CHAPTER II

LITERATURE REVIEW

2.1 Malaria

Malaria remains an important disease not only in tropical countries, but also in areas where malaria has been eradicate due to increase traveling to endemic countries. The causative agents in humans are four species of Plasmodium protozoa, including *P. falciparum, P. vivax, P. malariae* and *P. ovalae.* Of these, *P. falciparum* accounts for the majority of infections and is the most lethal. Malaria is transmitted by female anophelae mosquito with infections sporozoite injection into the blood stream during its blood meal (Figure 1). The sporozoites migrate to the liver, where they develop into merozoites which are released from the liver and infect red cells. Over a 48 hour period, the merozoites develop into rings, trophozoites and schizontes which burst out of the infected cells into new merozoites to begin a new cycle *P. falciparum* takes 48 hour to complete the cycle. The parasites tend to mature synchronously within the host, so that the simultaneous rupture of schizonts and release of merozoites are associated with the periodic onsets of high fever, which is the hallmark of the disease. Some of the merozoites, upon entry into new red cells, develop sexual forms of male and female gametocytes which are ready for mosquito infection. A sexual cycle develops in the mosquito and ends with spindle-shaped sporozoites which migrate to the salivary glands of the mosquito for disease transmission. It is the erythrocytic cycle that is directly responsible for the mortality and morbidity associated with malaria infections [6].

