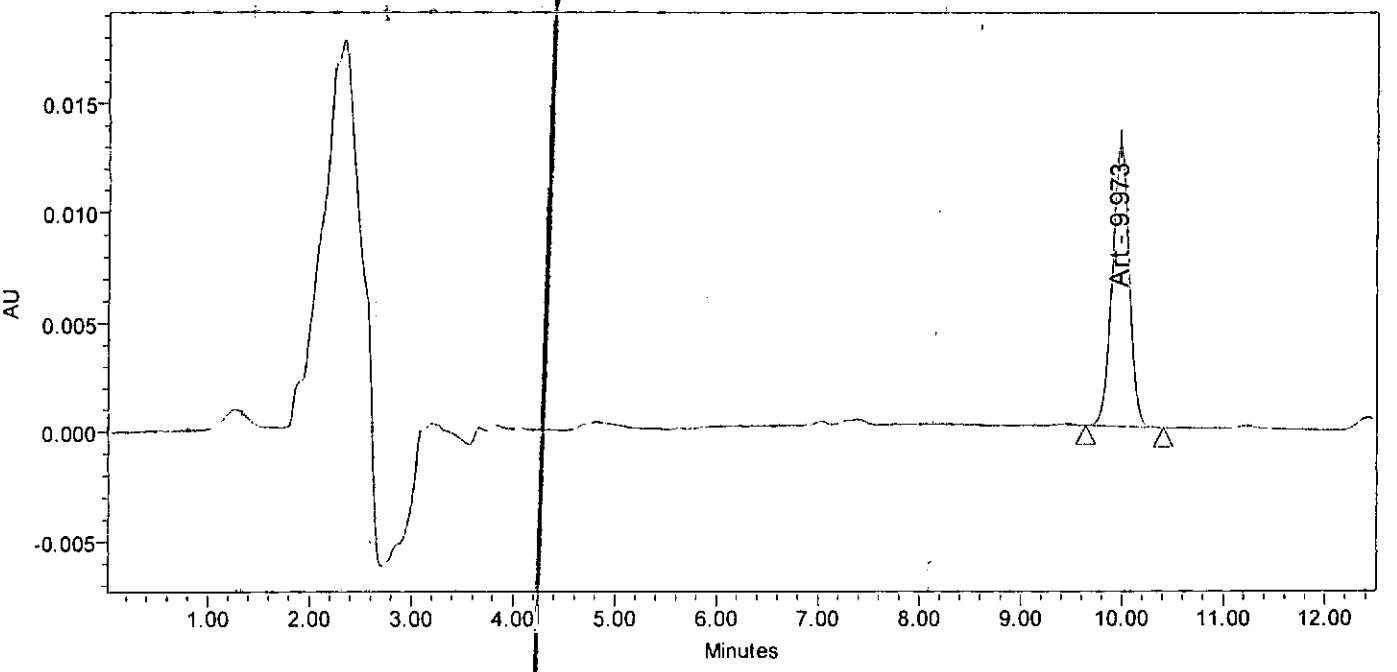
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	Std 005	Acquired By:	System
Sample Type:	Standard	Date Acquired:	5/23/2006 10:35:31 AM
Vial:	5	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	5/24/2006 9:08:16 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Art	9.973	150927	12956		

AT

St. 200 µg/ml

Empower software

Untitled

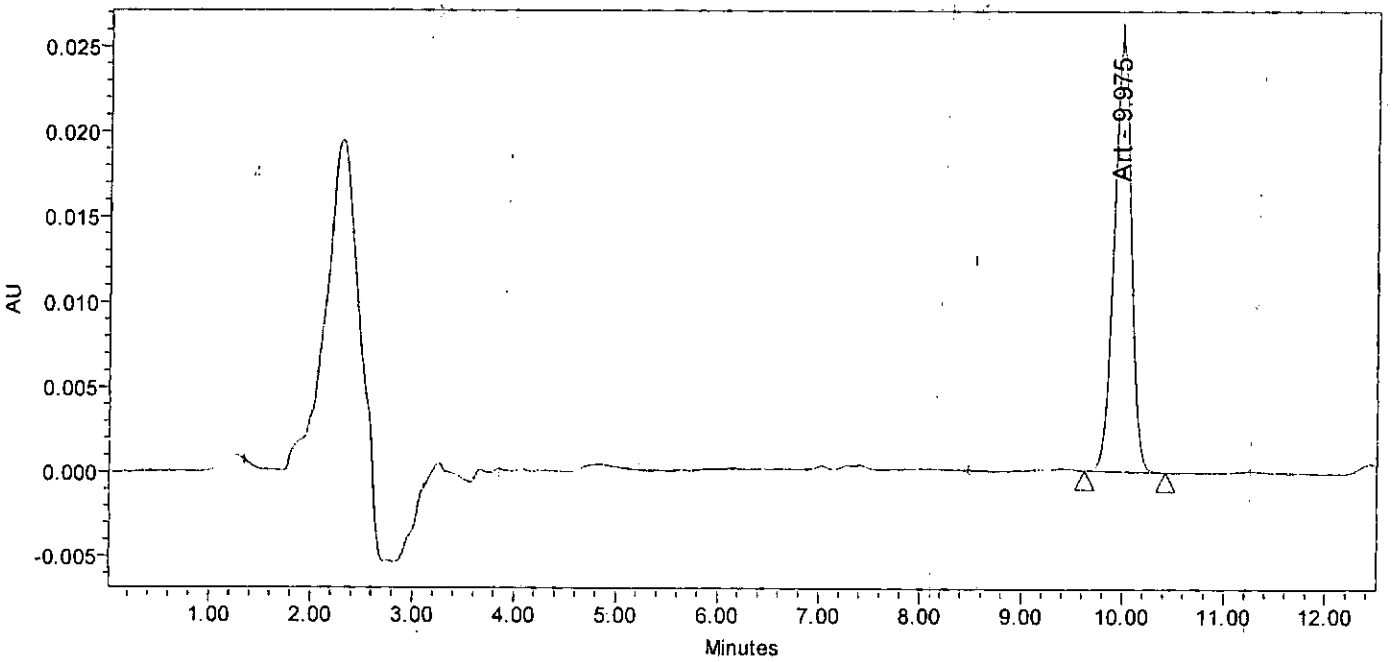
Reported by User: System

Project Name: ACT

SAMPLE INFORMATION

Sample Name:	Std 006	Acquired By:	System
Sample Type:	Standard	Date Acquired:	5/23/2006 10:48:55 AM
Vial:	6	Acq. Method Set:	ACT
Injection #:	1	Date Processed:	5/24/2006 9:08:16 AM
Injection Volume:	20.00 ul	Processing Method:	ACT
Run Time:	12.5 Minutes	Channel Name:	2487Channel 1
Sample Set Name:	ACT	Proc. Chnl. Descr.:	

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Art	9.975	300777	25563		

Report Method: Untitled

Printed 9:29:07 AM 5/24/2006

Page: 1 of 1

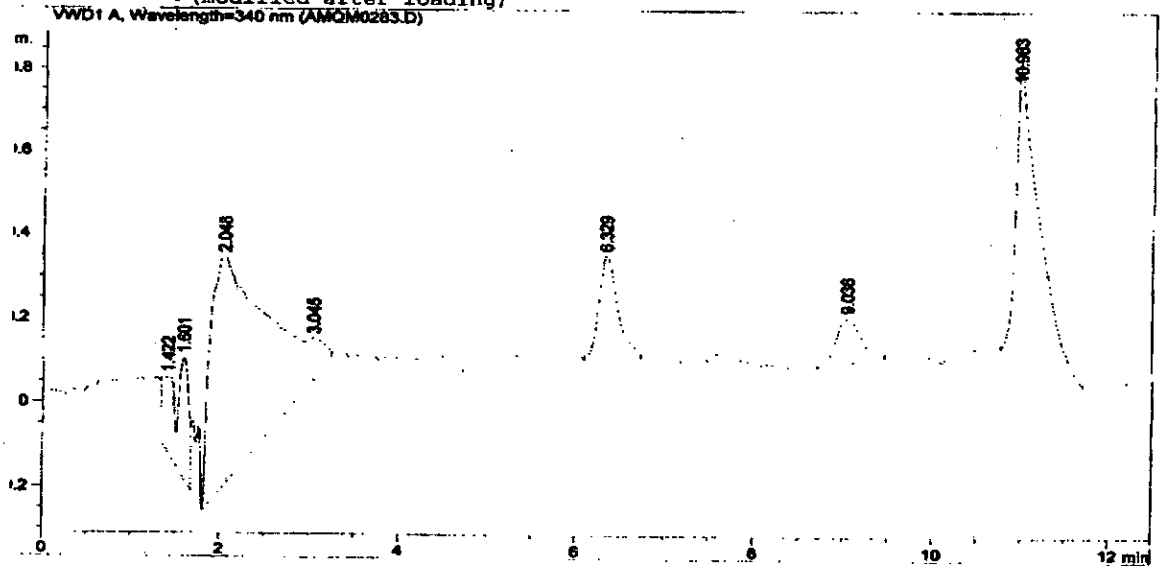
Std. AT 400 ng/ml

APPENDIX IIb: Shows the HPLC chromatograms of Amodiaquine, Chloroquine and Monodesethylamodiaquine in plasma of volunteers.

C:\HPCHEM\1\DATA\AMQM0283.D Sample Name: AMODIAQUINE

E PHASE:PO4/METHANOL(80/20).

tion Date : 11/23/2006 2:24:26 PM
e Name : AMODIAQUINE Location : Vial 1
Operator : AKINLEYE
d : C:\HPCHEM\1\METHODS\AKINAMO.M
changed : 11/23/2006 2:08:57 PM by AKINLEYE
(modified after loading)



Area Percent Report

d By : Signal
plier : 1.0000
ion : 1.0000

l 1: VWD1 A, Wavelength=340 nm

RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1.422	VV	0.1105	1.59078	1.79872e-1	3.4545
1.601	VV	0.1242	2.20799	2.88687e-1	4.7948
2.048	VV	0.4908	22.09620	5.43018e-1	47.9837
3.045	VV	0.1712	1.37145	9.93641e-2	2.9782
6.329	VP	0.2150	3.47976	2.43096e-1	7.5566
9.036	VV	0.2431	2.16898	1.10021e-1	4.7101
10.983	VP	0.2318	13.13421	7.24696e-1	28.5220
s :			46.04937	2.18875	

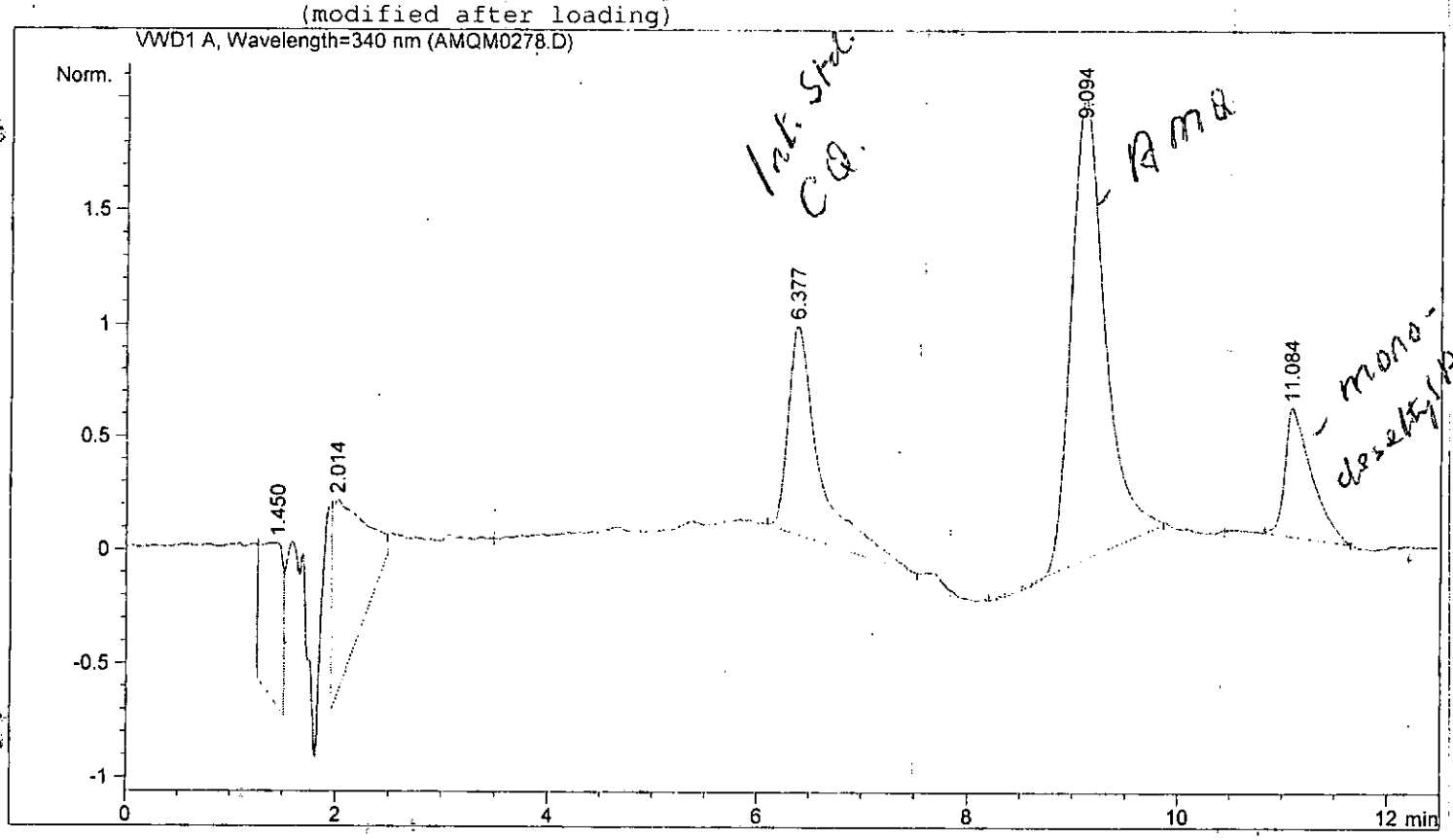
lts obtained with enhanced integrator!

*** End of Report ***

30.11.2006

32 MOBILE PHASE: PO4/METHANOL(80/20). AMA (Mesylate)

```
Injection Date   : 11/23/2006 1:05:18 PM
Sample Name      : AMODIAQUINE                    Location : Vial 1
Acq. Operator    : AKINLEYE
Method           : C:\HPCHEM\1\METHODS\AKINAMO.M
Last changed     : 11/23/2006 9:25:56 AM by AKINLEYE
                   (modified after loading)
```



Area Percent Report

```
Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
```

Signal 1: VWD1 A, Wavelength=340 nm

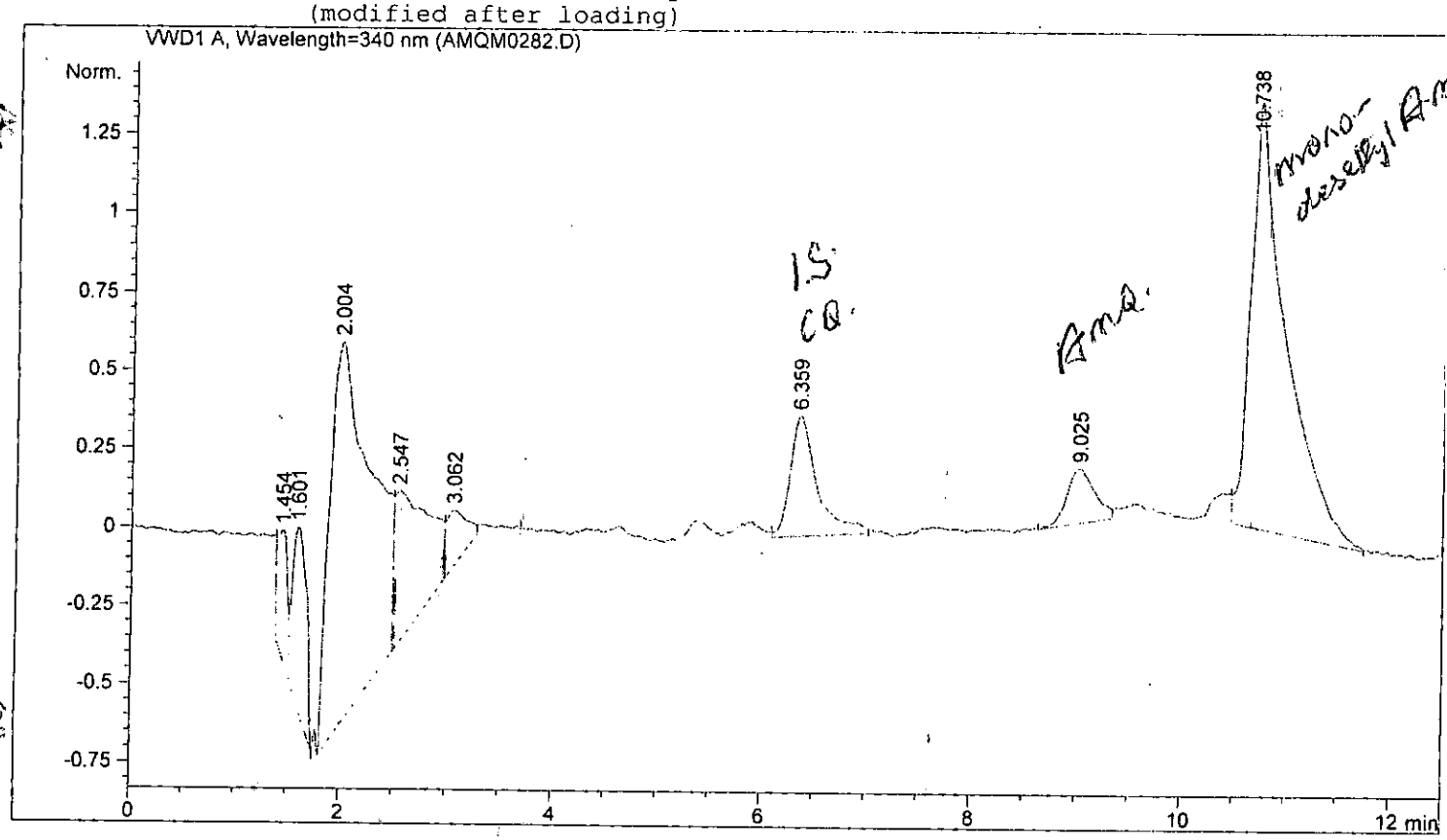
Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	1.450	VV	0.1781	9.94984	7.16379e-1	9.9489
2	2.014	VV	0.2298	15.67846	8.61484e-1	15.6769
3	6.377	BP	0.2891	19.02312	9.26331e-1	19.0213
4	9.094	VB	0.3266	45.26619	2.03379	45.2618
5	11.084	BB	0.2361	10.09206	5.68902e-1	10.0911
Totals :				100.00967	5.10688	

Results obtained with enhanced integrator!

