EX VIVO BLOOD SCHIZONTOCIDAL ACTIVITIES OF ARTEMISININ DERIVATIVES AGAINST *PLASMODIUM FALCIPARUM*

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Abstract. Serum samples collected at intervals from eight healthy volunteers after the administration of the six regimens of artemisinin derivatives were investigated for their *ex vivo* blood schizontocidal activities against K_1 strain *Plasmodium falciparum*. The regimens included single doses of (a) 300 mg oral artemether; (b) 300 mg intramuscular artemether; (c) 100 mg suppository artemether; (d) 300 mg oral artesunate (Guillin formulation); (e) 300 mg oral artesunate (Arenco formulation); (f) 300 mg oral dihydroartemisinin. Sera collected after various regimens of artemisinin derivatives showed distinct degree of *ex vivo* blood schizontocidal activities. Activity of sera after suppository dosing was remarkably low and variable comparing to the other two formulations (oral, intramuscular). Median values for A_{max} (the maximum activity normalized with dose) of sera from oral dosing were 2.4- and 118-fold, while AUA (the area under activity-time curve, normalized with dose) were 0.82- and 2,370-fold of that after the intramuscular and suppository dosing, respectively. Sera from artesunate-Arenco dosing exhibited significantly higher A_{max} and AUA (medians: A_{max} 12.4 *vs* 5.13 nmol/l/mg dose; AUA: 21.9 *vs* 8.8 nmol.h/ml/mg dose), compared to that from artesunate-Guillin dosing. Among the oral formulations of artemisinin derivatives investigated (artemether, artesunate, dihydroartemisinin), sera collected following a single dose of oral dihydroartemisinin exhibited lowest bioactivity (A_{max} 2.35 nmol/l/mg dose; AUA: 44 nmol.h/ml/mg dose).

INTRODUCTION

Malaria remains a major public health problem due to resistance of *Plasmodium falciparum* towards the currently used antimalarials (Wernsdorfer, 1994). The situation has drawn attention to the use of artemisinin and derivatives (artemether, artesunate, dihydroartemisinin etc) as possible alternative medicaments (Harinasuta and Karbwang, 1994). This group of compounds has been used successfully in the treatment of patients with multidrug resistant *P.falciparum* malaria, both in severe and uncomplicated cases (Myint *et al*, 1989; Bunnag *et al*, 1991; 1992; Karbwang *et al*, 1992; Li *et al*, 1994; Hein *et al*, 1996). Their onset of action is rapid; more than 95% of the parasitemia was cleared from peripheral blood within 24 hours after dosing. Due to this promising clinical efficacy and tolerability profiles of artemisinin derivatives, potential usage of the drugs in coping with the situation of multidrug resistant falciparum malaria has been increasing. Information on pharmacokinetics and pharmacodynamics (antimalarial activity) is essential in designing optimal therapeutic regimens of this group of antimalarials. The present study aimed to investigate the kinetics of *ex vivo* antimalarial activities (blood schizontocidal activities) of serum samples collected from healthy volunteers following single dose administrations of various regimens of artemisinin derivatives.

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MATERIALS AND METHODS

Malaria parasite

K_1 strain *P.falciparum* which is resistant to chloroquine and pyrimethamine was used as a test organism in this study. The parasite was originally isolated from a patient who was a resident of Kanchanaburi Province, western Thailand (Thaithong and Bealses, 1983). The parasites were routinely maintained in continuous culture (Trager and Jensen, 1976) in a 60 x 15 mm disposable plastic petri dishes (Nuncon®, Denmark), using RPMI 1640 medium containing *L*-glutamine (formula 430-1800; Gibco, New York), with 24 mM HEPES buffer (*N*-2-Hydroxyethyl-piperazine-*N*'-2-ethanesulfonic acid, Sigma), 32 mM $NaHCO_3$, and gentamicin (80 mg/l). The medium was supplemented with 10% (v/v) type-AB human serum obtained from Ramathibodi Blood Bank, Bangkok. The initial parasitemia was 0.5% with 6-8% cell suspension in a total volume of 4 ml. Prior to use, parasites were synchronized

with 5% *D*-sorbitol (Lombros and Vanderberg, 1979).