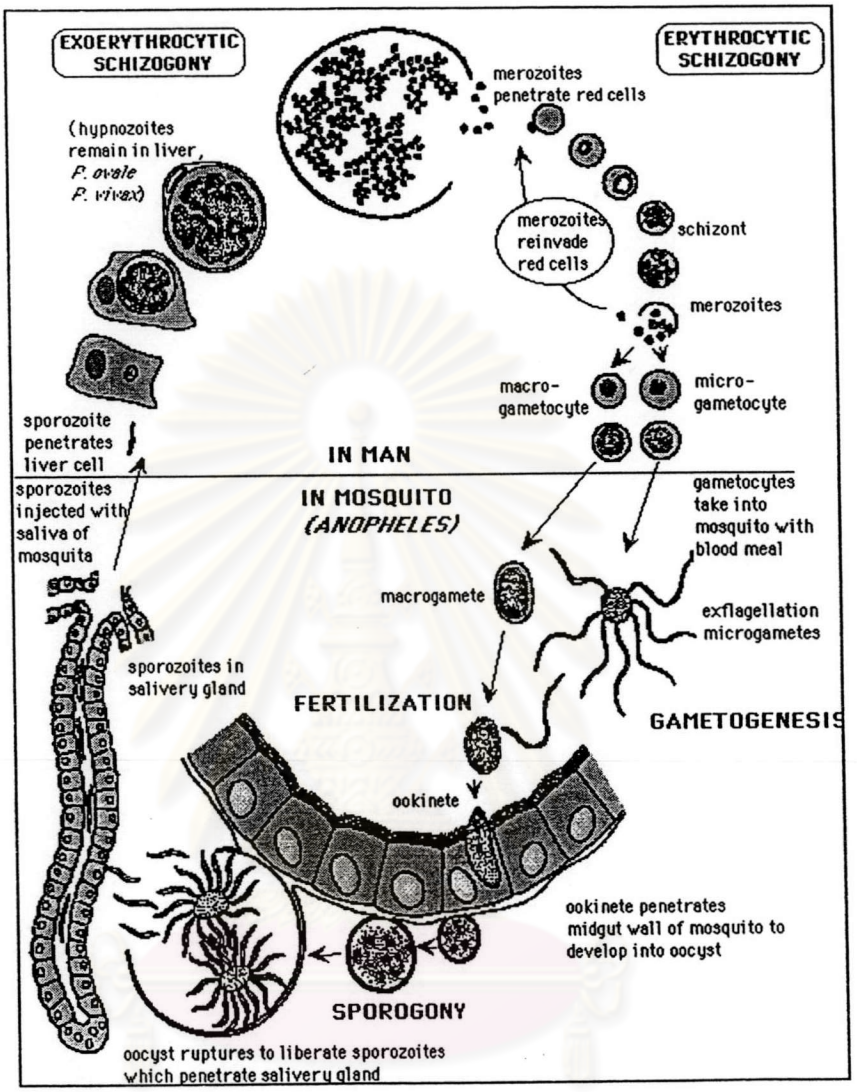


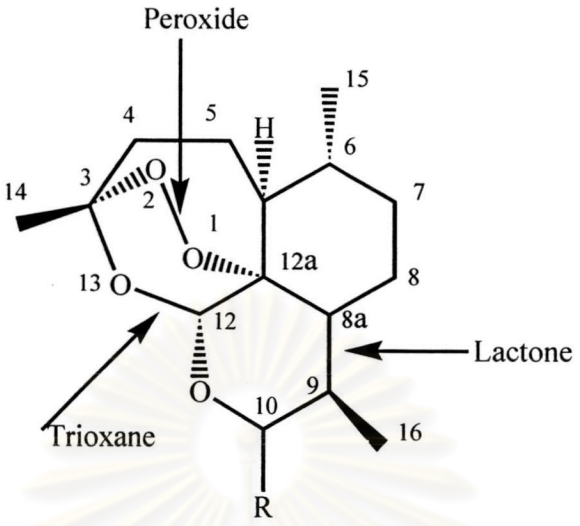
Figure 1 The malaria parasite life cycle. Disease occurs only as a result of the asexual blood stage after the parasite leaves the liver and begins to invade and grow inside red blood cell.

Practical, effective and safe drugs, insecticide and vaccines are still needed to combat malaria. In the 1950s, attempts to eradicate this scourge from most parts of the world failed, primarily because of the development of resistance to insecticides and malaria drugs. Since 1960, chloroquine-resistant and multidrug-resistant strains of *P. falciparum* have spread and the degree of resistance to the drugs of this most prevalent and dangerous plasmodial species has extremely increase [1].

Because of this emergence problem, the search for more potent antimalarial drugs has put on pace in the recent past. The Chinese drug "Qinghaosu" or artemisinin was brought in light. It was found to be a potent plasmodicidal agent and extensive clinical trials in china have revealed that, artemisinin has considerable promise for the treatment of drug-resistant malaria[7]. However, more potent derivatives of artemisinin are developed[3].

2.2 ARTEMISININ AND ITS DERIVATIVES

Artemisinin was developed from an ancient Chinese herbal remedy. *Artemisia anaua* – sweet wormwood or "qinghao" – was used by Chinese herbal medicine practitioners for at least 2000 years. In 1967, Chinese scientists screened a series of traditional remedies for drug activities, and found that extracts of qignhao had potent antimalarial, activity. In 1972, the active ingredient was purified and first named qignhao (essence of qignhao), and then later renamed artemisinin[8]. The initial studies conducted by Chinese scientists demonstrated that artemisinin and various derivatives were effective at nanomolar concentrations in vitro against *P. falciparum*. Scientists at the Walter Reed Army Institute of Research confirmed this [3]. Further studies are in progress. Figure 2 shows the chemical structure of artemisinin and its derivatives.



R:		
	=O	artemisinin
	-OH	dihydroartemisinin
	$-O-(C=O)(CH_2)_2COONa$	sodium artesunate
	$-O-CH_3$	artemether
	$-O-CH_2CH_3$	arteether
	$-O-CH_2C_6H_4COOH$	artelinate

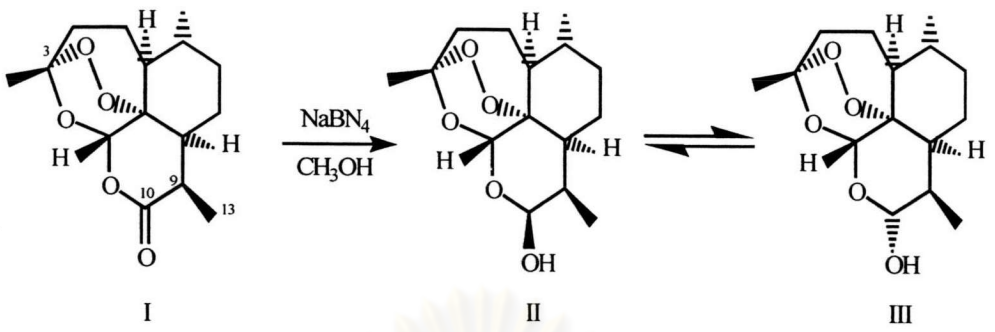
Figure 2 The chemical structure of artemisinin and its semi-synthetic derivatives

Artemisinin and its derivatives have reserved status in the World Health Organization (WHO) essential drug list; that means that they should be reserved for use only in area with multi-drug resistant malaria such as Southeast Asia[8]. The parasite and fever clearance rate with artemisinin and its derivatives are higher than with any other antimalarial drugs[9,10].