*** End of Report ***

33 MOBILE PHASE:PO4/METHANOL(80/20).

```
==========================================================================
Injection Date   : 11/23/2006 2:09:25 PM
Sample Name      : AMODIAQUINE                    Location : Vial 1
Acq. Operator    : AKINLEYE
Method           : C:\HPCHEM\1\METHODS\AKINAMO.M
Last changed     : 11/23/2006 2:08:57 PM by AKINLEYE
                   (modified after loading)
```

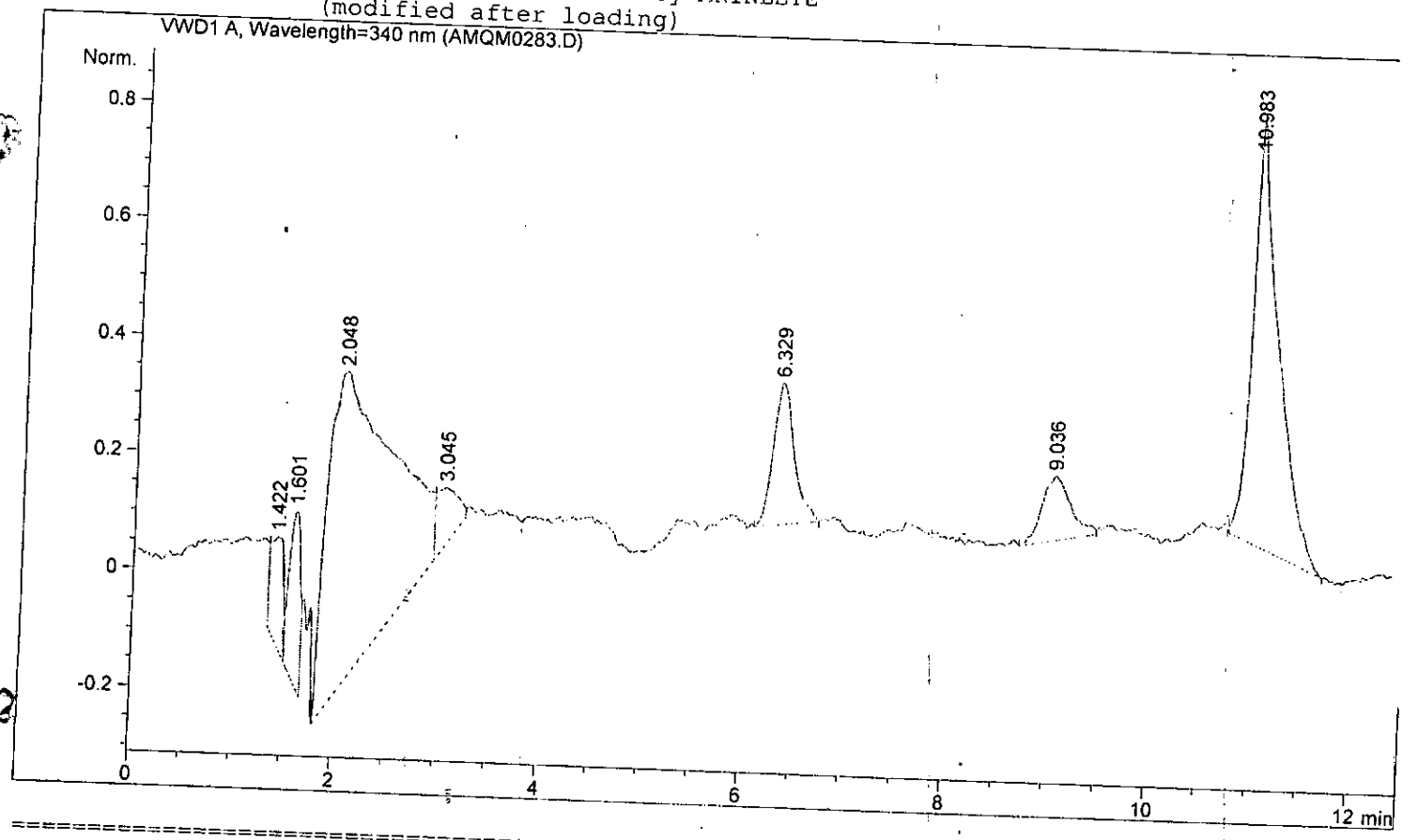



```
==========================================================================
                        Area Percent Report
==========================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: VWD1 A, Wavelength=340 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]      [min]  mAU   *s     [mAU   ]     %
----|-------|----|-------|---------|----------|--------|
   1   1.454 BV    0.0985    2.73866 4.08955e-1   2.9048
   2   1.601 VP    0.1450    5.45750 5.70806e-1   5.7886
   3   2.004 VV    0.3295   31.91515    1.22749  33.8514
   4   2.547 VV    0.2494    9.84457 4.89001e-1  10.4418
   5   3.062 VV    0.1700    2.25491 1.81180e-1   2.3917
   6   6.359 VV    0.2570    6.89489 3.84976e-1   7.3132
   7   9.025 VV    0.2309    3.20648 1.72917e-1   3.4010
   8  10.738 BV    0.2980   31.96806    1.35770  33.9075

Totals :                    94.28021    4.79303

Results obtained with enhanced integrator!
==========================================================================
                        *** End of Report ***
```

MOBILE PHASE:PO4/METHANOL(80/20).

Sample Name: AMODIAQUI

Injection Date : 11/23/2006 2:24:26 PM
Sample Name : AMODIAQUINE
Acq. Operator : AKINLEYE Location : Vial 1
Method : C:\HPCHEM\1\METHODS\AKINAMO.M
Last changed : 11/23/2006 2:08:57 PM by AKINLEYE
(modified after loading)



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: VWD1 A, Wavelength=340 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	1.422	VV	0.1105	1.59078	1.79872e-1	3.4545
2	1.601	VV	0.1242	2.20799	2.88687e-1	4.7948
3	2.048	VV	0.4908	22.09620	5.43018e-1	47.9837
4	3.045	VV	0.1712	1.37145	9.93641e-2	2.9782
5	6.329	VP	0.2150	3.47976	2.43096e-1	7.5566
6	9.036	VV	0.2431	2.16898	1.10021e-1	4.7101
7	10.983	VP	0.2318	13.13421	7.24696e-1	28.5220
Totals :				46.04937	2.18875	

Results obtained with enhanced integrator!

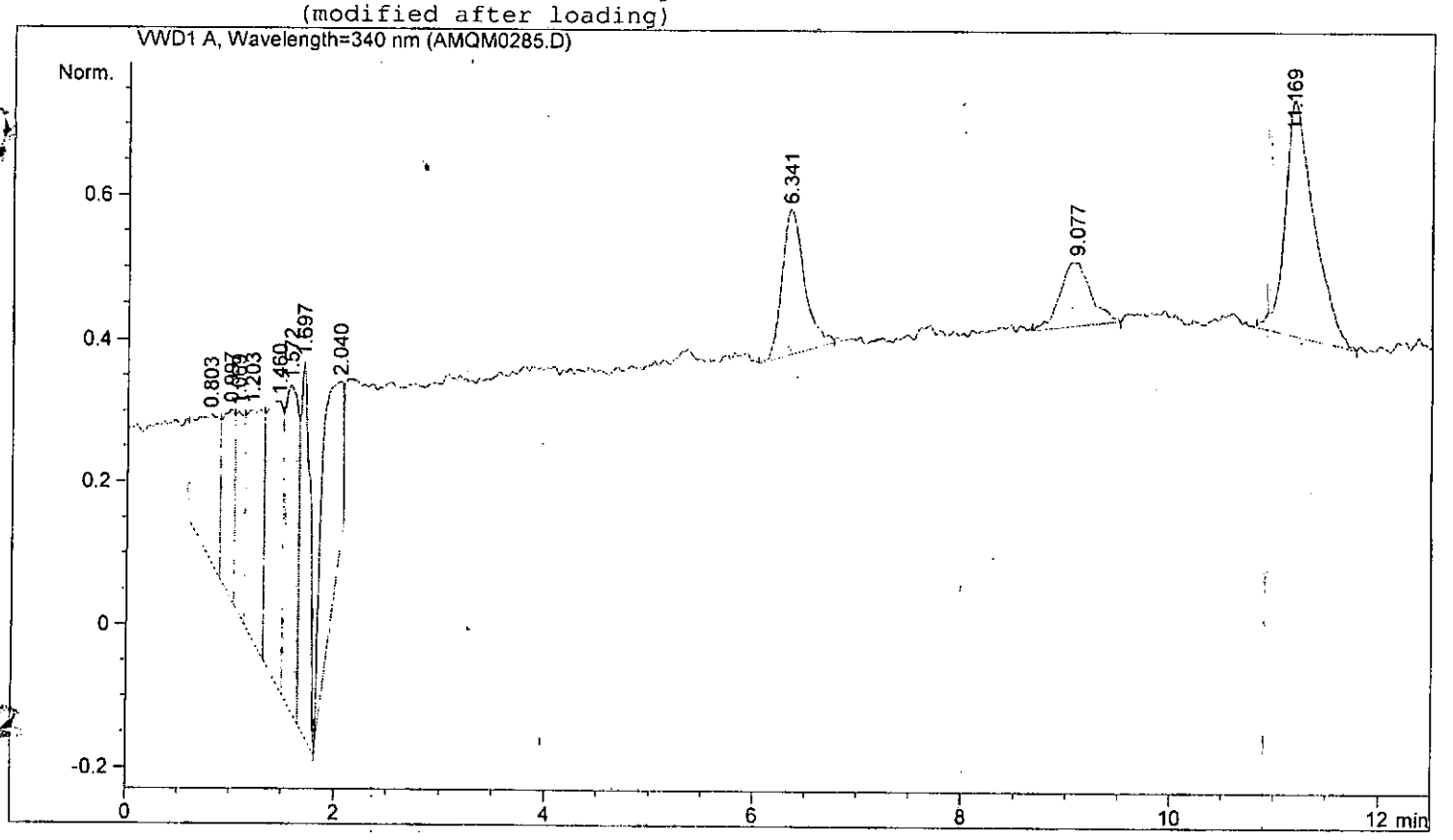
*** End of Report ***

30.11.2006

strument 1 11/23/2006 2:36:48 PM AKINLEYE

35 MOBILE PHASE:PO4/METHANOL(80/20).

```
Injection Date   : 11/23/2006 2:59:06 PM
Sample Name      : AMODIAQUINE                    Location : Vial 1
Acq. Operator    : AKINLEYE
Method           : C:\HPCHEM\1\METHODS\AKINAMO.M
Last changed     : 11/23/2006 2:08:57 PM by AKINLEYE
                   (modified after loading)
```



Area Percent Report

```
Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
```

Signal 1: VWD1 A, Wavelength=340 nm

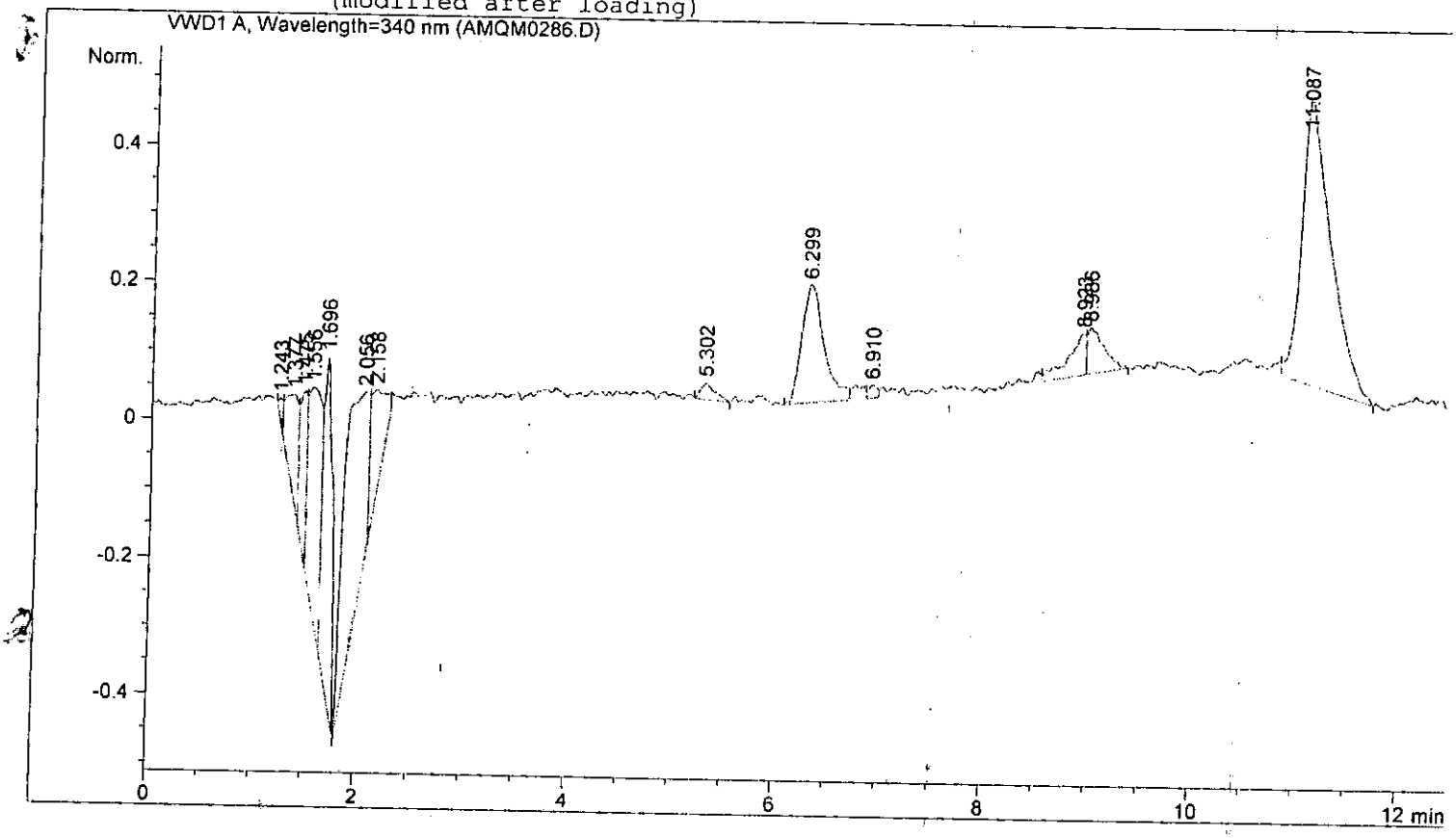
Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	0.803	VV	0.2040	3.36056	2.03867e-1	8.9274
2	0.997	VV	0.1012	2.05981	2.63948e-1	5.4719
3	1.069	VV	0.0769	1.63721	2.80202e-1	4.3493
4	1.203	VV	0.1399	3.62837	3.19103e-1	9.6389
5	1.460	VV	0.1315	4.17115	4.01039e-1	11.0808
6	1.572	VV	0.1210	3.96362	4.53248e-1	10.5295
7	1.697	VP	0.0864	3.33630	5.20496e-1	8.8630
8	2.040	VV	0.2211	4.33601	2.41568e-1	11.5188
9	6.341	VV	0.2073	2.99719	1.99503e-1	7.9621
10	9.077	BP	0.2535	1.80986	8.73001e-2	4.8080
11	11.169	BP	0.2640	6.34298	3.27192e-1	16.8503
Totals :				37.64308	3.29747	

Results obtained with enhanced integrator!