Preparation of parasitized erythrocytes

A 5% suspension of the infected RBCs (K_1 strain *P. falciparum*) with an initial parasitemia of 0.5-1% (>90% in ring form) was used for the assay. After removal of culture medium through centrifugation (1,000*g*, 7 minutes, 4°C), packed RBCs were resuspended in an equal volume of complete RPMI medium to make 50% (v/v) cell suspension. An appropriate 5% cell suspension with 0.5-1% parasitemia was prepared by diluting 50% cell suspension with normal O cells (50% v/v) and complete RPMI medium with AB-serum.

Serum samples from healthy volunteers after the administration of artemisinin derivatives

A series of serum samples (duplicate aliquots of 500 µl) were collected at intervals (at 0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 30, 48 and 72 hours after dosing), from 8 healthy Thai males following the administration of a single oral dose of various regimens of artemisinin derivatives as follows; (a) a single oral dose of 300 mg artemether (Artenam®, Arenco n.v., Belgium; 50 mg per tablet); (b) a single intramuscular injection of 300 mg artemether (Artemether injection, Arenco n.v., Belgium; 80 mg/ml per ampoule); (c) a single dose of artemether, given as a suppository dose of 100 mg (Arenco n.v., Belgium; 50 mg); (d) a single oral dose of 300 mg artesunate (Guillin Pharmaceuticals, People's Republic of China; 50 mg per tablet); (e) a single oral dose of 300 mg artesunate (Arenco n.v., Belgium; 50 mg per tablet); (f) a single oral dose of 300 mg dihydroartemisinin (Arenco n.v., Belgium; 100 mg per capsule).

The study was of a cross-over, randomized design, in which subjects were allocated to receive the six regimens of artemisinin derivatives on six separate occasions. The wash-out period between each drug administration was 7 days. Serum were obtained through centrifugation (1,200*g*, 10 minutes) immediately after clotting (2 hours), and stored at - 80°C until analysis.

Serum concentrations of artemether, artesunate and dihydroartemisinin (as an oral formulation or as an *in vivo* active metabolite of artemether and artesunate) were measured by high performance liquid chromatography with electrochemical detection, according to the methods of Karbwang *et al* (1997a) and Na-Bangchang *et al* (1998a). Coefficients of variation for all three compounds were below 10% at the concentration range of 10-800 ng/ml. The quantification limits for artemether, artesunate and dihydroartemisinin assay were 3, 3, and 2 ng/ml, respectively.

Assessment of *ex vivo* blood schizontocidal activities of the sera

The sera collected from all of the eight healthy subjects were investigated for their blood schizontocidal activities against K_1 strain *P. falciparum* using the standard *in vitro* microtechnique of Rieckmann *et al* (1978) with modifications. Serum samples collected at each instance were serially diluted with normal AB serum to the dilutions of 1:2, 1:4,1:6,1:8,1:12,1: 16, 1:20,1;24,1:28,1:32,1:36, 1:42, 1:56,1:64 and 1:128. The final volume in each well of the microtiter plates (96 wells, flat bottom, 8x12 matrix; Nuncon®, Denmark) was 100 µl. The innoculum in each triplicate well consisted of 80 µl of the prepared parasite cell suspension (0.5-1% parasitemia, 5% cell suspension, >90% ring stage), and 20 µl of the diluted sera from volunteers (or AB-serum in the control wells).

Evaluation of results

Following 48 hours of serum exposure (37°C, an atmosphere of 96% N_2, 1%O_2 and 3%CO_2 in a candle jar), a thin blood smear was made from each well, stained with Giemsa's stain and examined for the number and morphology of the ring, trophozoite, and schizont stage of *P.falciparum*. The assay was considered successful if at least 4 to 5-fold increase in number of parasitized cells in the control wells was achieved. The number of infected RBCs with normal appearance was counted per 10,000 RBCs.

The effect of drug-containing sera on the parasites were assessed microscopically for both a decrease in parasite density, and the viability of the remaining parasites. *Ex vivo* blood shizontocidal activities of serum samples collected from volunteers after the administration of various regimens of artemisinin derivatives were evaluated by using the maximum inhibitory dilution (MID) as an end-point. The MID was determined by noting the maximum dilution of drug-containing serum at which complete inhibitory effect on parasite growth/viability (at least 95% inhibition; IC_{95}) was produced. Activity was finally transformed to, and expressed as equivalent concentrations of dihydroartemisinin (DHA_{eq}: nmol/l), using serum drug concentrations (nmol/l), MID, and relative antimalarial potency of each drug as factors in calculation. The relative potency was determined from the ratio of IC_{50} of dihydroartemi sinin and the drug under investigation (artemether,