Chemistry

Artemisinin is a so-called sesquiterpene with a molecular weight of 282. Terpenes are compounds built up from isoprene units (C_5H_8). These are grouped according to the number of double isoprene units. A sesquiterpene thus contains 15C-atoms. It is a tetracyclic structure with a trioxane ring and a lactone ring. The trioxane ring contains a peroxide bridge, the active moiety of molecule [9](Figure 2).

Artemisinin dissolved in aportic solvents and stable up to a temperature of 150 °C . It is not very soluble in either water or oil. This and its short elimination half-life ($t_{1/2}$) led to search for derivatives that had improved pharmaceutical properties as well as better antimalarial activity. Chemical modification of artemisinin is a difficult task because of the chemical reactivity of the peroxide linkage. However, it was found that the carbonyl group could be reduced by sodium borohydride ($NaBH_4$) without affecting the peroxide and yielded DHA (Figure 3), a potent antimalarial compound. With DHA as an intermediate, other active derivatives were synthesized [11]. The most important derivatives are currently artesunate, water-soluble sodium salt of succinic acid ester and the lipophillic esters arteether and artemether.



I II III

Figure 3 Conversion of artemisinin (I) to DHA (II, III). Treating artemisinin (I) with $NaBH_4$ in methanol yielded a mixture of α- and β-DHA (II, III)[11].

Arteether is the β-anomer of the ethyl ether of DHA[12]. It has become an increasingly important new drug candidated as a treatment for the erythrocytic stages of chloroquine-resistant stain of *P. falciparum* and cerebral malaria. Ether is the good storage stability and highly soluble in oils lipophilic property, arteether is accumulate in brain tissue and thus is useful for treatment of cerebral malaria [9,13].

Artemether is the methyl ether derivative of dihydroartemisinin and has similar chemical and physical properties as arteether[12]. This compound has been synthesized to enhance the antimalarial activity and solubility of artemisinin. It is used in China as a solution in oil for intramuscular administration in humans [3].

Artesunate, a water-soluble derivative, is prepared by treating DHA with succinic anhydride in the presence of 4-dimethylamino-pyridine. Administrated intravenously, theis compound showed about 5.2 folds more potent than artemisinin against both chloroquine-resistant and chloroquine-sensitive stain of *P. berghei*. Artemisinin tablets and injection are registered in Brazil, Ghana, China, Burma, Vietnam and Thailand [3].

Mechanism of action

The peroxide function in artemisinin and related compounds are vital for antimalarial activity. The killing of malarial parasite by artemisinin and its derivatives is mediated by the production of cytotoxic compounds such as free radicals and reactive aldehydes. This is evidenced by the following observation that artemisinin derivatives lacking the endoperoxid bridge are devoid of antimalarial activity. Similarly, antioxidants (free-radical scavengers) such as α-tocopherol, catalase, ascorbate and reduced glutathione block antimalarial activity [3,14]. Most free-radical-generating drugs cause " oxidant damage" by producing oxygen free radicals, such as superoxide, which then cause indiscriminate damage to the cell. Artemisinin derivatives have been shown to affect parasites very differently from other oxidant drugs. Instead of reacting with oxygen and producing large quantities of oxygen containing free radicals such as superoxide and $^{\bullet}OH$, artemisinin itself becomes a free radical in a reaction catalyzed by iron.

The biochemical action of artemisinin depends on two sequential steps (Figure 4) [14]. In the first step, artemisinin compounds are activated by haeme or molecular iron. The reductive, iron-mediated, cleavage of the endoperoxide bridge involves the transfer of an electron from ferrous iron (Fe [II]) and the formation of Fe [IV]. The endoperoxide bridge is cleaved first; this followed by intermolecular electronic rearrangements which produce carbon-centered radicals. The carbon-centered free radical may be important in the mechanism of action. A highly electrophillic epoxide, a potent alkylating agent, is also formed after the cleavage, as is a 1, 5-diketone [3]. In the second step is alkylation. After the drug is converted to a reactive free radical it can then form covalent bonds with proteins[15]. There is a marked and reproducible reaction between artemisinin derivatives and several proteins when malaria-infected erythrocytes are exposed to artemisinin derivatives. Malaria-specific proteins are with molecular masses of 25, 32, 42, 50, 65 and > 200 kDa[14,16]. The identities of the artemisinin target proteins await elucidation [3].