*** End of Report *** 239

MOBILE PHASE:PO4/METHANOL(80/20).

```
Injection Date   : 11/23/2006 3:13:00 PM
Sample Name      : AMODIAQUINE                    Location : Vial 1
Acq. Operator    : AKINLEYE
Acq. Method      : C:\HPCHEM\1\METHODS\AKINAMO.M
Last changed     : 11/23/2006 2:08:57 PM by AKINLEYE
                   (modified after loading)
Analysis Method  : C:\HPCHEM\1\METHODS\AKINAMO.M
Last changed     : 11/23/2006 3:21:32 PM by AKINLEYE
                   (modified after loading)
```



Area Percent Report

```
Sorted By        :      Signal
Multiplier       :      1.0000
Dilution         :      1.0000
```

Signal 1: VWD1 A, Wavelength=340 nm

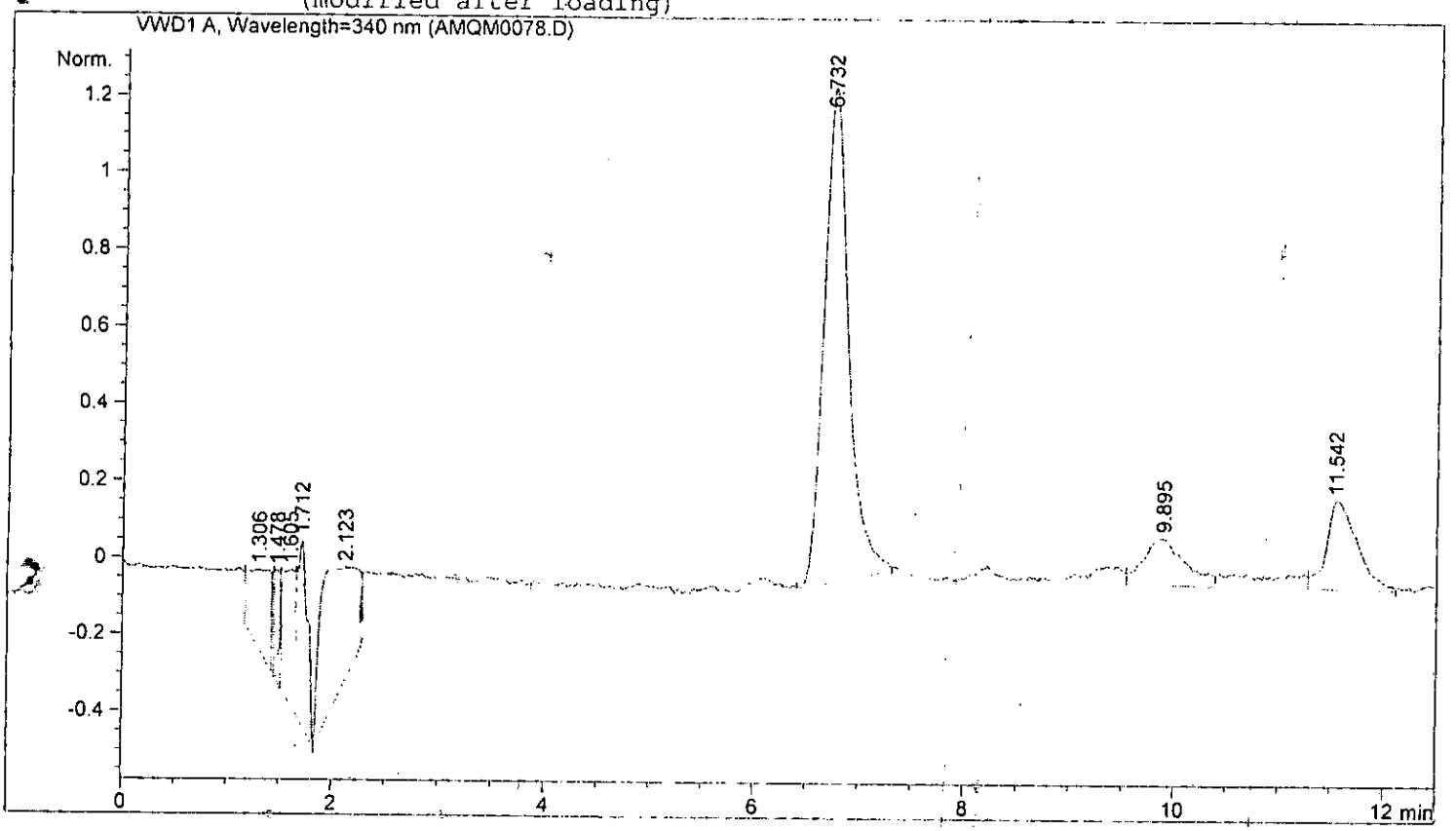
Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	1.243	BV	0.0447	1.07800e-1	3.50071e-2	0.3991
2	1.377	VV	0.0965	1.10701	1.49713e-1	4.0981
3	1.475	VV	0.0638	1.08224	2.35438e-1	4.0065
4	1.556	VV	0.1235	2.82604	3.06681e-1	10.4619
5	1.696	VP	0.0849	2.97624	4.64574e-1	11.0180
6	2.056	VV	0.2355	4.93895	2.60850e-1	18.2839
7	2.158	VB	0.1090	1.38135	1.63111e-1	5.1137
8	5.302	VP	0.1212	2.28911e-1	2.40678e-2	0.8474
9	6.299	PV	0.1950	2.56564	1.71457e-1	9.4980
10	6.910	VV	0.0904	1.06949e-1	1.86024e-2	0.3959
11	8.923	VV	0.1480	7.34230e-1	6.02385e-2	2.7181
12	8.986	VB	0.1381	7.39732e-1	6.74953e-2	2.7385
13	11.087	BP	0.2605	8.21744	4.17037e-1	30.4208
Totals :				27.01252	2.37427	

240

MOBILE PHASE BUFFER:METHANOL 80:20 pH 2.8 COLUMN 250 X 4.6MM

==========

Injection Date : 10/26/2006 11:08:59 AM
Sample Name : AMODIAQUINE Location : Vial 1
Acq. Operator : AKINLEYE
Acq. Method : C:\HPCHEM\1\METHODS\AKINAMO.M
Last changed : 10/26/2006 11:07:24 AM by AKINLEYE
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\AKINAMO.M
Last changed : 10/26/2006 3:48:27 PM by AKINLEYE
(modified after loading)



==========

Area Percent Report

==========

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: VWD1 A, Wavelength=340 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	1.306	VB	0.1810	3.09497	2.07798e-1	6.5555
2	1.478	BV	0.0537	1.15614	2.90422e-1	2.4488
3	1.605	VV	0.1088	2.99071	3.53777e-1	6.3347
4	1.712	VP	0.0890	3.19332	4.81352e-1	6.7638
5	2.123	VV	0.3201	7.86353	2.96063e-1	16.6559
6	6.732	VB	0.2436	20.88175	1.28389	44.2300
7	9.895	VV	0.3430	3.39538	1.20089e-1	7.1918
8	11.542	VV	0.2613	4.63598	2.27242e-1	9.8195
Totals :				47.21178	3.26063	

Results obtained with enhanced integrator!

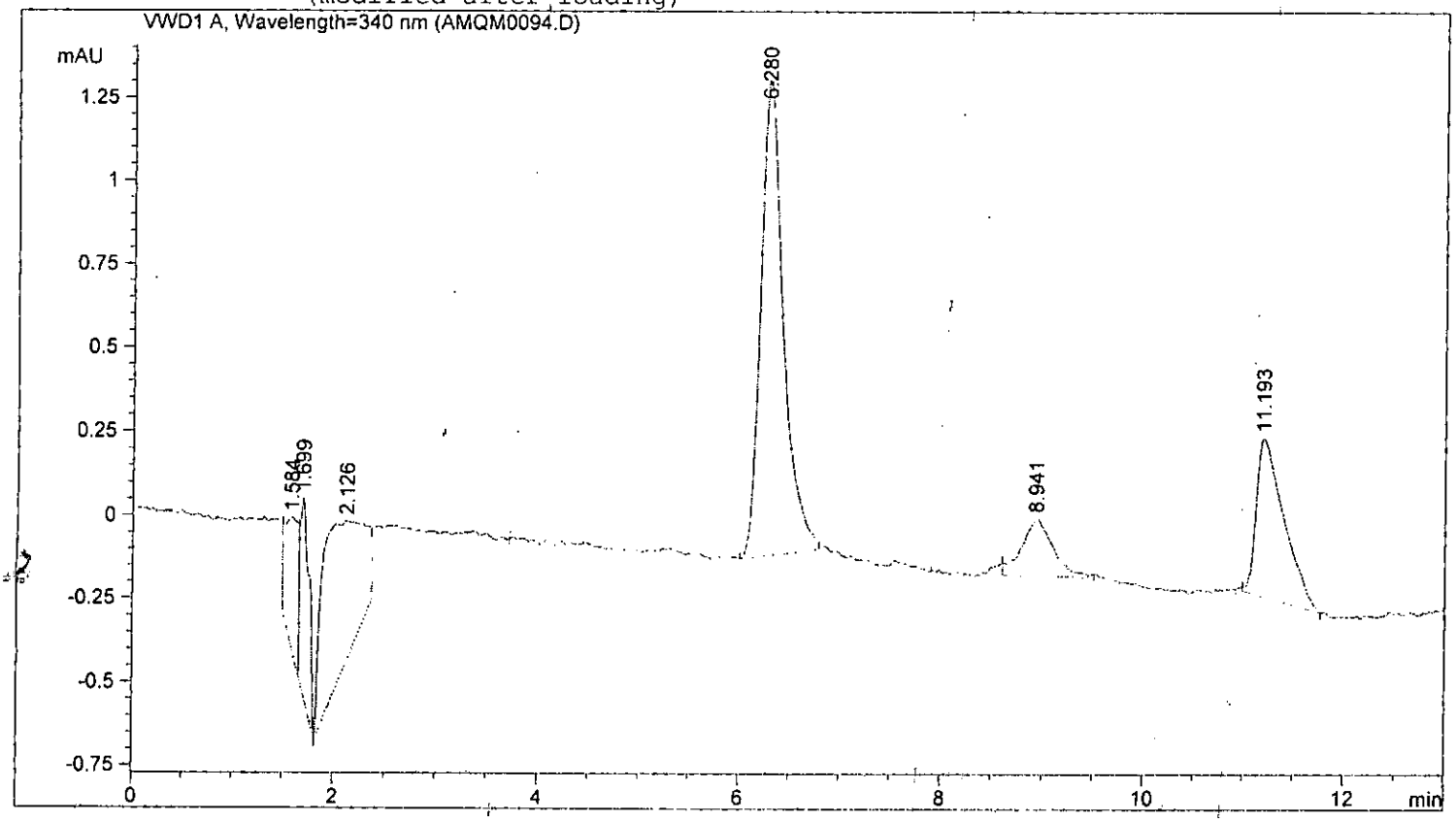
==========

241

*** End of Report ***

MOBILE PHASE BUFFER:METHANOL 80:20 pH 2.8 COLUMN 250 X 4.6MM

Injection Date : 10/26/2006 3:11:20 PM
Sample Name : AMODIAQUINE Location : Vial 1
Acq. Operator : AKINLEYE
Acq. Method : C:\HPCHEM\1\METHODS\AKINAMO.M
Last changed : 10/26/2006 12:46:36 PM by AKINLEYE
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\
Last changed : 11/9/2006 2:17:43 PM
(modified after loading)



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: VWD1 A, Wavelength=340 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	1.584	BV	0.1283	3.69975	3.89408e-1	6.6934
2	1.699	VP	0.0844	3.72071	5.85109e-1	6.7314
3	2.126	VV	0.3678	12.78304	4.23934e-1	23.1266
4	6.280	PB	0.2335	22.26187	1.42291	40.2753
5	8.941	BB	0.2731	3.71695	1.71569e-1	6.7246
6	11.193	BB	0.2667	9.09193	4.72434e-1	16.4488
Totals :				55.27426	3.46537	

Results obtained with enhanced integrator!

*** End of Report ***

242

39 MOBILE PHASE BUFFER:METHANOL 80:20 pH 2.8 COLUMN 250 X 4.6MM

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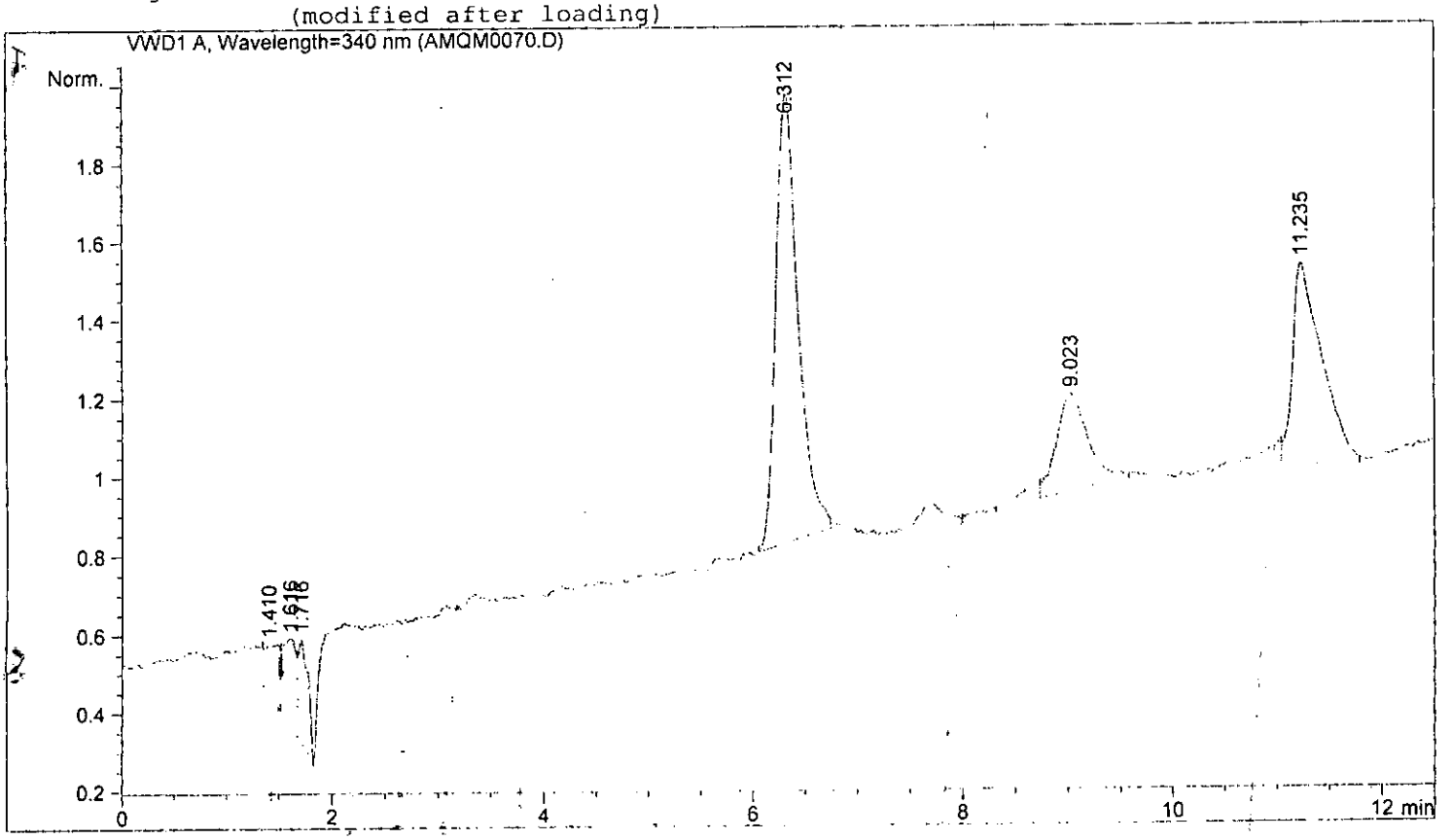
Injection Date : 10/25/2006 3:03:39 PM
Sample Name : AMODIAQUINE Location : Vial 1
Acq. Operator : AKINLEYE
Method : C:\HPCHEM\1\METHODS\AKINAMO.M
Last changed : 10/25/2006 12:08:28 PM by AKINLEYE
(modified after loading)



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Area Percent Report

==========

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: VWD1 A, Wavelength=340 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	1.410	BV	0.1371	1.39738	1.29918e-1	3.7561
2	1.616	VV	0.1186	1.91981	2.28064e-1	5.1604
3	1.716	VP	0.0849	1.70436	2.66232e-1	4.5812
4	6.312	PB	0.2218	17.07186	1.14642	45.8883
5	9.023	BB	0.2871	5.18258	2.46923e-1	13.9305
6	11.235	VB	0.2558	9.92709	5.20850e-1	26.6835
Totals :				37.20307	2.53840	

Results obtained with enhanced integrator!

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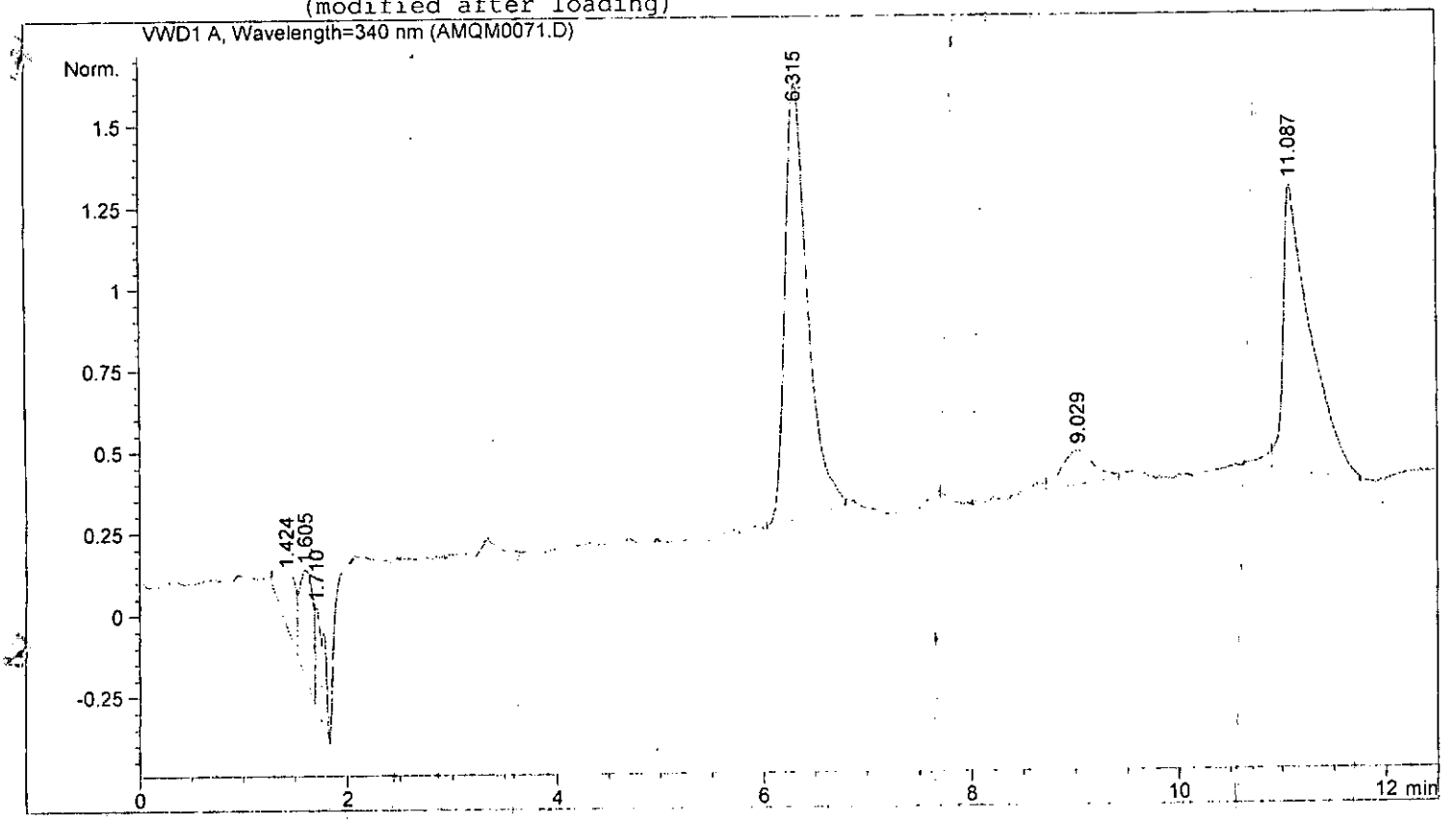
*** End of Report ***

Instrument 1 10/25/2006 3:15:23 PM AKINLEYE Page 1 of 1

three 2 hours.