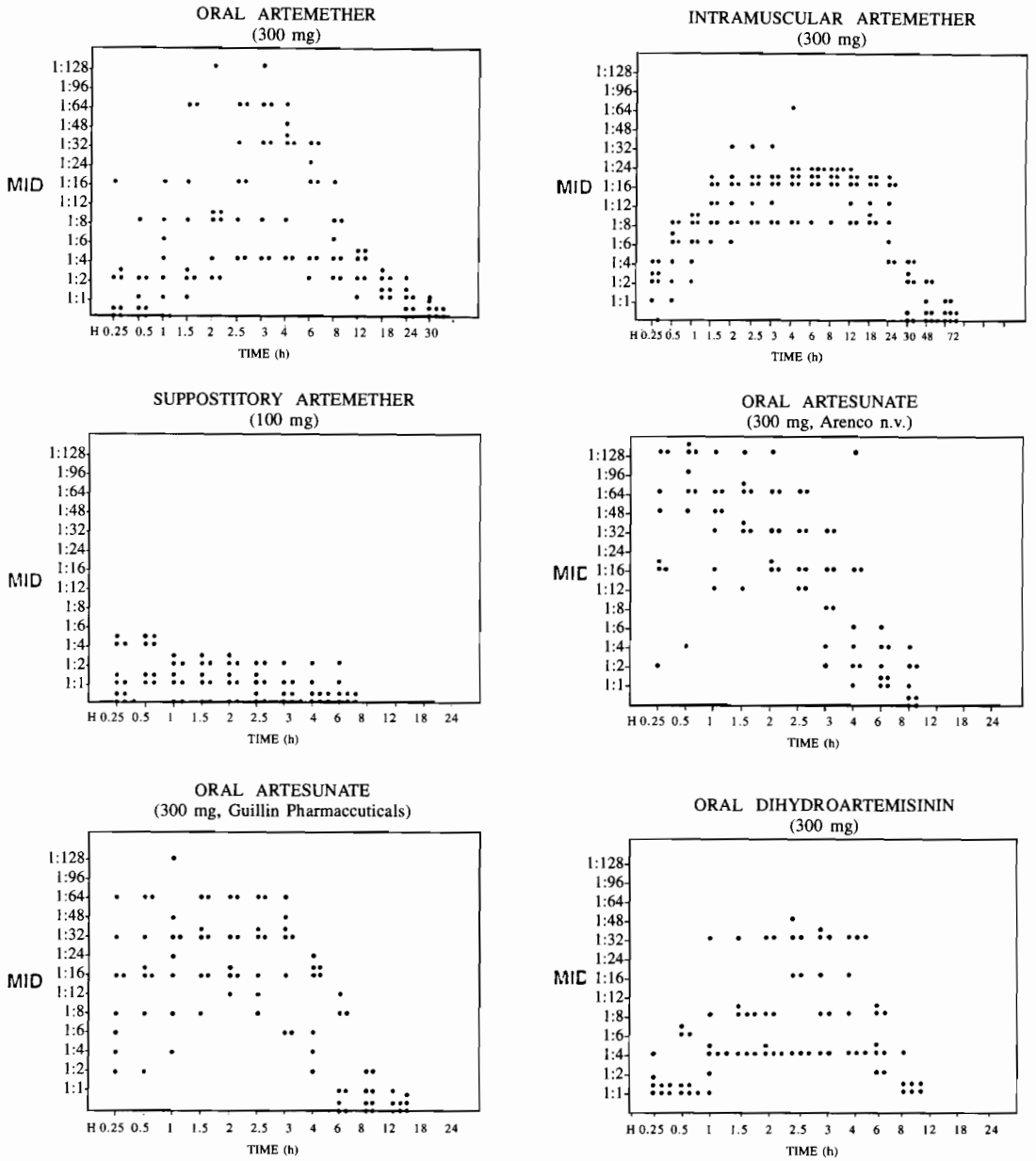

Fig 1–Patterns of MIDs of sera obtained from 8 volunteers following a single doses of artemether, artesunate and dihydroartemisinin.

artesunate) against K_1 strain *P. falciparum in vitro* (IC_{50}: artemether: 0.914 nmol/l; artesunate: 0.131 nmol/l; dihydroartemisinin 0.181 nmol/l). Overall activity (DHA_{eq}) of serum after artemether or artesunate dosing was the combined activity of the parent compounds themselves and the *in vivo* active metabolite–dihydroartemisinin.