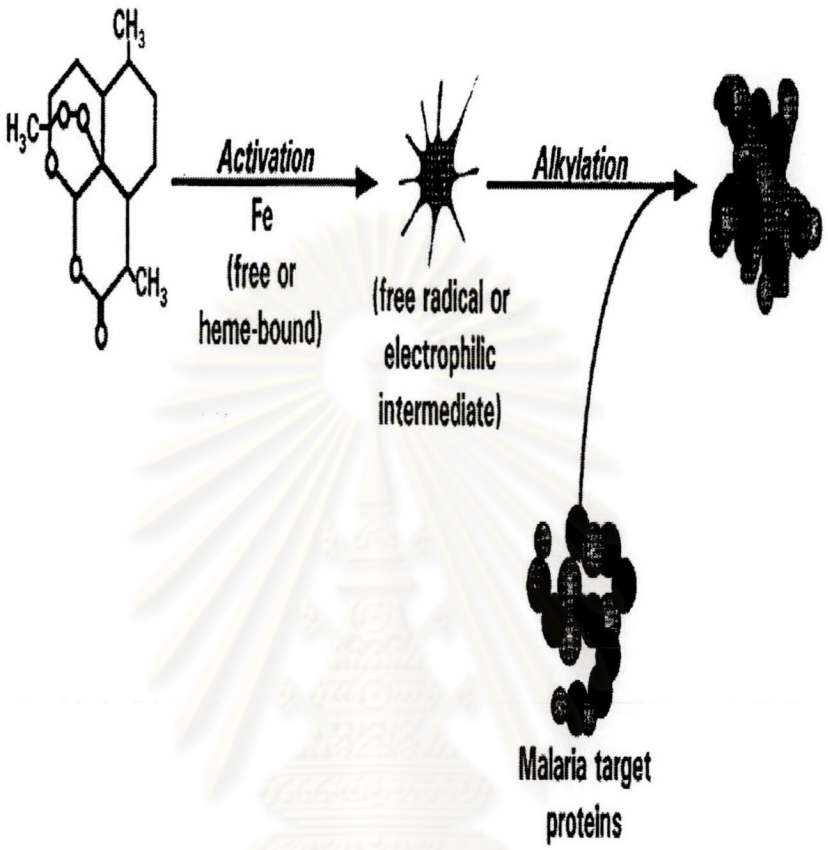


Figure 4 Model of action of artemisinin derivatives

Antimalarial efficacy

Artemisinin, artemether and artesunate are rapidly acting blood schizontocidal antimalarials against chloroquine sensitive and chloroquine resistant falciparum as well as vivax malaria. The quickly arrest the ring or the trophozoite development and also prevent pathological sequel. Fever subsides and parasites are cleared rapidly. Defervescence occurs within 2-3 days after drug administration. Ninety percent clearance of asexual erythrocytic parasitemia is usually observed within 4 h. These compounds can clear parasitemia faster than any other antimalarial in uncomplicated malaria. However, in severe malaria the speed of parasite clearance is similar to that observed with quinine. The rapidity of fever and parasite clearance is not dose-dependent, but total does and duration of drug administration does have an influence on the cure rate. The longer the duration of treatment and/or the lesser the severity of the infection, the higher is the cure rate [12]. No sporontocidal effect of artemisinin has been observed [16]. So far no in vitro resistance has been described, marking them also effective in the treatment of multi-resistant malaria. A disadvantage of the artemisinin drugs is the occurrence of recrudescence when given in short course monotherapy regimens [17].

Pharmacokinetics

In vivo, the liver is the main site of metabolism of artemisinin and its derivatives. Metabolism occurs both in liver microsomes and the cytosol. Metabolism leads to the formation of more polar metabolites, which are more water-soluble and more easily excreted after metabolic transformation. Artemether, arteether, artenate and artesunate yield DHA as a major metabolite that is held responsible for the antimalarial activity[18,19]. Reduction of the peroxide bridge is the most important step in the metabolic pathway. The metabolites all lack the peroxide moiety and are therefore devoid of antimalarial activity [14].

Artemisinin is metabolized into 4 inactive metabolites namely deoxyartemisinin, DHA, 9, 10-dihydrodeoxyartemisinin and 'crystal 7' which were extracted from urine (Figure 5) [20].