40 MOBILE PHASE BUFFER:METHANOL 80:20 pH 2.8 COLUMN 250 X 4.6MM

Injection Date : 10/25/2006 3:18:20 PM
Sample Name : AMODIAQUINE Location : Vial 1
Acq. Operator : AKINLEYE
Method : C:\HPCHEM\1\METHODS\AKINAMO.M
Last changed : 10/25/2006 12:08:28 PM by AKINLEYE
(modified after loading)



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: VWD1 A, Wavelength=340 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	1.424	BV	0.1506	1.89654	1.55958e-1	4.2392
2	1.605	VV	0.1398	2.96931	3.30381e-1	6.6371
3	1.710	VV	0.0498	1.05298	3.10441e-1	2.3536
4	6.315	PB	0.2285	20.39159	1.33968	45.5797
5	9.029	BV	0.2605	2.33712	1.07665e-1	5.2240
6	11.087	BB	0.2399	16.09078	8.78582e-1	35.9664
Totals :				44.73833	3.12271	

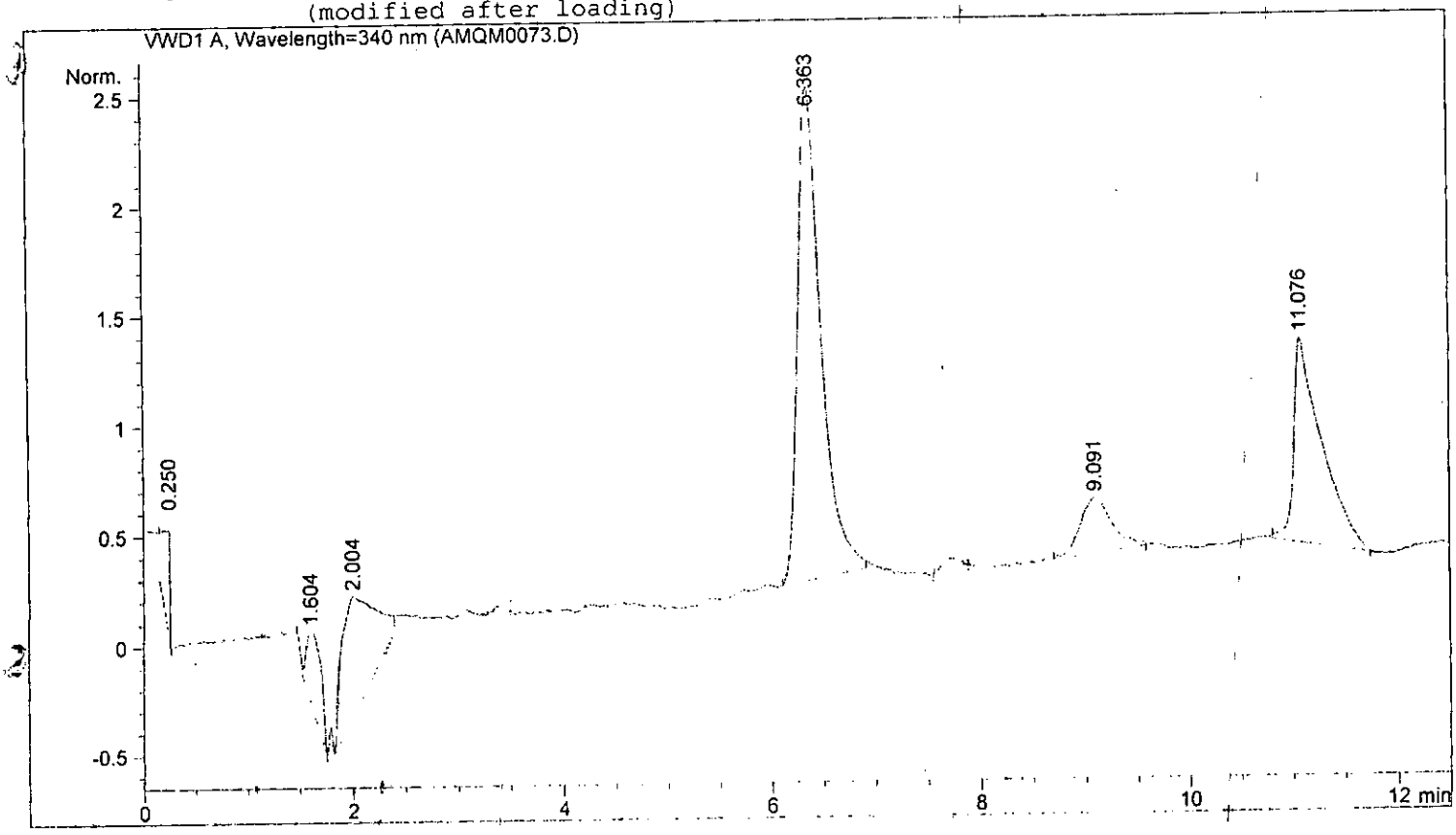
Results obtained with enhanced integrator!

*** End of Report ***

MOBILE PHASE BUFFER:METHANOL 80:20 pH 2.8 COLUMN 250 X 4.6MM

41

Injection Date : 10/25/2006 3:48:07 PM
Sample Name : AMODIAQUINE Location : Vial 1
Acq. Operator : AKINLEYE
Method : C:\HPCHEM\1\METHODS\AKINAMO.M
Last changed : 10/25/2006 12:08:28 PM by AKINLEYE
(modified after loading)



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: VWD1 A, Wavelength=340 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	0.250	BP	0.0656	2.17549	5.52247e-1	2.9048
2	1.604	BP	0.1652	3.63497	3.29208e-1	4.8536
3	2.004	VB	0.2640	11.49265	5.43782e-1	15.3455
4	6.363	BB	0.2354	34.96724	2.24688	46.6898
5	9.091	PB	0.2694	5.28150	2.50350e-1	7.0521
6	11.076	BB	0.2428	17.34077	9.33945e-1	23.1542
Totals :				74.89262	4.85642	

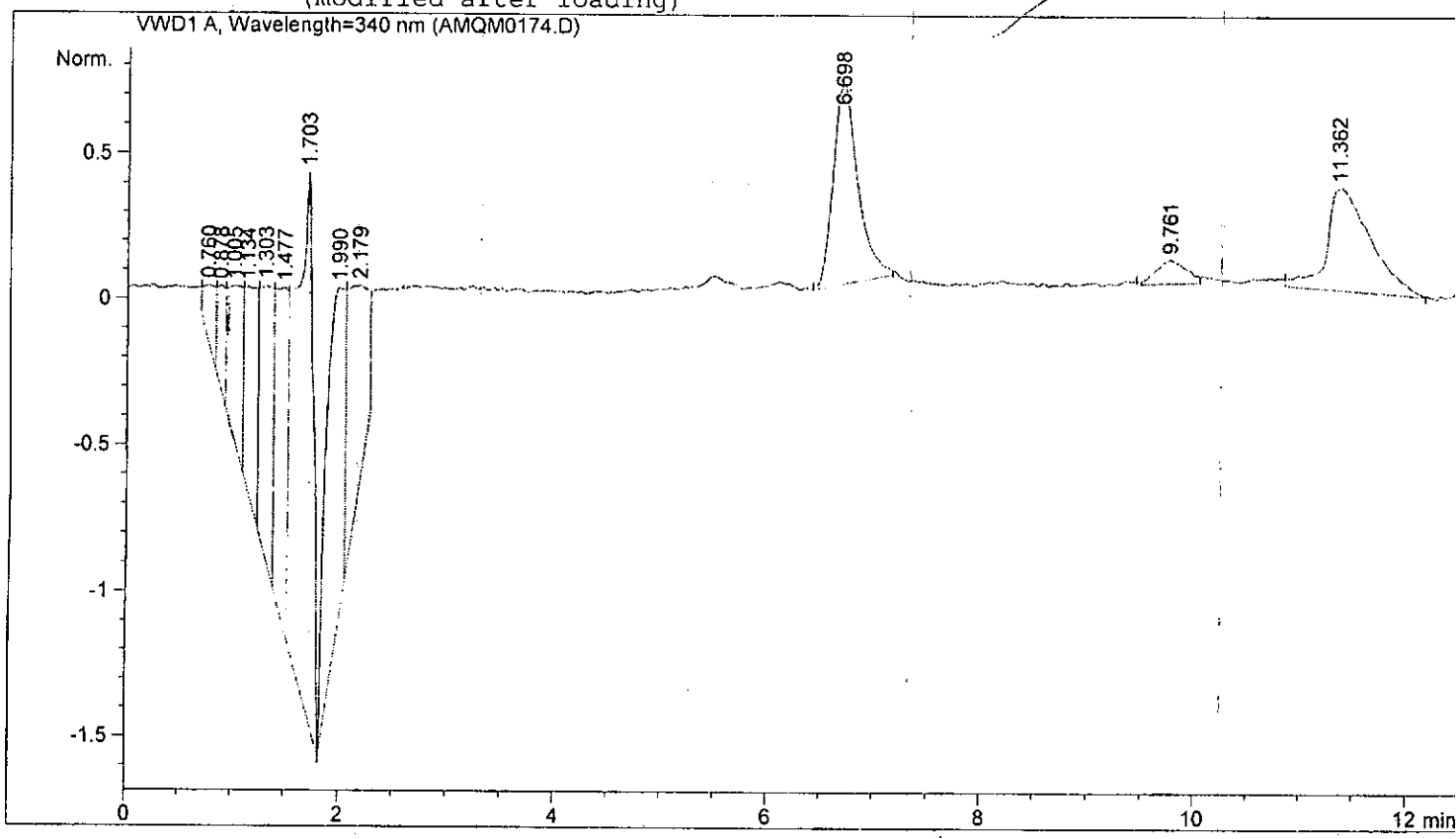
Results obtained with enhanced integrator!

*** End of Report ***

42

MOBILE PHASE BUFFER:METHANOL 80:20 pH 2.8 COLUMN 250 X 4.6MM

Injection Date : 11/3/2006 10:10:29 AM
Sample Name : AMODIAQUINE Location : Vial 1
Acq. Operator : AKINLEYE
Acq. Method : C:\HPCHEM\1\METHODS\AKINAMO.M
Last changed : 11/3/2006 8:43:38 AM by AKINLEYE
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\AKINAMO.M
Last changed : 11/10/2006 3:20:55 PM by
(modified after loading)



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

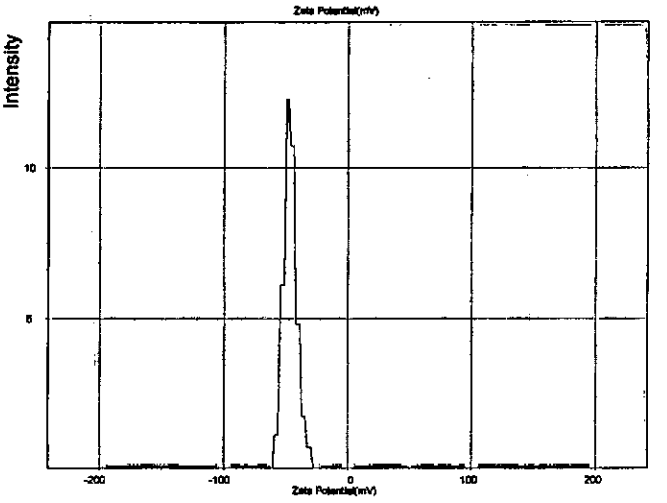
Signal 1: VWD1 A, Wavelength=340 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	0.760	VV	0.1110	1.62269	1.85101e-1	1.6129
2	0.878	VV	0.0774	1.88574	3.42204e-1	1.8744
3	1.005	VV	0.1244	5.21494	5.13167e-1	5.1836
4	1.134	VB	0.1106	5.89744	6.85575e-1	5.8620
5	1.303	BV	0.1108	8.03554	9.18835e-1	7.9873
6	1.477	VV	0.1025	9.07585	1.14698	9.0214
7	1.703	VP	0.1503	21.99032	1.85087	21.8583
8	1.990	VV	0.1925	14.16377	1.16709	14.0787
9	2.179	VV	0.1729	9.64402	6.91394e-1	9.5861
10	6.698	VB	0.2390	11.18621	6.83585e-1	11.1190
11	9.761	BV	0.2525	1.63643	8.12154e-2	1.6266
12	11.362	VB	0.3437	10.25104	3.55535e-1	10.1895
Totals :				100.60401	8.62156	

Instrument 1 11/10/2006 3:20:59 PM

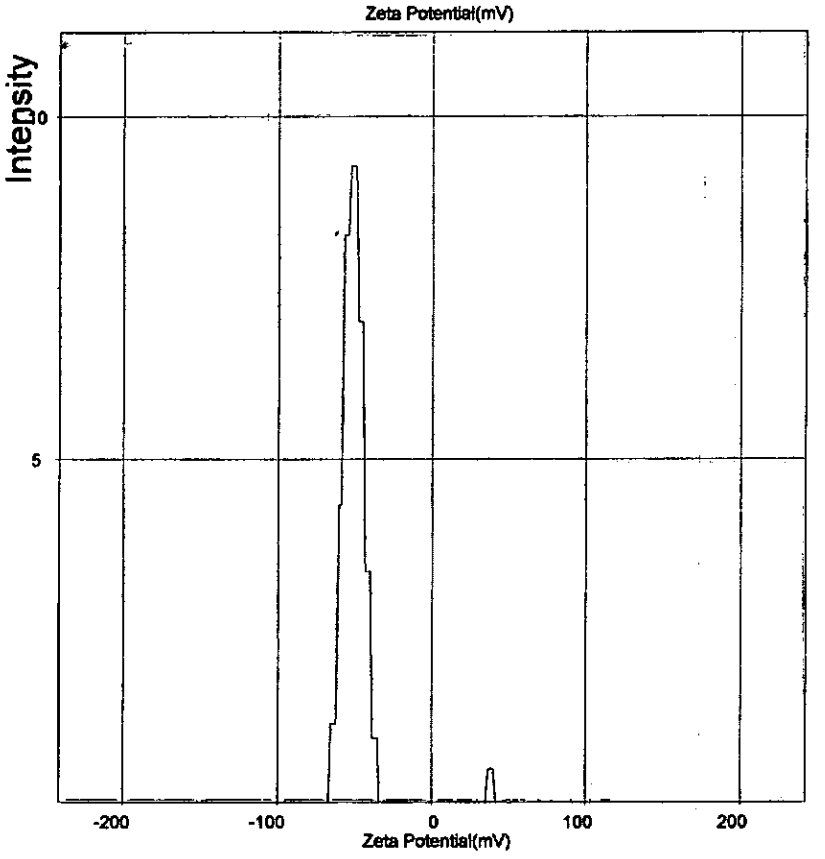
C 8HR

APPENDIX IIIa: Shows the Photon Correlation Spectroscopic Charts of DNA/DAB16 Complexes (DENDRIPLEXES) for zeta potential and particle size measurements.



Run	Pos.	KCps	Mob.	Zeta	Width	Time
1	50.0	1310.3	-3.661	-46.2	1.6	10:58:24
2	50.0	1298.9	-3.693	-46.6	2.7	11:00:40
3	50.0	1298.6	-3.670	-46.3	1.6	11:02:55
Average		1302.6	-3.675	-46.3	1.9	
+/-		6.7	0.016	0.2	0.6	

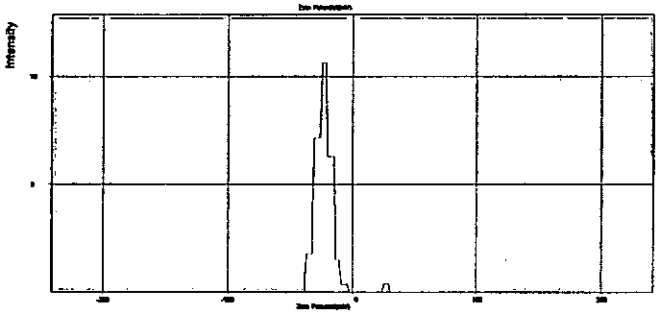
Zeta Potential of Standard Solution



Run	Pos.	KCps	Mob.	Zeta	Width	Time
1	50.0	1208.1	-3.581	-45.2	1.6	11:16:37
2	50.0	1177.9	-3.633	-45.8	16.2	11:18:39
3	50.0	1159.5	-3.702	-46.7	19.2	11:20:41
Average		1181.9	-3.639	-45.9	12.3	
+/-		24.5	0.061	0.8	9.4	

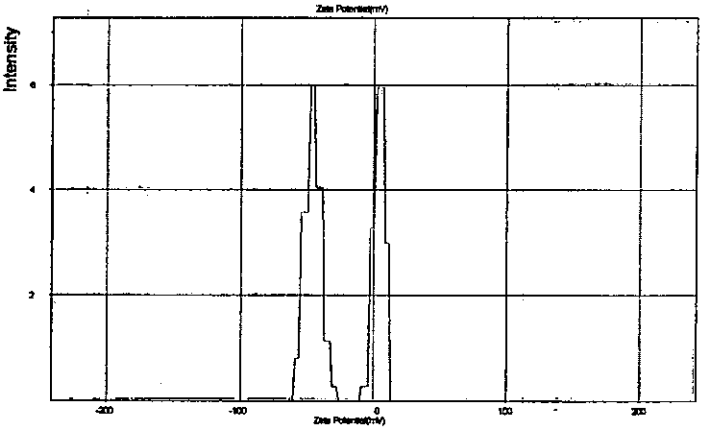
Standard 2

Sample 25/8(1)