The time courses of *ex vivo* blood schizontocidal activity of sera following the administration of all derivatives/formulations were analyzed by model-independent method. A_{max} (the maximum blood schizontocidal activity), t_{Amax} (the time to maximum activity) and TD (the time from the first until the last observed activity) were directly obtained from data, and AUA (area under the activity-time curve) was calculated using linear trapezoidal rule.

Comparison of the *ex vivo* serum blood schizontocidal activities from various drug administrations

was done by Kruskal-Wallis test, and significant difference between paired-data was performed using Wilcoxon signed rank test. The significance of the statistical tests was set at $p < 0.05$.

RESULTS

Minimum inhibitory dilution

No inhibitory effect was observed in the undiluted sera collected prior to the administration of all drug regimens, as well as in the control wells. In most cases, activities of sera obtained following the administration of all derivatives/formulations were first detected at the early time of 15 minutes after dosing. In wells of complete inhibition, all parasites were inhibited at the ring stage. Wells that exhibited incomplete inhibition (at higher serum titer), contained mixed stages of both live and dead parasites; only a few normal mature schizonts and ring stage parasites were observed together with some dead trophozoites and dead schizonts.

Artemether: Marked variation of MIDs was noted for sera collected after oral artemether dosing, particularly during the period of maximum activity *ie* at hours 1.5-4 (Fig 1a). Higher MIDs (1:128 *vs* 1:32) but shorter duration of activity (12-30 *vs* 30-72 hours) was noted with sera from oral compared to intramuscular dosing (Fig 1a, 1b). Following the lower dose of artemether (100 mg) administered as suppository formulation, markedly lower MIDs (1:1 to 1:4) was observed. (Fig 1c).

Artesunate: Similar patterns of MIDs were observed in sera obtained following the administration of 300 mg of the two pharmaceutical formulations of oral artesunate. Sera from artesunate-Arenco dosing however, exhibited higher range of maximum MIDs (1:32 to 1:128 *vs* 1:12 to 1:64) compared to that after artesunate-Guillin dosing (Fig 1d, 1e).

Dihydroartemisinin: Fig 1f depicts activities of the sera obtained from volunteers after the administration of 300 mg dose of oral dihydroartemisinin. Maximum MIDs of sera ranged between 1:16 and 1:48, and at hour 8, almost all showed no activity.

Ex vivo blood schizontocidal activity

Ex vivo blood schizontocidal activities of sera (expressed as DHAeq) obtained after various derivatives/formulations are summarized in Fig 2 and Table 1.

Artemther: When normalized with dose, sera after suppository dosing exerted lowest bioactivity (median A_{max}: 0.58 nmol/l/mg dose; AUA: 0.01 nmol.h/l/mg dose) and shortest duration of activity (median TD: 12 hours), comparing to the other two formulations of artemether (oral, intramuscular). Median values for A_{max} of sera from oral dosing were 2.4- and 118-fold, while AUA were 0.82- and 2,370-fold of that after the intramuscular and suppository dosing, respectively.