Only a very small fraction of the administered is excreted unchanged in feces and urine, regardless of the route of administration [21].

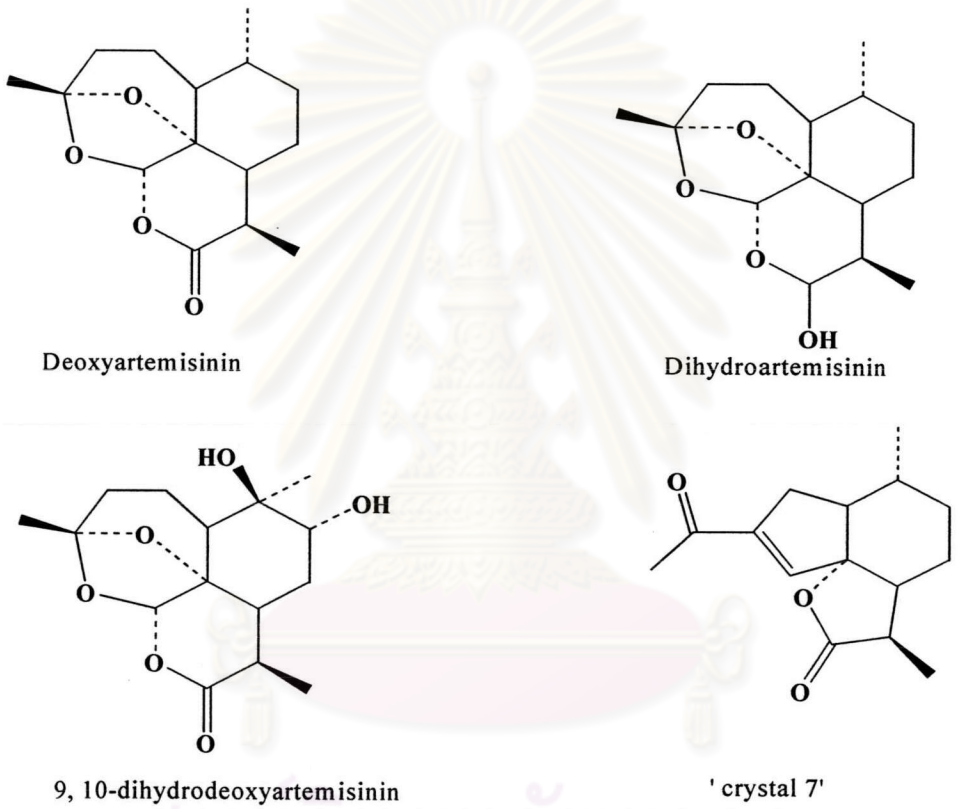


Figure 5 Metabolites from artemisinin, isolated from human urine

Toxicity and adverse effects

Toxicological studies in animals have shown that toxicity of artemisinin and its derivatives is much less than that of quininlines such as chloroquine, quinine, mefloquine and primaquine [12,14]. The acute lethal doses in rodents cause a neurological syndrome and sings of cardiotoxicity. DHA also causes haemolysis, but less than artemether[9,14]. Artemisinin and its derivatives are reported to be embryotoxic in laboratory animals [3,10].

The toxicity of artemisinin derivatives to neuronal cells may also be iron dependent, since haeme potentiates the toxicity of artemisinin derivatives to neuroblastoma cells *in vitro*[22,23]. The toxic effect of artemisinin derivatives on neurons involves protein alkylation. Thus, the mechanism of neurotoxicity is similar in many aspects to the mechanism of antimalarial action [9].

Side effects reported thus far include nausea, vomiting, diarrhea, dizziness, sinus bradycardia, and acute psychoses. In studies with healthy volunteers, no side-effects have been reported after oral or rectal administration of artemisinin.. No symptomatic side effects from artemisinin suppositories or local effects such as anal irritation have been reported from a clinical trial with children[18]. Limited drug use during human pregnancy has shown no apparent toxicity but, because of reported embryotoxicity in rat and mice, at present these compounds are contraindicated in pregnancy [3,10,24].

Other biological activities

Artemisinin and its derivatives have been tested for possible therapeutic in other systems as well because of its unusual chemical structure. They are active against *Schistosoma mansoni* and *Schistosoma japonica in vitro* and in experimental animal. This is of mechanistic interest, since shistosomes, like malaria parasites. Artemisinin, its derivative DHA and arteether have been found to exhibit suppression of humoral responses at high doses levels but no effect on delayed hypersensitivity to sheep erythrocytes [14].