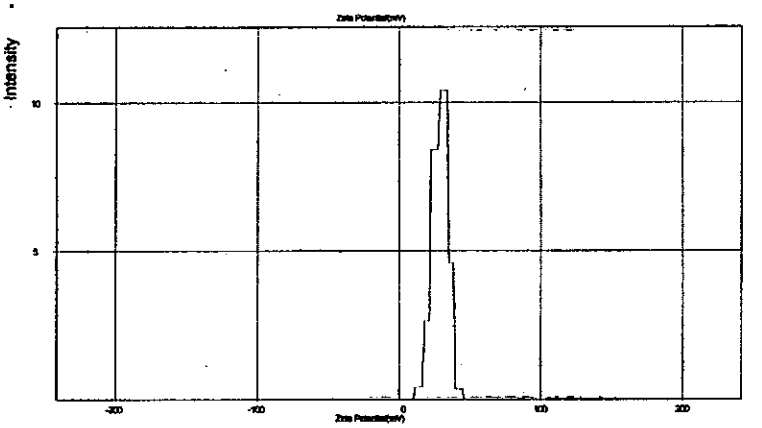
Run	Pos.	KCps	Mob.	Zeta	Width	Time
1	50.0	392.8	-1.382	-22.0	23.9	14:53:11
2	50.0	691.3	-1.011	-16.1	20.4	15:00:09
3	50.0	1050.2	-1.341	-21.4	9.4	15:07:06
Average		711.4	-1.245	-19.9	17.9	
+/-		329.2	0.203	3.2	7.6	

25/8(2)



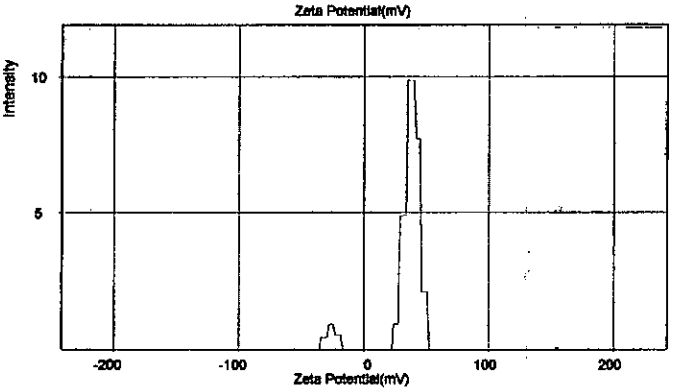
Run	Pos.	KCps	Mob.	Zeta	Width	Time
1	50.0	1194.5	-1.319	-21.0	12.1	15:35:56
2	50.0	1186.4	-1.378	-22.0	9.5	15:41:36
3	50.0	1183.2	-1.475	-23.5	22.6	15:47:15
Average		1188.0	-1.391	-22.2	14.8	
+/-		5.8	0.079	1.3	6.9	

25/15



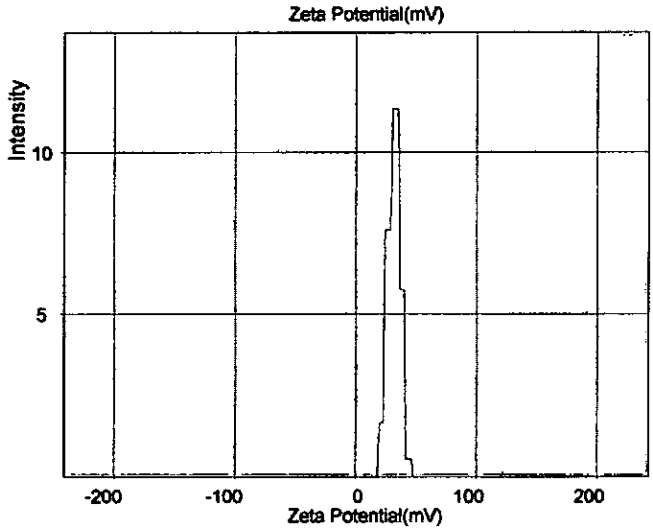
Run	Pos.	KCps	Mob.	Zeta	Width	Time
1	50.0	1055.9	1.346	21.8	23.0	14:55:26
2	50.0	1095.7	1.161	18.8	25.1	14:58:25
3	50.0	1118.0	1.297	21.0	13.4	15:01:25
Average		1089.9	1.268	20.6	20.5	
+/-		31.4	0.096	1.6	6.2	

25/48



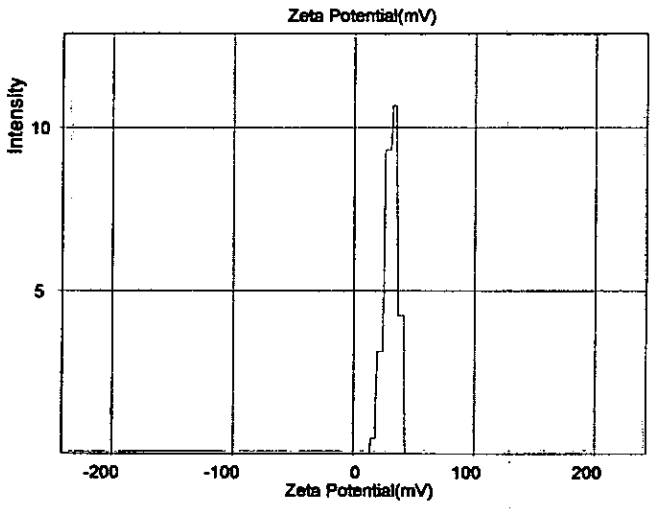
Run	Pos.	KCps	Mob.	Zeta	Width	Time
1	50.0	699.8	1.645	26.7	16.0	08:47:37
2	50.0	850.7	1.537	24.9	20.3	08:53:02
3	50.0	1051.6	1.411	22.9	26.8	08:59:45
Average		867.4	1.531	24.8	21.0	
+/-		176.5	0.117	1.9	5.5	

25/80



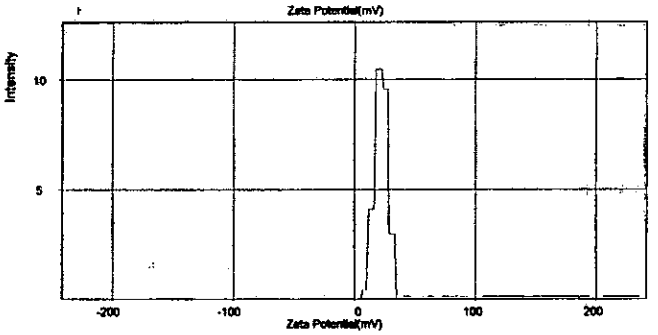
Run	Pos.	KCps	Mob.	Zeta	Width	Time
1	50.0	917.6	1.637	26.6	28.3	19:17:00
2	50.0	968.2	1.547	25.1	22.3	19:22:26
3	50.0	1136.3	1.673	27.2	26.6	19:27:50
Average		1007.4	1.619	26.3	25.8	
+/-		114.5	0.065	1.1	3.0	

25/120



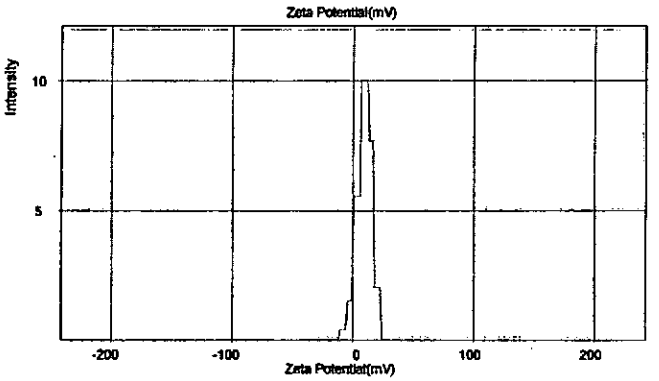
Run	Pos.	KCps	Mob.	Zeta	Width	Time
1	50.0	1294.2	1.637	26.6	2.0	17:34:53
2	50.0	1308.3	1.584	25.7	2.0	17:36:05
3	50.0	1318.1	1.692	27.5	2.0	17:37:16
Average		1306.9	1.638	26.6	2.0	
+/-		12.0	0.054	0.9	0.0	

25/750



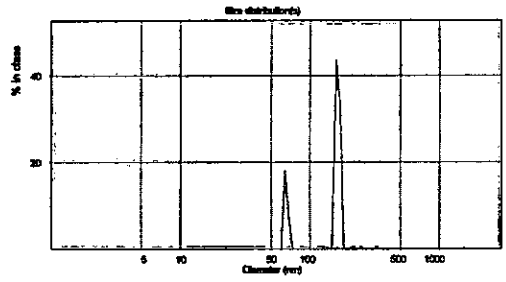
Run	Pos.	KCps	Mob.	Zeta	Width	Time
1	50.0	1218.9	2.155	35.0	2.0	13:17:24
2	50.0	1213.4	2.390	38.8	17.6	13:21:56
3	50.0	1203.3	2.240	36.4	28.3	13:26:28
Average		1211.8	2.262	36.7	15.9	
+/-		7.9	0.119	1.9	13.2	

50/16



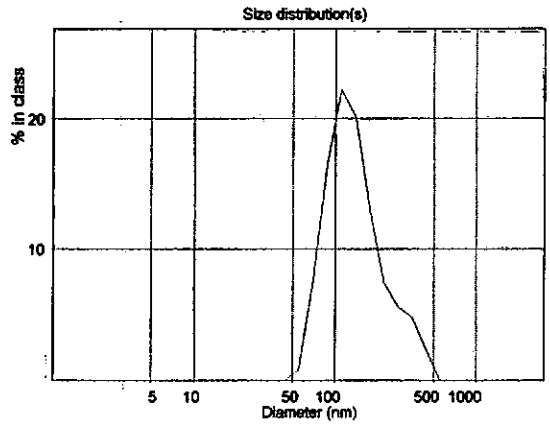
Run	Pos.	KCps	Mob.	Zeta	Width	Time
1	50.0	1063.2	0.576	9.3	2.0	19:13:52
2	50.0	1098.4	0.740	12.0	2.0	19:14:56
3	50.0	1186.6	0.594	9.6	2.0	19:15:59
Average		1116.1	0.637	10.3	2.0	
+/-		63.6	0.090	1.5	0.0	

25/15(1)



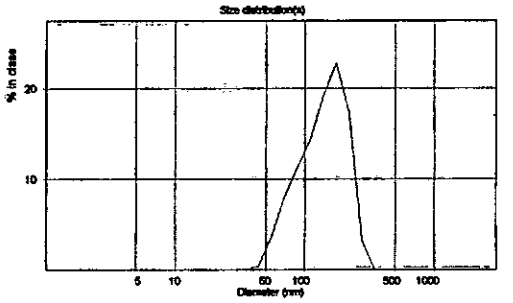
Run	Angle	KCps.	ZAve	Poly	Fit	Time
1	90.0	266.7	123.1	0.171	0.000130	14:14:26
2	90.0	266.6	123.7	0.179	0.000133	14:19:42
3	90.0	268.0	125.2	0.168	0.000175	14:25:01
Average		267.1	124.0	0.173		
+/-		0.8	1.1	0.006		

25/15(2)



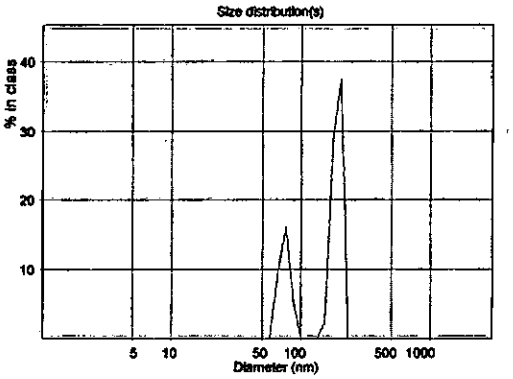
Run	Angle	KCps.	ZAve	Poly	Fit	Time
1	90.0	266.8	125.6	0.171	0.000157	14:31:34
2	90.0	267.8	124.7	0.173	0.000152	14:36:33
3	90.0	267.3	124.8	0.177	0.000148	14:41:37
Average		267.3	125.1	0.174		
+/-		0.5	0.5	0.003		

25/15(3)



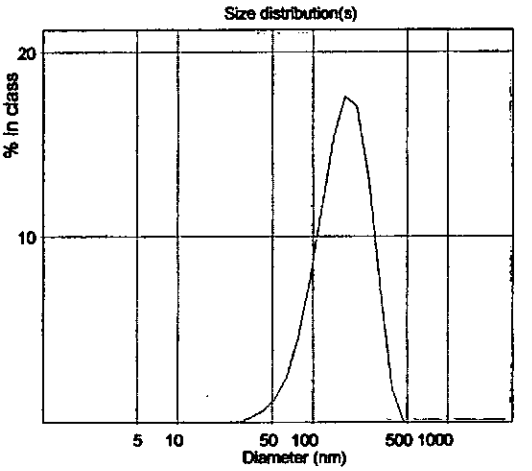
Run	Angle	KCps.	ZAve	Poly	Fit	Time
1	90.0	265.7	124.7	0.165	0.000127	14:48:23
2	90.0	264.4	123.4	0.195	0.000146	14:53:25
3	90.0	265.5	124.2	0.178	0.000136	14:58:27
Average		265.2	124.1	0.180		
+/-		0.7	0.7	0.015		

25/24(1)



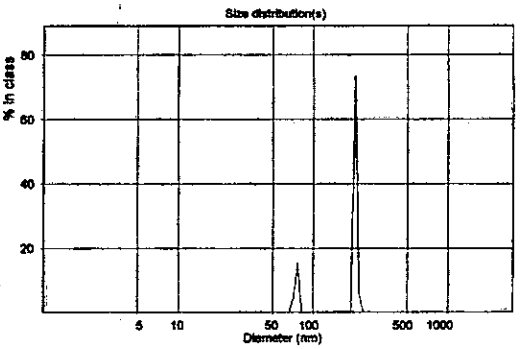
Run	Angle	KCps.	ZAve	Poly	Fit	Time
1	90.0	244.3	130.5	0.268	0.000194	15:08:12
2	90.0	243.6	131.0	0.240	0.000220	15:13:31
3	90.0	244.3	132.6	0.244	0.000122	15:18:50
Average		244.1	131.4	0.251		
+/-		0.4	1.1	0.015		

25/40(1)



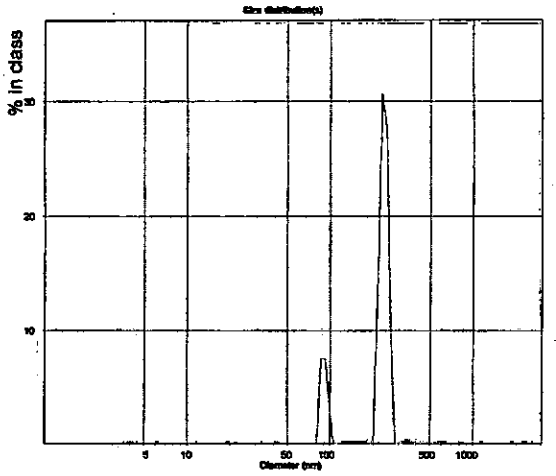
Run	Angle	KCps.	ZAve	Poly	Fit	Time
1	90.0	394.5	147.8	0.250	0.000117	17:00:04
2	90.0	395.0	148.5	0.250	0.000077	17:04:40
3	90.0	391.7	149.5	0.240	0.000113	17:09:17
Average		393.7	148.6	0.247		
+/-		1.8	0.8	0.005		

25/48(1)



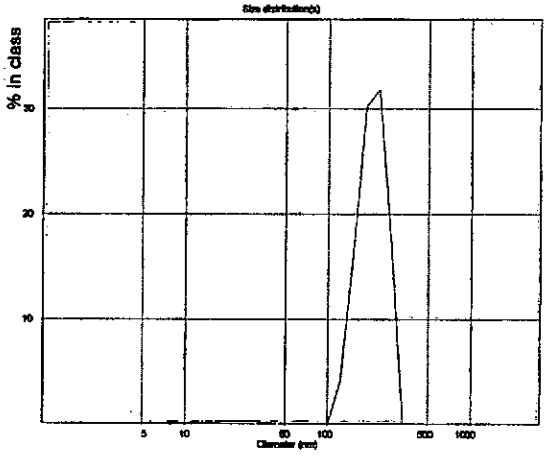
Run	Angle	KCps.	ZAve	Poly	Fit	Time
1	90.0	434.7	147.1	0.231	0.000124	17:50:24
2	90.0	437.1	148.3	0.218	0.000126	17:55:01
3	90.0	436.6	149.3	0.228	0.000093	17:59:38
Average			436.1	148.2	0.226	
+/-		1.2	1.1	0.007		

25/80(1)



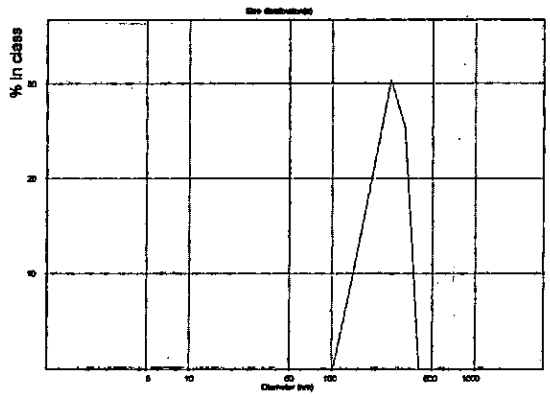
Run	Angle	KCps.	ZAve	Poly	Fit	Time
1	90.0	370.0	195.2	0.174	0.000136	10:10:49
2	90.0	374.6	183.3	0.223	0.000090	10:15:52
3	90.0	379.1	180.2	0.208	0.000119	10:20:51
Average		374.6	186.2	0.202		
+/-		4.5	7.9	0.025		

25/120(1)