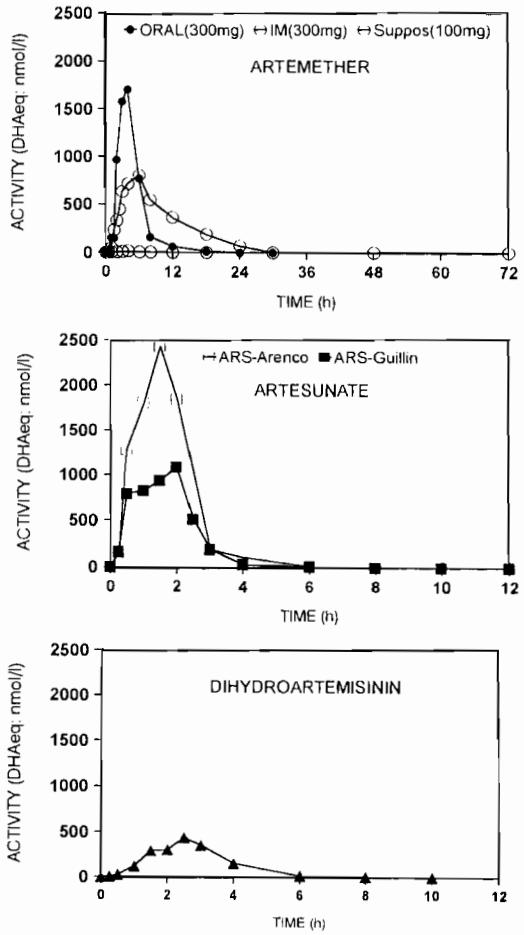

Fig 2–*Ex vivo* blood schizontocidal profiles of sera obtained after the administrations of artemether, artesunate and dihydroartemisinin.

Artesunate: Sera from artesunate-Arenco dosing exhibited significantly higher A_{max} and AUA (normalized with dose), compared to that from artesunate-Guillin dosing.

Oral dihydroartemisinin in relation to oral formulations of artemether and artesunate: Sera collected following a single dose of oral dihydroartemisinin showed lowest bioactivity among the oral formulations of artemisinin derivatives investigated. However, due to large variation, statistically

significant differences were found only when compared with the activity following artesunate-Arenco dosing (higher A_{max} and AUA with artesunate-Arenco).

DISCUSSION

Ex vivo blood schizontocidal activity profiles of sera obtained from volunteers after the administration of all regimens of artemisinin derivatives under investigation generally, coincided with their pharmacokinetic profiles (Karbwang *et al*, 1997b; Na-Bangchang *et al*, 1997; 1998b). The rapid onset and short duration of activity were characterized by their rapid absorption and elimination kinetic characteristics. The activity observed under the *ex vivo* condition closely mimics what occurs *in vivo*, in which bioactivity as a whole, is a composite of the pharmacokinetics and pharmacodynamics (blood schizontocidal activity) of both the parent drug and the active plasma metabolite. At present, dihydroartemisinin is the only identified active metabolite of all artemisinin derivatives (Lee and Hufford, 1990), which possesses antimalarial activity approximately 3-4 times as potent as the parent drugs (Basco *et al*, 1993). Since all artemisinin derivatives are extensively metabolized in liver to this compound, entire bioactivity is determined to a great degree by the rate and extent of this biotransformation process. It is interesting to note that, certain degree of activity still persisted in some serum samples collected at the time when serum levels of artemether, artesunate or dihydroartemisinin fell below the limits of quantification (2-3 ng/ml). This persistent activity was probably accounted for by other unidentified active metabolite(s).

Despite the fact that several formulations and generics of different artemisinin derivatives have been used extensively for the treatment of malaria,

Table 1

Ex vivo blood schizontocidal activities of sera obtained from volunteers following the administration of various regimens of artemisinin derivatives (presented as median and range).

	Artemether*			Artesunate (oral)**		Dihydroartemisinin
	Oral	Suppository	Intramuscular	Arenco	Guillin	(oral)***
A_{max} (nmol/l)	2,050	17.5	841	3,710	1,540	704
	(215-3,730)	(7.2-88)	(52-1,210)	(1,130-10,700)	(438-2,900)	(129-4,520)
A_{max}	6.83	0.058[a]	2.8[b]	12.4[f]	5.13	2.35[h]
(nmol/l/mg dose)	(0.72-12.43)	(0.024-0.29)	(0.17-4.0)	(3.77-35.67)	(1.46-9.67)	(0.43-15.1)
t_{Amax} (h)	3	4	6[c]	1.5	1.5	2.5
	(2.5-4)	(3-4)	(3-6)	(0.25-2.5)	(0.5-2.5)	(1-3)
AUA	7,096	108	8,687	6,574	2,646	1,322
(nmol.h/ml)	(1,145-11,450)	(28-3,456)	(6,720-11,969)	(3,827-13,372)	(1,310-4,202)	(213-8,002)
AUA	23.7	0.01[d]	29	21.9[g]	8.8	4.4[i]
(nmol.h/ml/mg dose)	(4.8-38.2)	(0.004-0.32)	(22.4-39.9)	(12.75-44.57)	(4.37-14)	(0.7-26.7)
t_{lag} (h)	0.5	0.5	0.25	0.25	0.25	0.25
	(0.25-0.5)	(0 5-1)	(0.25-0.5)	(0.25-0.55)	(0.25-0.25)	(0.25-0.25)
TD (h)	21	12[e]	36	7	7	10[j]
	(8-48)	(8-18)	(24-96)	(4-12)	(6-8)	(8-12)