Artemisinin derivatives have immunosuppressive activity and also, potentially anticancer activity. The activity of artemisinin against cancer cells was potentate if the cancer cells were first loaded with iron by exposure to transferrin[25-27]. The derivatives of DHA, used as antimalaria, had a somewhat more potent cytotoxicity than artemisinin [28,29]. In addition, the endoperoxide moiety only causes inhibition of the tumor cell proliferation, whereas an exocyclic methylene group fused to the lactone ring is responsible for clonogenic cell death.

The concentrations or doses of artemisinin derivatives which are necessary for these alternate activities *in vitro* and *in vivo* are substantially higher those required for antimalarial activity. Therefore, antimalarial endoperoxides are not likely to be useful for other therapeutic purposes. However, directed-synthesis programs might lead to endoperoxides with useful anti-infective or anticancer activity [3].

2.3 Dihydroartemisinin

DHA, a hemiacetal, is the major derivative of artemisinin. Sodium borohydride reduction of artemisinin yields DHA, as a mixture of diastereoisomers (Figure 6). It has used primarily as a semisynthetic compound for derivatization. In addition, it is also the biologically active metabolite of derivatives of artemisinin.

Physico-chemical properties

The chemical name of DHA is [(3*R*, 5a*S*, 6*R*, 8a*S*, 9*R*, 10*S*, 12*R*, 12a*R*]-Decahydro-3, 6, 9-trimethyl-3, 12-epoxy-12*H*-pyrano- [4, 3-j]-1, 2-benzodioxepin-10-ol. The other name is artenimol. Molecular formula of DHA is $C_{15}H_{24}O_5$, molecular weight is 284.4 and the melting point at 151-153 °C. It is a colorless needle or a white or almost white crystalline powder, practically insoluble in water, slightly soluble in acetonitrile, ethanol and dichloromethane. It should be kept in a well-closed container, protected from light and stored in a cool place [30].

Initial *in vitro* studies on laboratory-adapted clones of *P. falciparum* have shown that DHA is more active than other antimalarial drugs [31]. In addition, DHA is equally active against the chloroquine-sensitive and chloroquine-resistant falciparum

malaria [32]. Base on the observation that DHA is a highly active metabolite of artemisinin derivatives and that it is well absorbed by oral administration.

DHA has been evaluated in clinical trial in Thailand for the treatment of falciparum malaria during 1994-1995. It is a potent antimalarial drug that can reduce parasitaemia by 90 % within 24 hours of administration and gave a 90 % cure rate. All the artemisinin derivatives are metabolized rapidly to the active metabolite DHA. The use of DHA instead of the substitute compounds, artesunate or artemether, has advantages such as easy to produce with less synthetic step and, thus, a low cost [33].

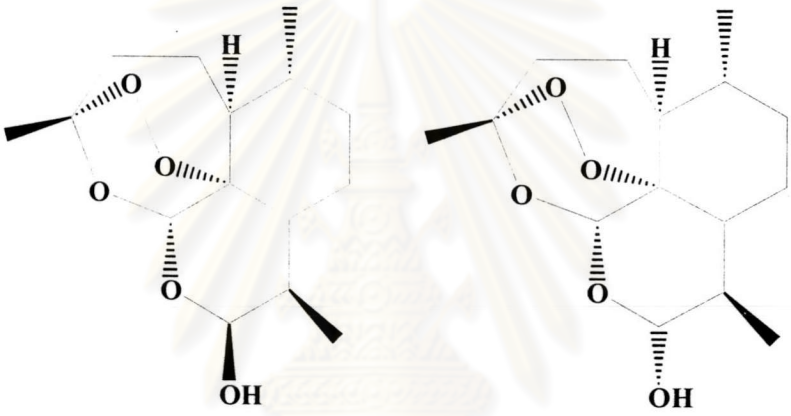


Figure 6 The chemical structure of DHA

So this led to an interest to develop DHA into new antimalarial drug. However, it was found that artemisinin and its derivatives have been shown to undergo a variety of unusual rearrangement reaction under thermal, basic and acidic condition [34-39].