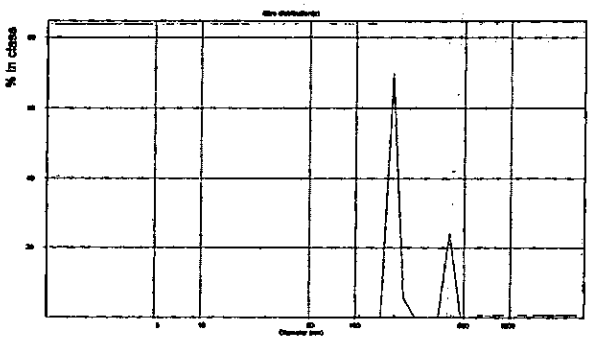


Run	Angle	KCps.	ZAve	Poly	Fit	Time
1	90.0	258.1	202.1	0.182	0.000095	11:11:06
2	90.0	258.3	202.0	0.133	0.000122	11:16:29
3	90.0	259.5	203.1	0.136	0.000123	11:21:49
Average		258.6	202.4	0.150		
+/-		0.8	0.6	0.028		

25/250

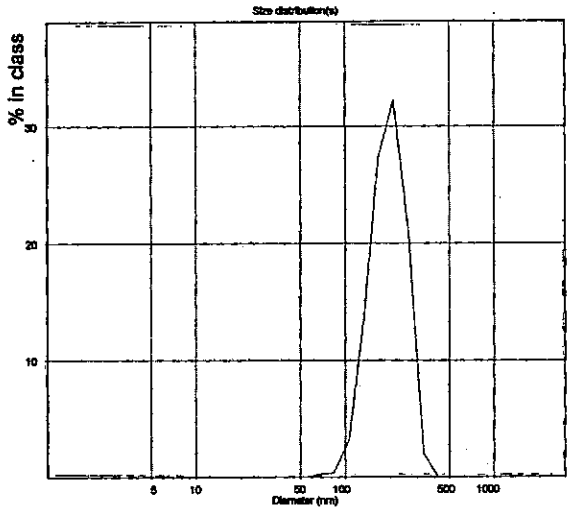


	Run	Angle	KCps.	ZAve	Poly	Fit	Time
1	90.0	253.6	227.4	0.152	0.000113		12:54:31
2	90.0	252.6	227.0	0.155	0.000123		12:59:50
3	90.0	255.7	225.7	0.121	0.000124		13:05:09
Average			254.0	226.7	0.143		
+/-		1.6	0.9	0.019			



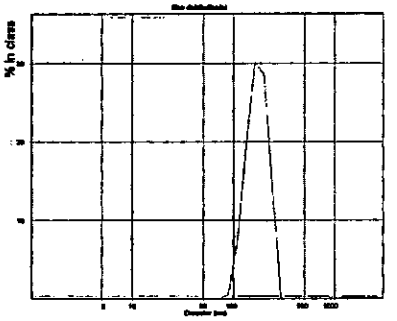
25/750(1)

Run	Angle	KCps.	ZAve	Poly	Fit	Time
1	90.0	220.3	220.9	0.073	0.000129	13:14:46
2	90.0	220.7	222.2	0.063	0.000191	13:20:06
3	90.0	219.6	221.3	0.098	0.000124	13:25:27
Average		220.2	221.4	0.078		
+/-		0.6	0.7	0.018		



50/16(2)

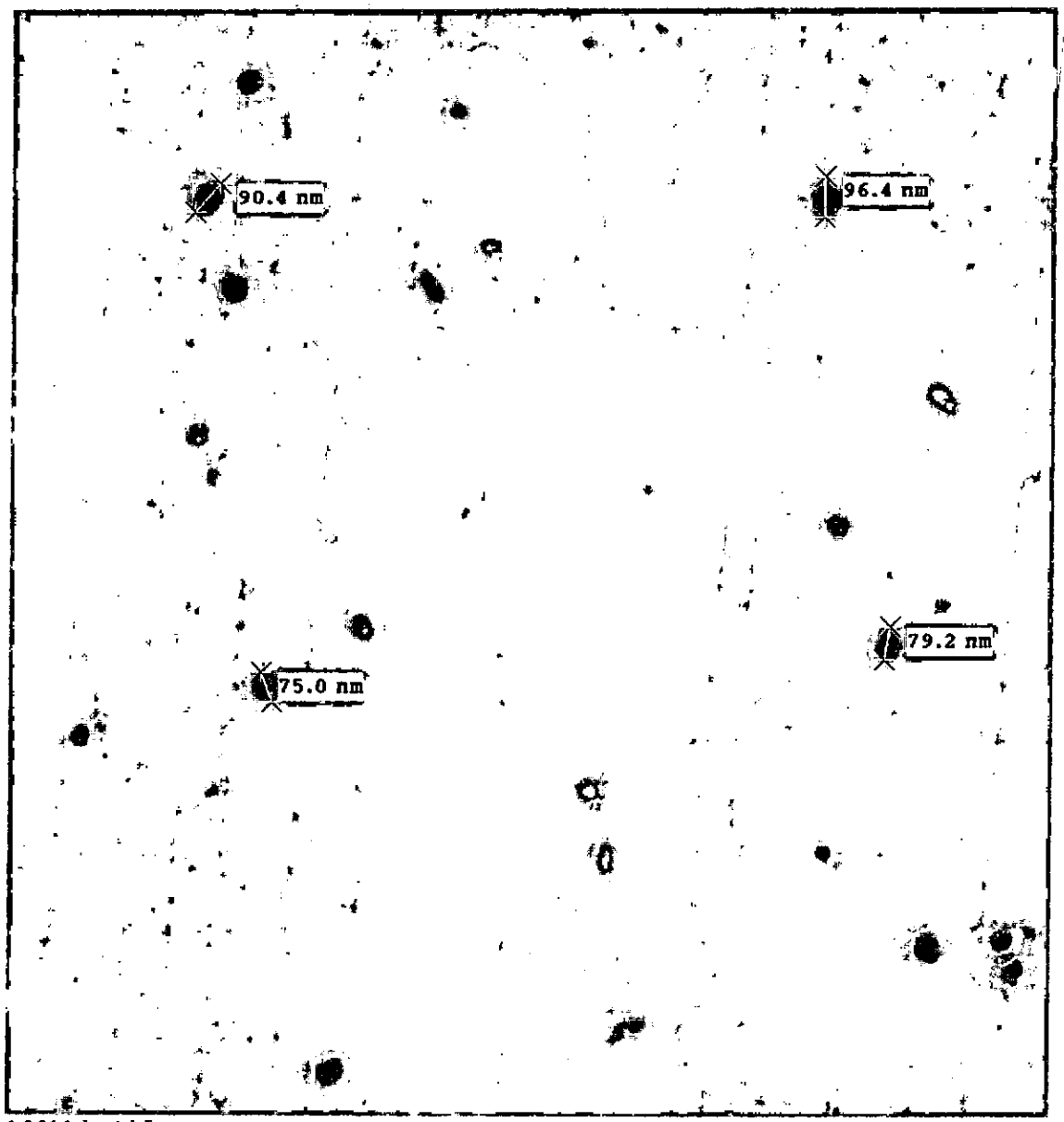
Run	Angle	KCps.	ZAve	Poly	Fit	Time
1	90.0	273.2	185.5	0.118	0.000107	15:07:06
2	90.0	270.2	187.1	0.112	0.000095	15:12:10
3	90.0	272.3	188.1	0.103	0.000156	15:17:14
Average		271.9	186.9	0.111		
+/-		1.5	1.3	0.007		

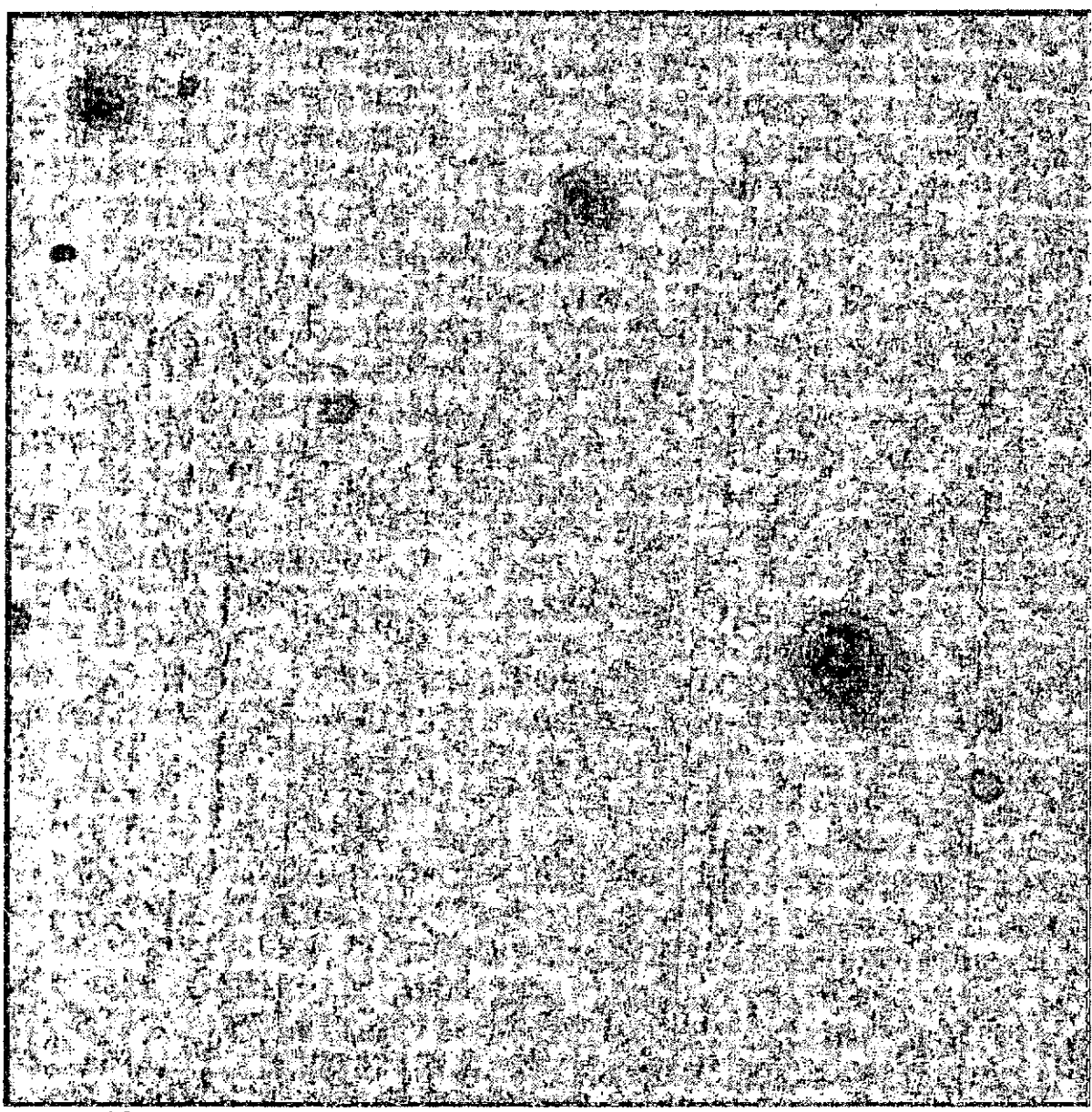


50/500(3)

Run	Angle	KCps.	ZAve	Poly	Fit	Time
1	90.0	207.2	167.3	0.121	0.000139	18:32:50
2	90.0	209.4	168.5	0.149	0.000159	18:38:13
3	90.0	209.6	167.8	0.123	0.000151	18:43:36
Average			208.7	167.9	0.131	
+/-		1.3	0.6	0.016		

APPENDIX IIIb: Shows Transmission Electron Micrograph of Dendriplexes





16611a.tif
50/64
1661.1 1 UA
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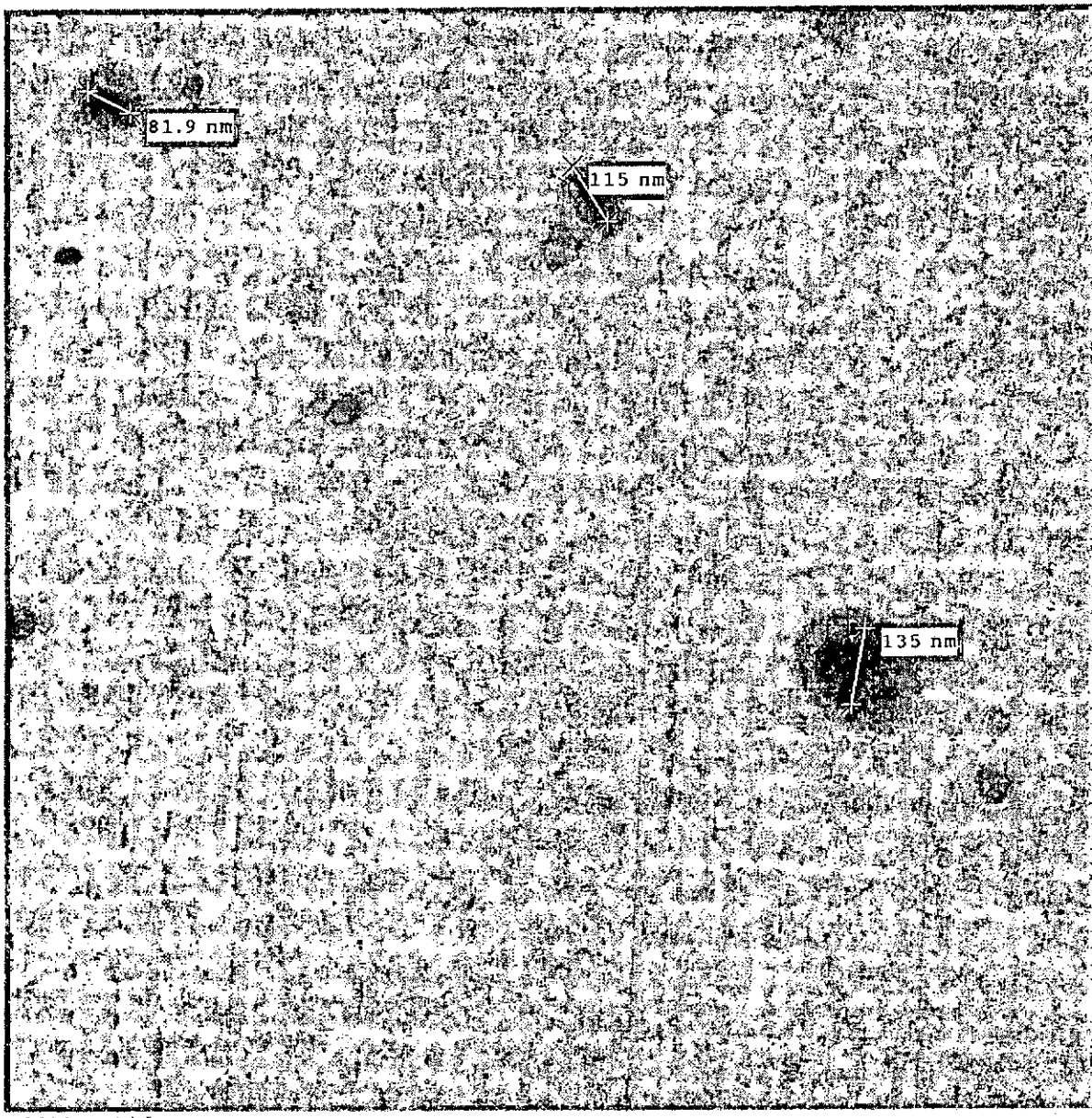
10:59 04/30/07

100 nm
HV=120kV
Direct Mag: 46000x



16611ax.tif
50/64
1661.1 1 UA
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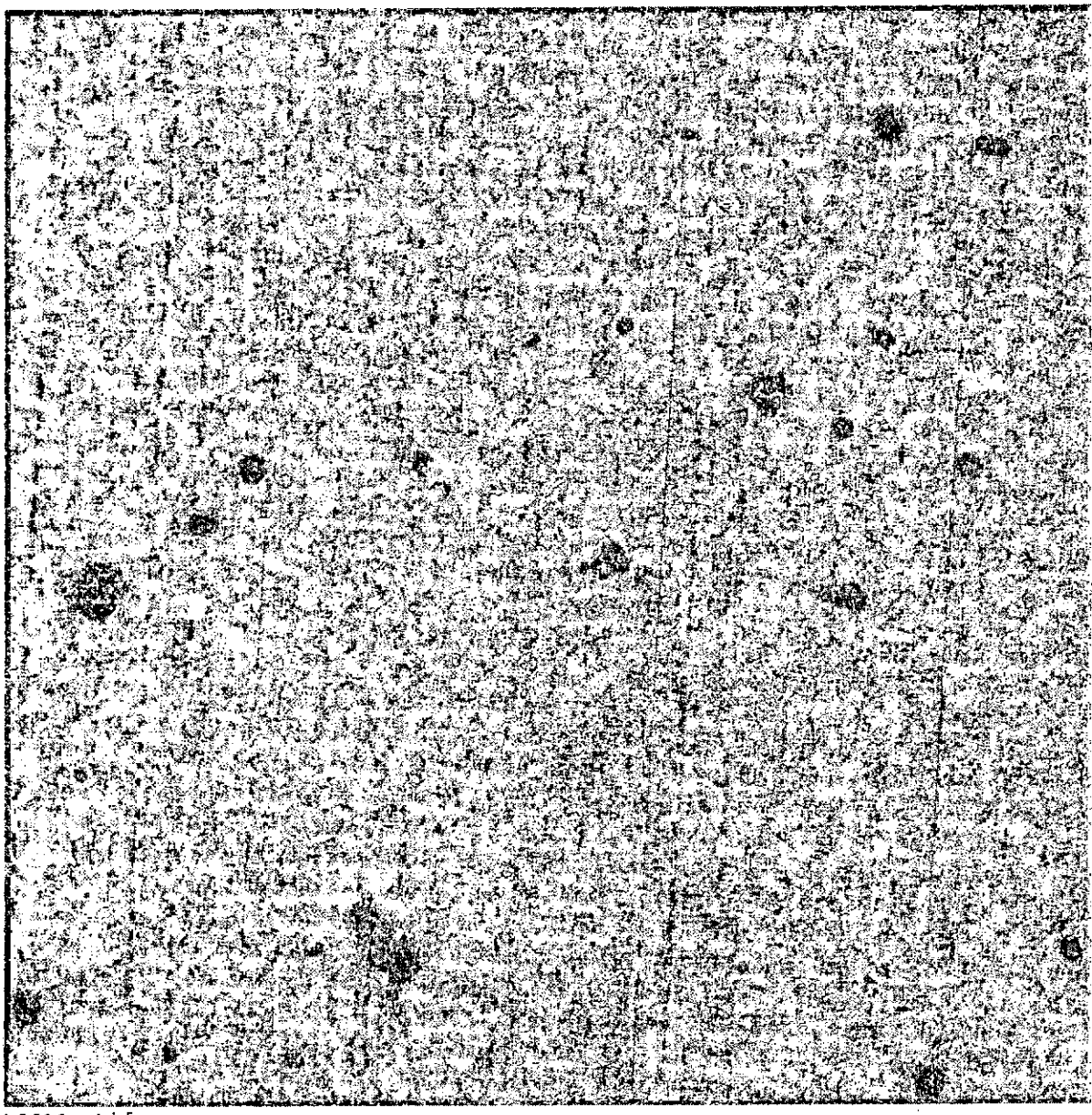
10:59 04/30/07