*Statistics for comparison of artemether

[a]Significantly different from oral (p 0.0078, C.I. 0.82, 10.1) and intramuscular artemether (p 0.0078, C.I. 1.41, 3.43)

[b]Significantly different from oral (p 0.002, C.I. 1. 8.7)

[c]Significantly different from oral (p 0.027, C.I. 0, 3.5) and suppository artemether (p 0.033, C.I. 0.5, 3)

[d]Significantly different from oral (p 0.0078, C.I. 5.55, 33.21) and intramuscular altemether (p 0.0078, C.I. 23.4, 37.6)

[e]Significantly different from oral (p 0.0078, C.I. 0.026, 0.3) and intramuscular artemether (p 0.012, C.I. 11,60)

**Statistics for comparison of artesunate

[f]Significantly different from altesunate-Guillin (p 0.039, C.I. 2.55, 19.7)

[g]Significantly different from artesunate-Guillin (p 0.0078, C.I. 6.23, 24.9)

***Statistics for comparison of oral dihydroartemisinin, oral artemether, oral artesunate

[h]Significantly different from artesunate-Arenco (p 0.02, C.I. 0.7, 1.5)

[i]Significantly different from artesunate-Arenco (p 0.0078, C.I. 1.2, 3.9)

[j]Sinificantly different from oral artemether (p 0.017, C.I. 6, 25) and artesunate-Guillin (p 0.0097, C.I. 2, 4)

very little is known about their relative bioavailability or bioequivalence. The present study has well demonstrated the applicability of the *ex vivo* model, in addition to the standard pharmacokinetic method, in evaluating bioavailability and bioequivalence of artemisinin derivatives. The results, in conjunction with the previous findings (Karbwang *et al*, 1997b; Na-Bangchang *el al*, 1997; 1998b) suggest that the pharmaceutic preparations and generics influenced pharmacokinetics/antimalarial activity profiles of artemisinin derivatives. Sera collected after the three pharmaceutical formulations of artemether *ie* oral, intramuscular and suppository formulations exhibited distinct profiles of *ex vivo* blood schizontocidal activity. Activity of sera after suppository dosing (normalized with dose) was remarkably low and variable comparing to the other two formulations. Overall blood schizont inhibitory activities (reflected by AUA) of sera collected after intramuscular and oral dosing were similar, although lower A_{max} and more prolonged t_{Amax} were observed with the first. In the previous assessment of pharmacokinetics/ bioavailability of oral and intramuscular artemether (Karbwang *et al*, 1997b), relatively higher maximum plasma concentration and systemic exposure of artemether, but lower maximum plasma concentration of the metabolite–dihydroartemisinin were found following intramuscular artemether. This indicates that integrated pharmacokinetics/ pharmacodynamics of the two active moieties (artemether and dihydroartemisinin) following the administration of these two artemether formulations, eventually resulted in insignificant difference in bioactivity.

With respect to artesunate, the results indicate bio-inequivalence of the two pharmaceutical formulations based on the *ex vivo* bioactivity (AUA), the observation of which was again, in accordance with that of the pharmacokinetic evaluation (Na-Bangchang *et al*, 1998). Pharmacokinetically, relatively lower rate and extent of artesunate absorption, but greater rate and extent of dihydroartemisinin formation were observed. *Ex vivo* investigation yet, revealed superior bioactivities of sera obtained following dosing with oral artesunate formulated by Arenco n.v., compared to that of Guillin Pharmaceutical. This observation signifies the crucial contribution of dihydroartemisinin to overall antimalarial activity following oral artesunate dosing.

Dihydroartemisinin is the active metabolite of all artemisinin derivatives, and besides, the intermediate product in the synthesis of these compounds. The results show that A_{max} and AUA of sera obtained following the adminsitration of oral dihydroartemisinin formulation was notably lower than oral artemether or artesunate. Oral artemether or artesunate is probably preferable to oral dihydroartemisinin, as they resulted in sera with combined antimalarial activity of the parent drugs and dihydroartemisinin. Furthermore, unlike oral dihydroartemisinin, sustainable high serum bioactivity was achieved from the administration of the two derivatives due to the gradual release of the parent compounds to dihydroartemisinin.

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