Stability

Thermal decomposition of DHA was studies by Lin, et al. 1986[40]. DHA, a sodium borohydride reduction product of aretmisinin, was heated in a round-bottom flask for 3 minutes in an oil bath preheated to 190 °C. Upon cooling to room temperature, the brown mixture was separated by a silica gel column using 15% EtOAc/$CHCl_3$ as eluent to give desoxyartemisinin and a preponderant decomposition product consisting of 2 epimer **4a**, (2S, 3R, 6S)-2-(3-oxobutyl)-3-mehyl-6-[(R)-2-propanal]-cyclohexanone, and **4b**, (2S, 3R, 6R)-2-(3-oxobutyl)-3-mehyl-6-[(R)-2-propanal]-cyclohexanone.

The stability of DHA suppositories was reported by Chaipakdee, et al. 1999[41]. It was found that DHA-Suppocre AM and Suppocre AP suppository formulations stored at 45 °C for 90 days, the color of this particle change from white to yellow. The DHA content was determined by HPLC with UV detection. It can be seen that the percent remaining of DHA in suppository formulation stored at 30 °C was higher than that in suppository stored at 45 °C and followed the first-order kinetic (Figure 7). The sequence of DHA degradation was attributed to be hydrophillicity of suppository bases.

The stability of DHA powder was discussed by Sirichotbundit, et al. 1999[42]. It was detected that the long time storage in stress conditions, 45 °C and 75% RH, the more intense in yellow was of DHA powder. Likewise, the chemical content of DHA progressively decreased from the week 8 onwards. At the time of week 10, 12 and 16 the decrease in drug content was proportional to the time. The drug content was about 90% of the initial amount after storage under 45 °C, 75% RH and 4 months (Figure 8).

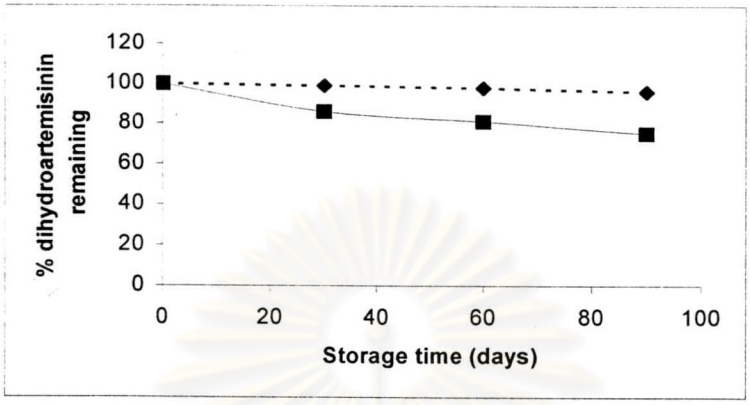


Figure 7 Percent remaining of DHA in Suppocire AP suppository after storage at 30 °C (--♦--) and 45 °C (-■-) for 90 days.

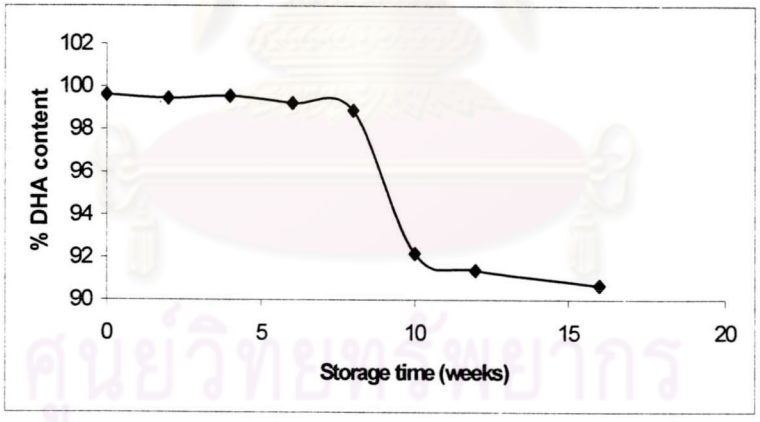


Figure 8 Stability of DHA powder conducted under 45 °C and 75% RH

To date, the stability study of DHA was investigated by the Government Pharmaceutical Organization (GPO) [43]. It was found that stability of DHA is affected by the stored temperature and not affected by type of containers, glass and plastic, used. The content stored in container at 40 °C (75% RH) was found to decrease with time from 99.44% w/w at the initial month to about 91% w/w at 6th month. The sharp increase of total degradation products was found from DHA stored in both containers reaching almost 10 % from the initial month at 6th month. At 25 °C (60% RH), the DHA content slightly decreased about 2% w/w at 6th month. The decreased rate of DHA content and increased rate of total degradation products at 25 °C were slower than at 40 °C. At 8 °C (30% RH), the DHA content at 12th month was not significantly different from initial month. A small increase of total degradation products of less than 0.5% was detected from DHA stored (Figure 9).