100 nm
HV=120kV
Direct Mag: 46000x



16611c.tif
50/64
1661.1 1 UA
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11:01 04/30/07

500 nm
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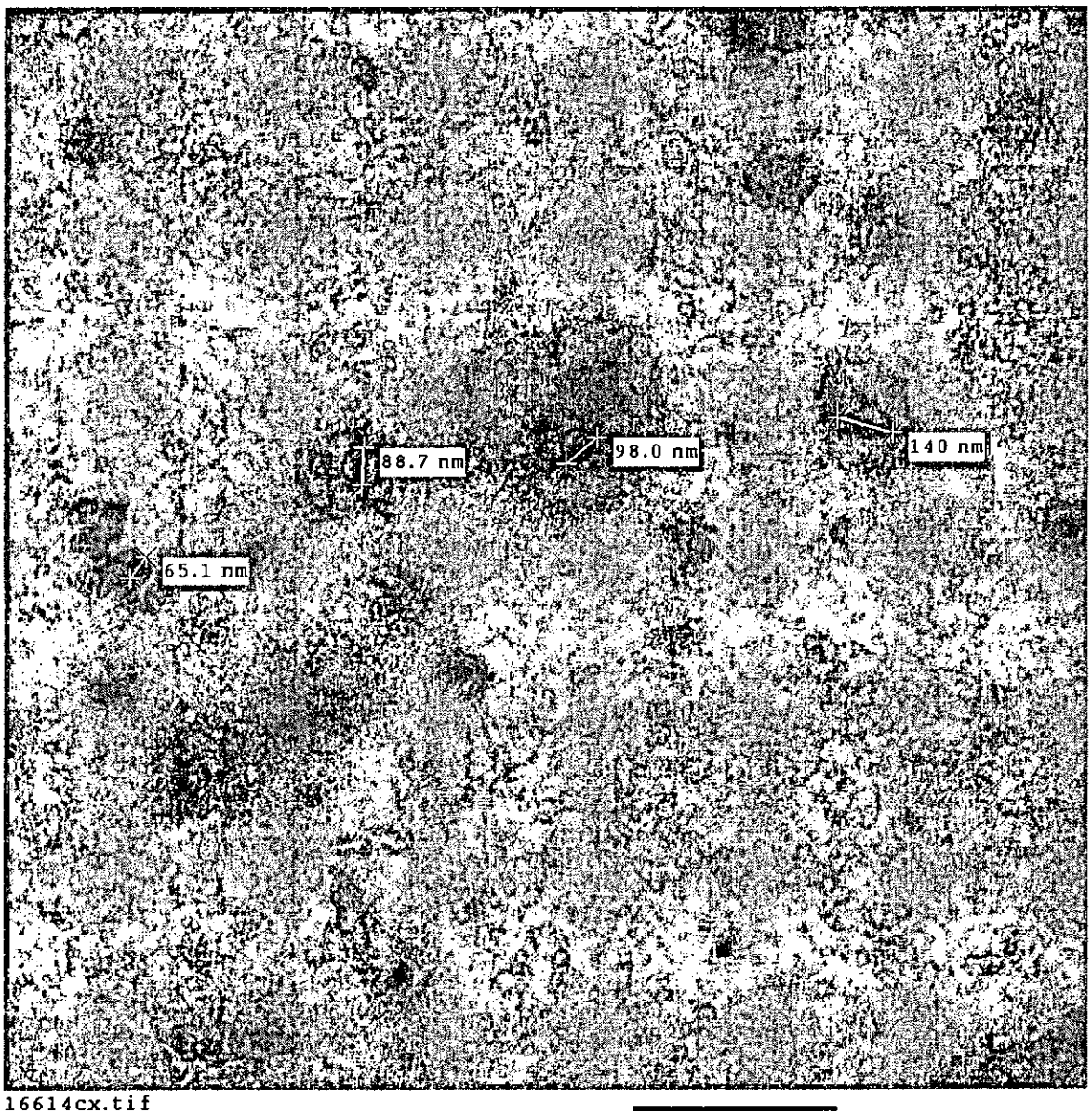

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100/128
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11:44 04/30/07

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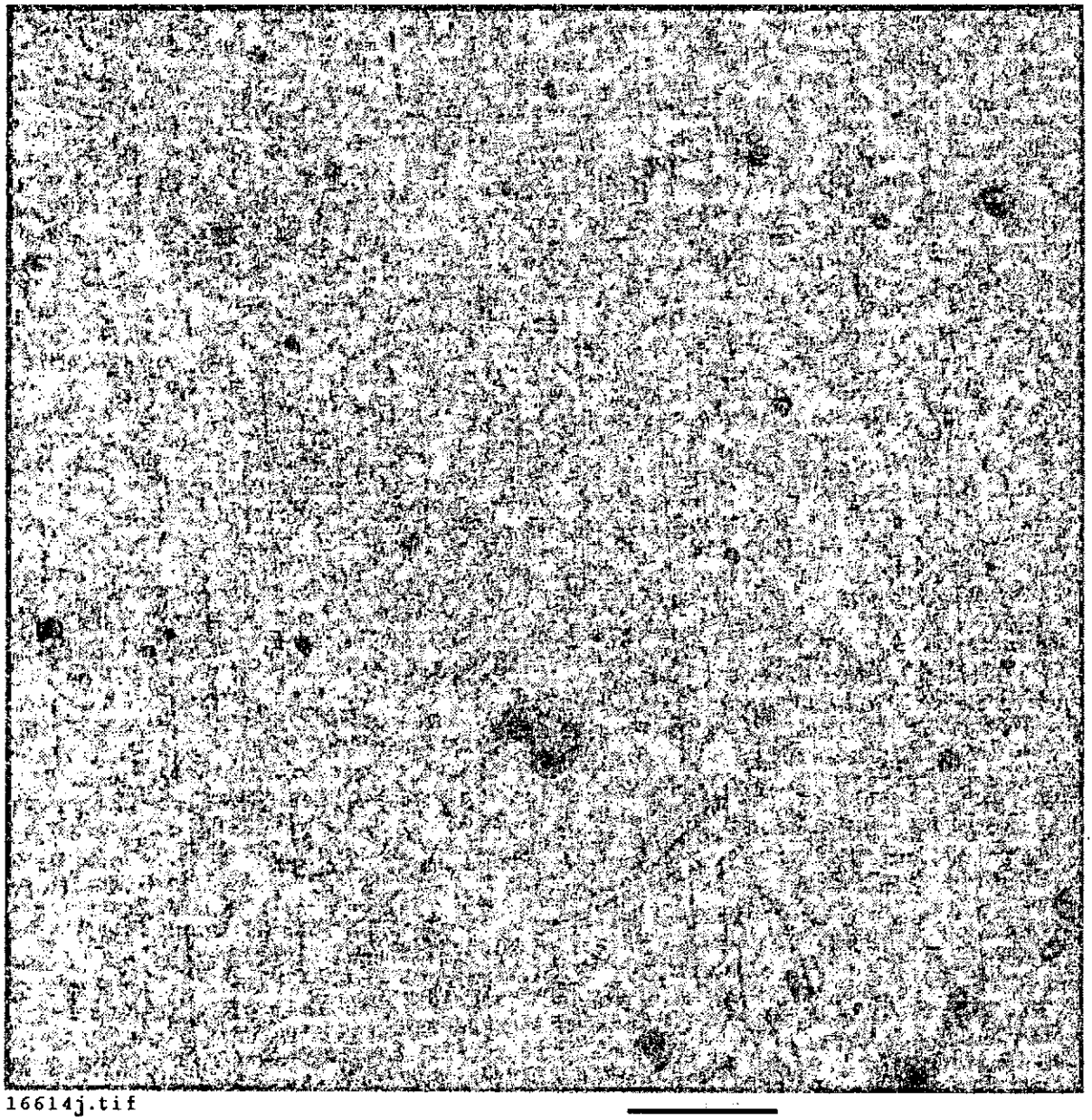
Direct Mag: 33000x



16614j.tif
100/128
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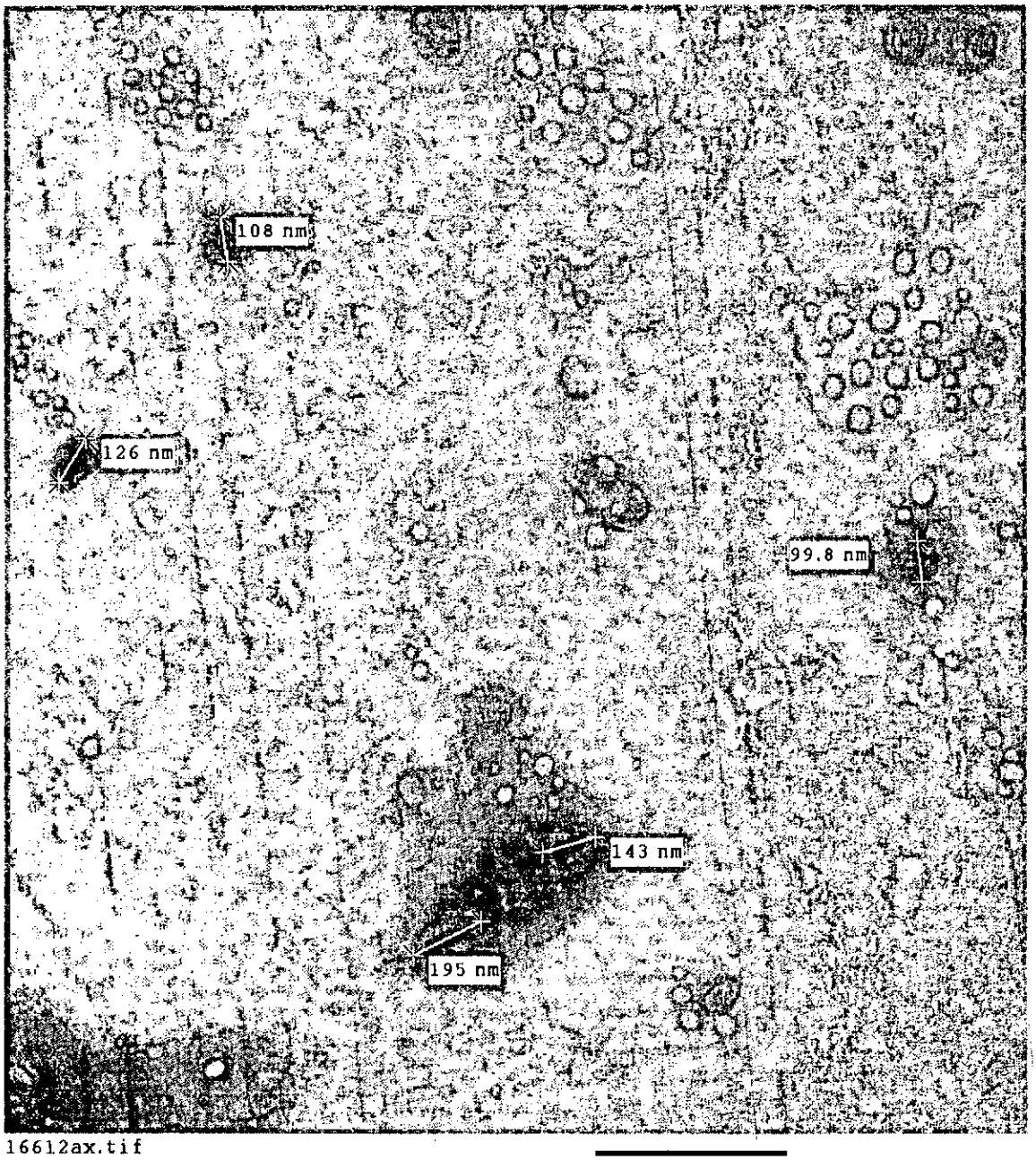
11:53 04/30/07

500 nm
HV=120kV
Direct Mag: 24500x



16612ax.tif
250/320
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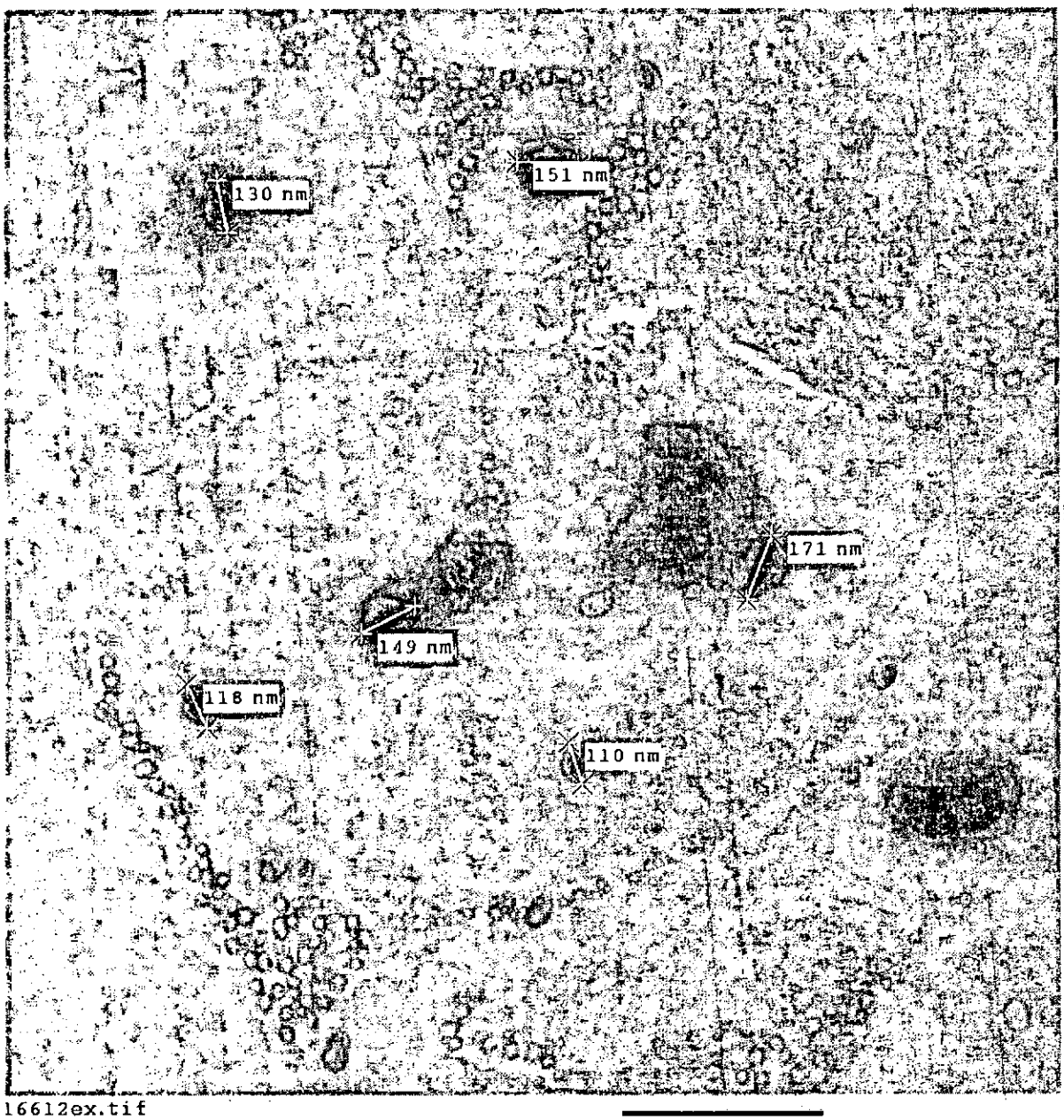
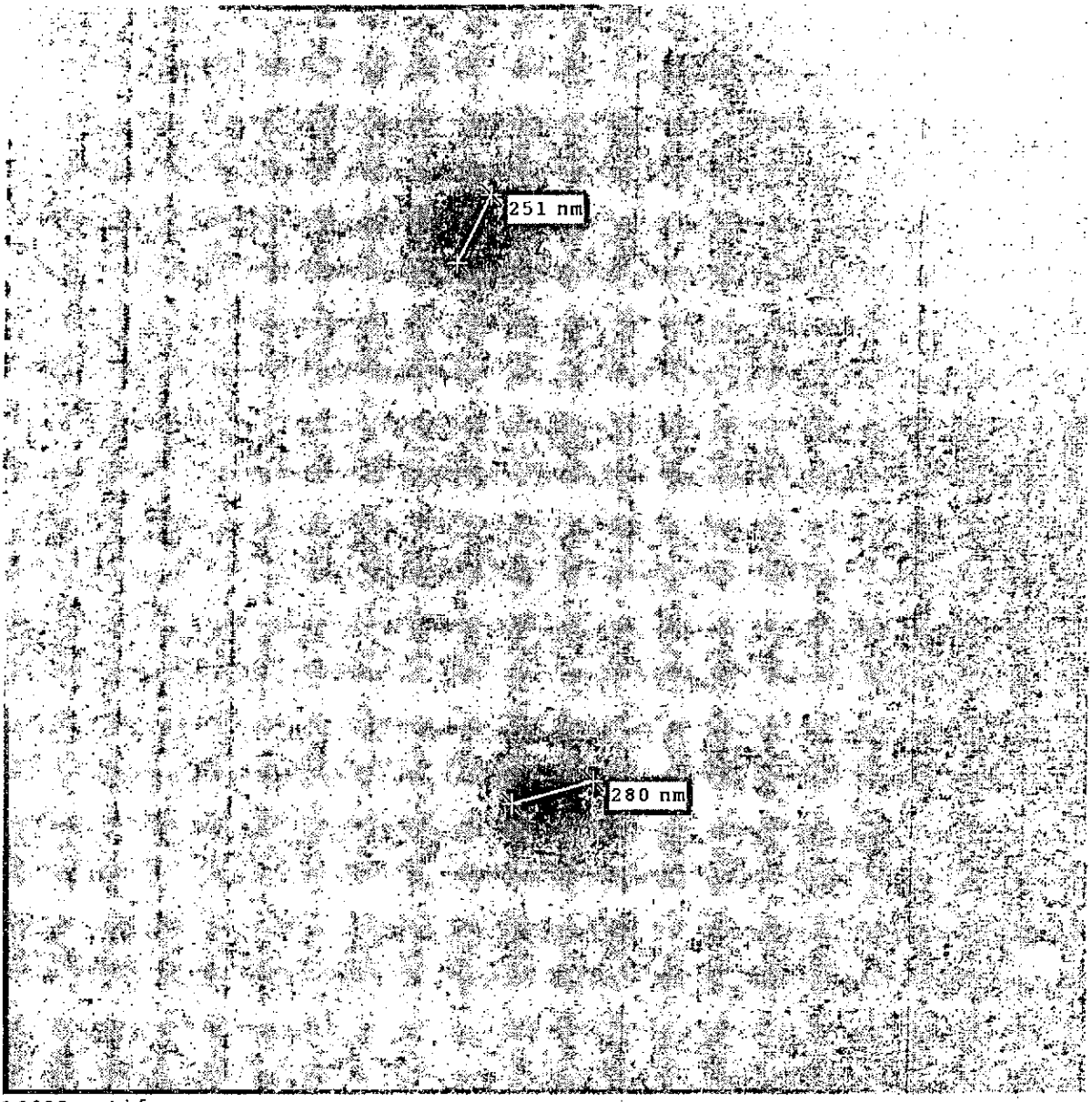
Print Mag: 76100x @ 203 mm
11:06 04/30/07