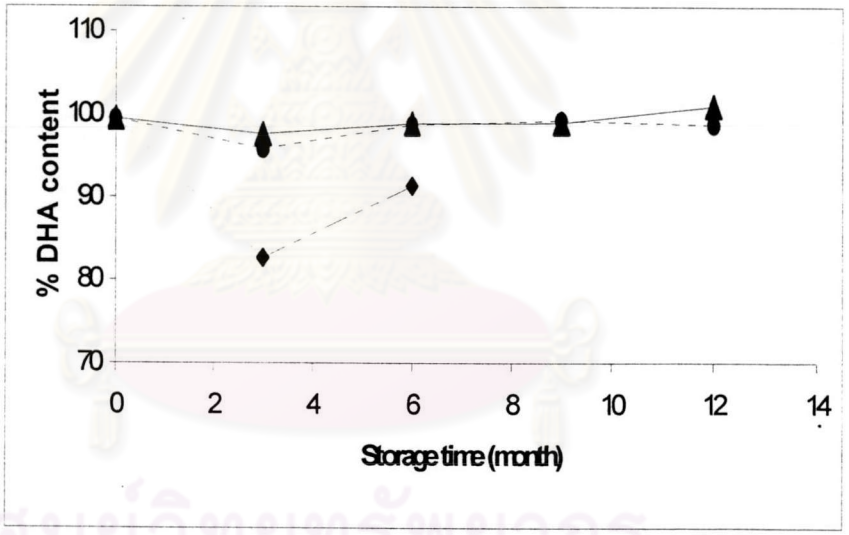


Figure 9. Effect of stored temperature on the content of DHA in glass container at 8 °C , 30% RH (–▲–), 25 °C , 60% RH (-●-), 40 °C ,75% RH (-♦-)

Analytical procedure

Analytical method to assay artemisinin and related compounds are difficult because the molecule is unstable and do not contain fluorescent chromophore. They have UV spectra with absorbance < 220 nm except for artelinic acid, an aromatic group at C10, gives ultraviolet absorption at 278 nm. However, a method for determination at artemisinin and derivatives using HPLC with UV detection have been developed[14].

The closely related sesquiterpene lactone endoperoxide artemisitene, which may occur together with artemisinin in the plant, can be assayed simply and selectively using reversed-phase HPLC with UV detector. This useful for the routine analysis of artemisinin to check its purity and can also be used for preparative scale purification of these compounds [14].

Aravind, et al. 1989[44] reported micellar solubilization of β-arteether using a reversed-phase HPLC with UV detection at 216 nm. The separation was conducted on a reversed-phase C_8 column (10 μm, 30 cm x 4.6 mm.). The mobile phase consisted of 60% acetonitrile and 40% water with a flow rate of 1.5 ml/min. Under this chromatographic condition, β-arteether had a retention time of 9.0 min.

The detection at low wavelength region has its obvious drawbacks, especially with the plant extract and biological fluids. Therefore, there is a need for its modifications or derivation for a favorable spectrometric detection. Acid or base hydrolysis of artemisinin and its derivatives producing an ultraviolet-chromophore have been employed, but this approach lack specificity. This method was simple to operate. Chiableam, et al.[45] reported that after treatment with 6.4% sodium hydroxide solution at 60 ºC for 45 min, DHA which has a UV absorption spectra at 237 nm.

For the measurement of artemisinin and its derivatives in biological fluids several methods have been reported including chemical assay, thin layer chromatography, GC, RIA and HPLC. HPLC methods involved acid or base hydrolysis to a UV absorbable decomposition product and the assay with reductive electrochemical detection using either thin layer mercury amulgum, dropping mercury or glassy carbon electrode. Among the available HPLC methods, HPLC with

reductive electrochemical detection provides excellent sensitivity, with a limit of detection in the order of 5-10 μg/l, as well as specificity since the compounds are not decomposed before analysis. The sample solution is essential for the sensitive and stable operation of the electrochemical detector in the reductive mode.

For metabolic studies, thermospray high-performance liquid chromatography (HPLC)-mass spectrometry is currently being used. This method is highly sensitive and can detect nanogram level of the compound [14].