500 nm
HV=120kV
Direct Mag: 33000x



16612ex.tif
250/320
1661.2 1 UA
Print Mag: 76100x @ 203 mm
11:10 04/30/07

500 nm
HV=120kV
Direct Mag: 33000x



16603ax.tif
250/320
1660.3 1 UA Stain
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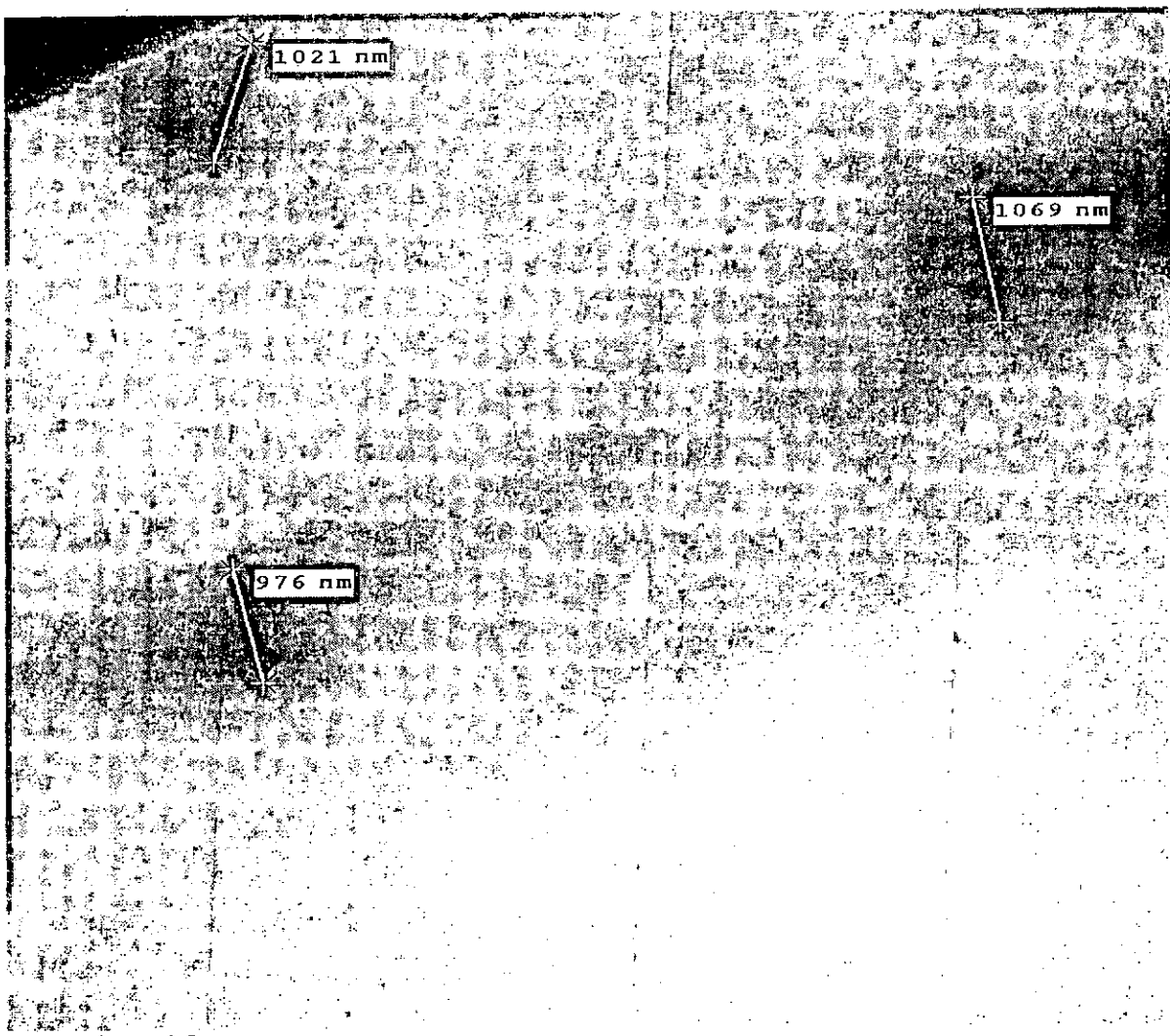
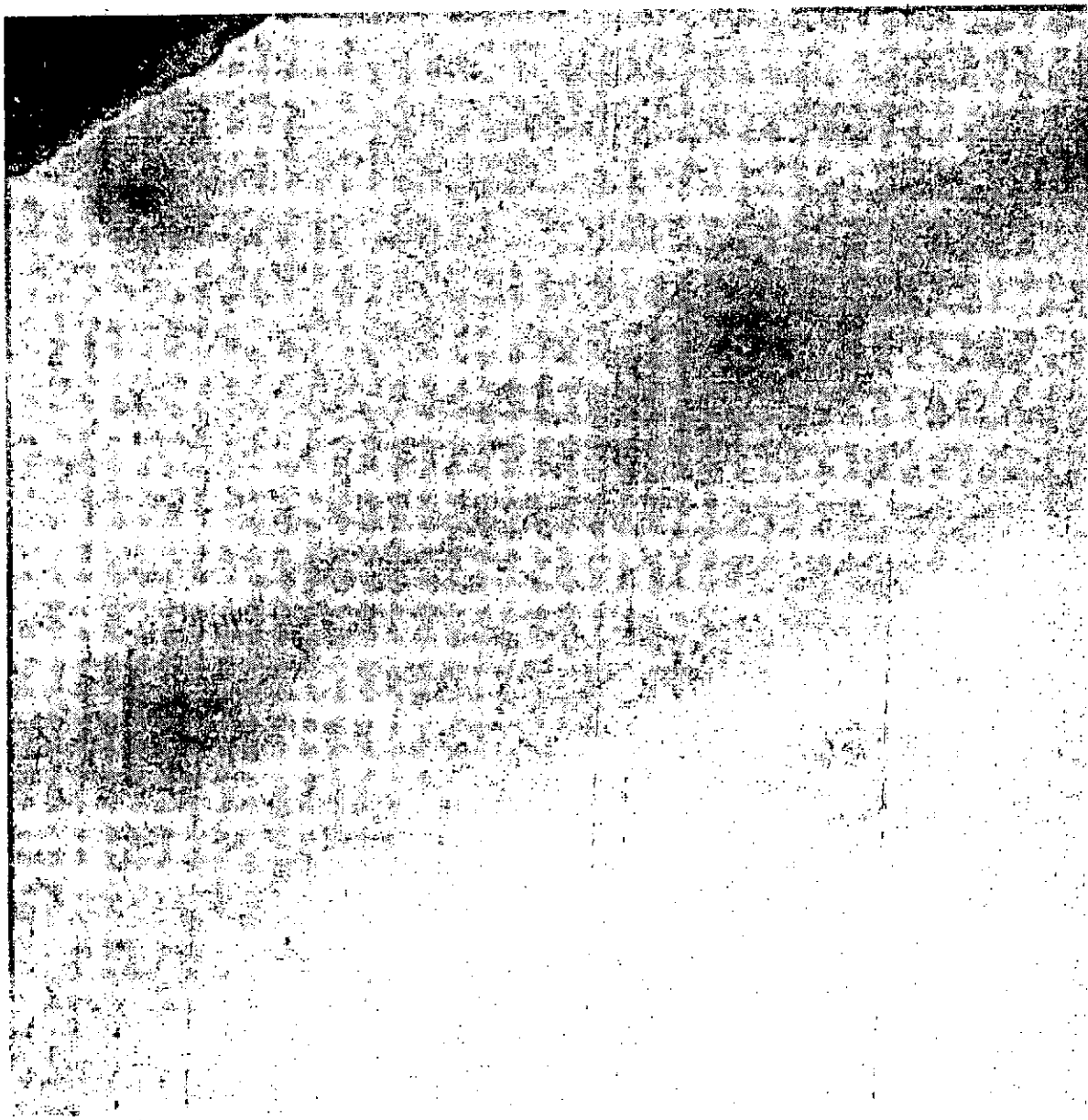
11:49 04/25/07

500 nm
HV=120kV
Direct Mag: 24500x



16613bx.tif
250/2400
1661.3 1 UA
Print Mag: 22300x @ 203 mm
11:15 04/30/07

500 nm
HV=120kV
Direct Mag: 9700x



16613b.tif
250/2400
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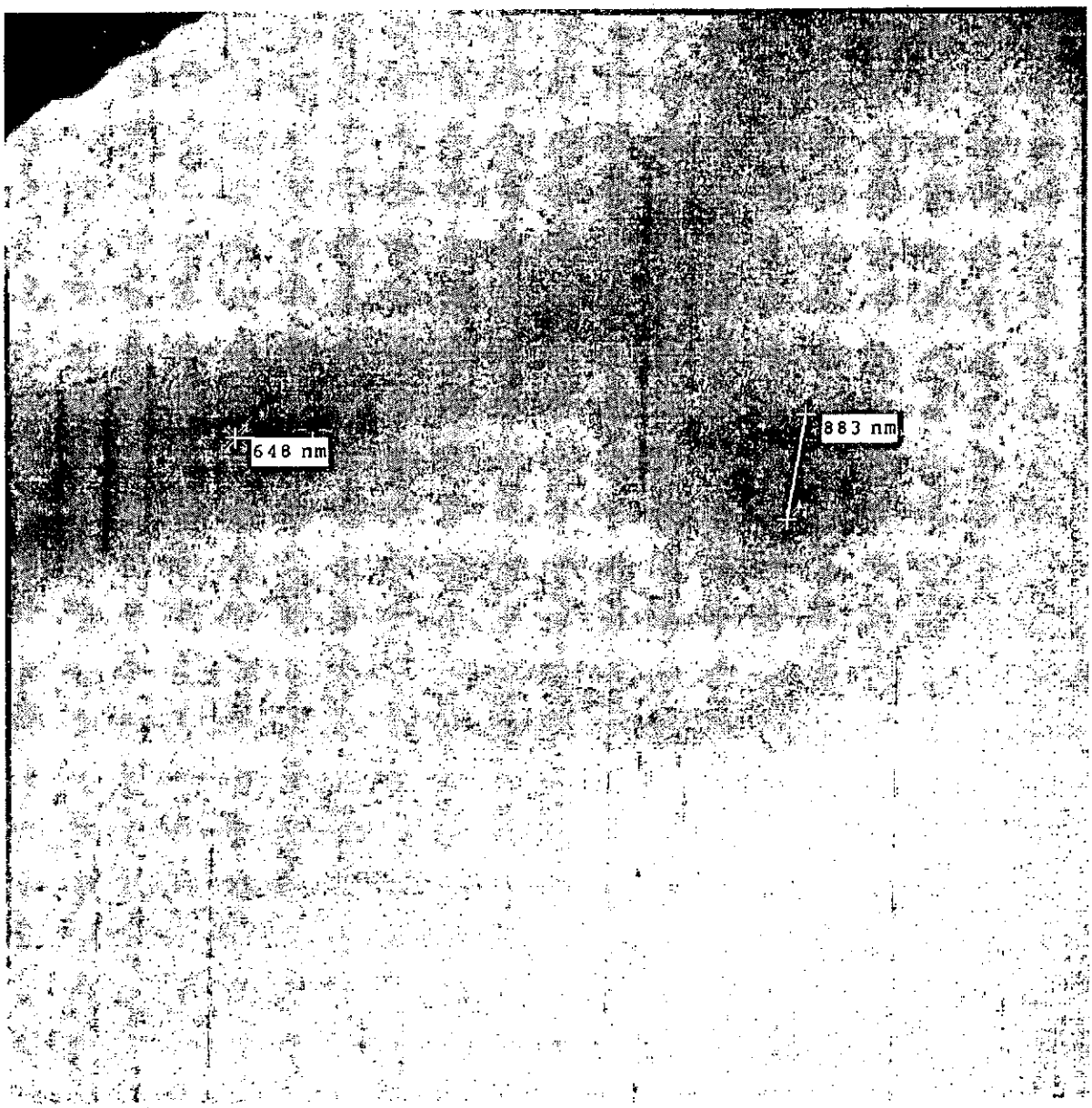
11:15 04/30/07

500 nm
HV=120kV
Direct Mag: 9700x



16613ax.tif
250/2400
1661.3 1 UA
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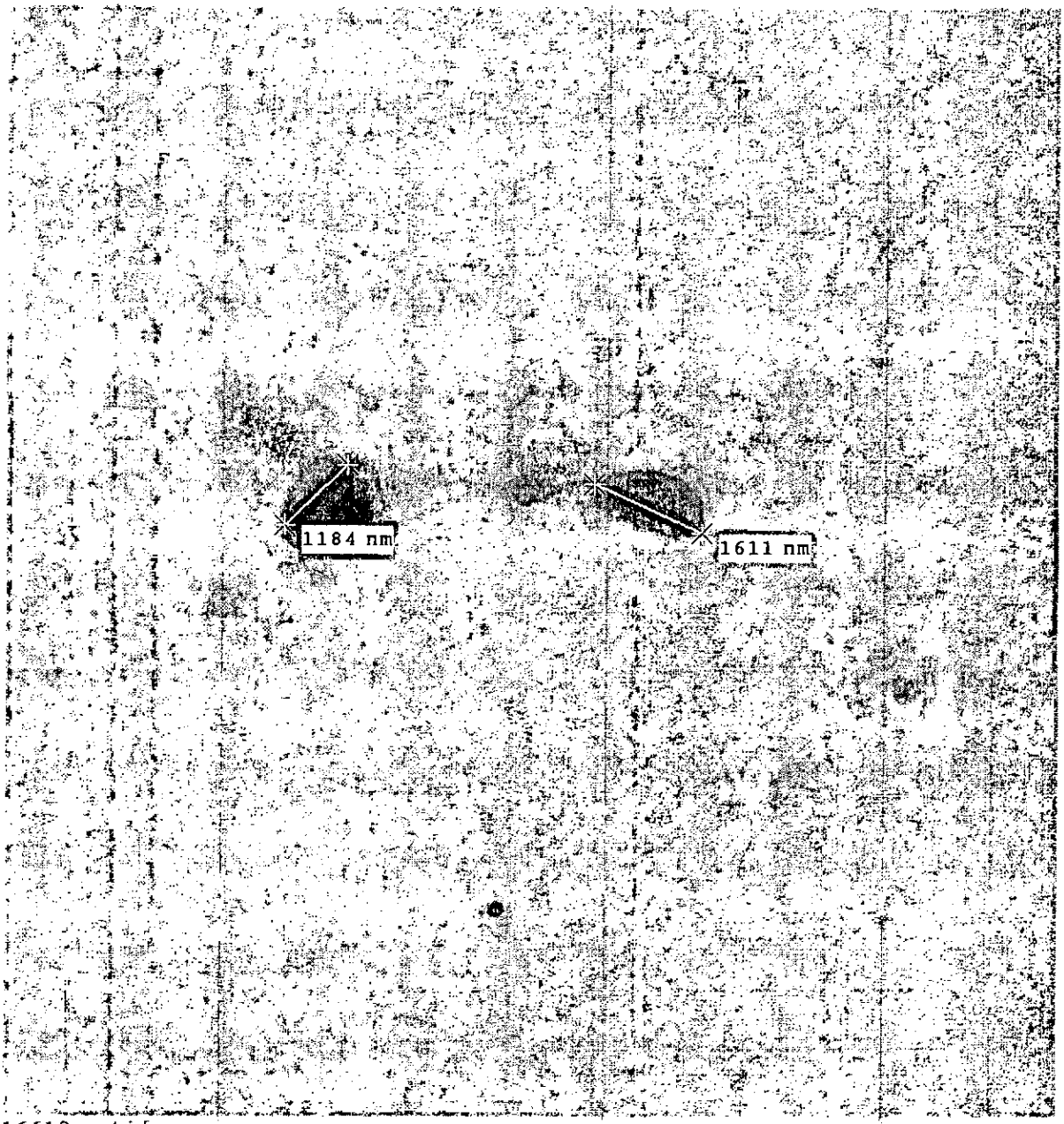
11:14 04/30/07

500 nm
HV=120kV
Direct Mag: 9700x



16613cx.tif
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11:16 04/30/07

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HV=120kV
Direct Mag: 5800x