ァリでタンザニアの国立公園を訪ねており、同地は日本人旅行者も多いことから、研究班でも治療薬剤を保管する必要性があると判断した。

本症の治療薬については、旅行者感染症を対象とする欧州のサーベイランスネットワーク[4]から入手先を把握し、スラミン、メラルソプロール、エフロールニチンの3種類を確保することができた。

スラミンは、ペンタミジンとともに、病変が中枢神経系に及んでいない時期(病初期)の治療薬である。メラルソプロールは、元来、ガンビア型、ローデシア型両者のすべての病期に使用可能であるが、実際は病変が中枢神経系に及んでいる時期(病後期)に使われる。しかし、毒性が非常に強いのが難点である。エフロールニチンはガンビア型のみ適応となり、その病後期に使われるが、メラルソプロールより副作用が少なく耐薬性は優れている。

5．クリプトスポリジウム症

通常、免疫健常者では自然治癒するが、HIV感染などの免疫不全者では重症化することから、有効な治療薬が望まれていた。ニタゾキサニドはニトロチアゾールベンザミド系薬で、エジプトなどでの治験で免疫健常者(主に小児)での本症による下痢に優れた効果が示されている。さらに、本薬剤はジアルジア、赤痢アメーバ、トリコモナスなどの原虫や、回虫、鞭虫、肝蛭などの蠕虫、さらにはクロストリジウム属、バクテロイデス属、ヘリコバクター・ピロリなどの細菌にも広く効果を示す。研究班でも最近、本薬剤の輸入・保管を開始したが、当面は、HIV感染者などの免疫不全者におけるクリプトスポリジウム症のみを対象とする。しかし、成人あるいは免疫不全者での治験は世界でも例数が少ないため[5]、本剤の効果や安全性が確立されているとは言えない。

おわりに

本稿で述べた輸入感染症は寄生虫症、しかもほとんどが原虫症であるが、輸入感染症全体の病原体としては他の寄生虫、真菌、細菌、リケッチア、ウイルスなど多彩である。輸入感染症は一般の臨床医にはなじみが薄く、診断を誤ったり、不適切な治療が行われがちである。また、治療薬には国内未発売のものが多い。国内で輸入感染症の診療が適切に行われるために、本研究班のネットワークが活用されることを望む。

本稿は、創薬等ヒューマンサイエンス総合研究事業(KH42074)の研究成果を含む。

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熱帯熱マラリアに対するアトバコン/プログアニル合剤（マラロン®）の使用経験

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Key Words：熱帯熱マラリア，マラリア，アトバコン/プログアニル合剤，マラロン®

はじめに

新規抗マラリア薬であるアトバコン/プログアニル合剤（マラロン®，アトバコン250mg・プログアニル100mg/錠）は，他の抗マラリア薬と異なる作用機序，すなわち原虫のミトコンドリア内の電子伝達系チトクローム bc1 複合体レベルでの阻害作用を有するアトバコンと葉酸拮抗薬のプログアニルの合剤で，薬剤耐性熱帯熱マラリアに対し高い忍容性と有効性が海外で報告されている[1)~7)]。しかしわが国において，治療経過を踏まえ本剤の治療効果や副作用について検討した研究はほとんど報告されていない。

そこで，1994～2003年に本学で診療した熱帯熱マラリア患者の中で，推定感染地から薬剤耐性熱帯熱マラリアが推定され，本剤を治療に使用した17例について retrospective に解析した。

対象と方法

対象は，合併症のない熱帯熱マラリア患者（以下，軽症例）15例，重症熱帯熱マラリア患者（以下，重症例）2例で，重症度の判定はWHO重症マラリア基準（2000年）を参考にした[8)]。症例は，全例とも本学受診当日に血液塗抹ギムザ染色標本，アクリジンオレンジ染色標本の鏡検およびマラリア抗原検出法を用いて診断し，治療を開始した。治療開始後は4週間にわたり経過を観察し，再燃の有無を確認した。

マラロン®は，創薬等ヒューマンサイエンス総合研究事業「熱帯病に対するオーファンドラッグ開発研究」班から入手し，1日1回4錠，3日間，食事または乳製品とともに同じ時間に投与した。軽症例に対しては本剤を急性期治療として使用し，重症例に対してはキニーネ注射剤投与により病状改善した後に経口療法として本剤に切り換えた。

Clinical Trial of Atovaquone/Proguanil Against Falciparum Malaria

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表1　アトバコン/プログアニル合剤（マラロン®）使用患者の患者背景

	合併症のない熱帯熱マラリア (n=15)	重症熱帯熱マラリア (n=2)
性　別	男 14：女 1	男 2：女 0
平均年齢 (範囲)	32.7歳 (20～42)	38.5歳 (19～58)
推定感染地	サハラ以南熱帯アフリカ 15	サハラ以南熱帯アフリカ 2
国　籍	日本人 9：外国人 6	日本人 2：外国人 0
渡航目的	(日本人) 観光 5, 業務 3, ﾎﾞﾗﾝﾃｨｱ 1 (外国人) 帰省 4, 日本への観光 2	観光 1, 業務 1
抗マラリア薬予防内服	有 3 (全例不適正内服), 無 12	有 1 (不適正内服), 無 1
発症後当院受診までの平均日数 (範囲)	3.8日 (0～16)	6.5日 (5～8)
本剤開始時の平均体温 (範囲)	38.9℃ (38.4～40.3)	――
本剤開始時の末梢血平均原虫寄生率 (範囲)	0.43% (<0.01～2.4)	――
本剤開始時の末梢血平均原虫数 (範囲)	21272/μL (472～112320)	――
重症マラリアの合併症	――	脳症 2

軽症例，重症例について，臨床効果，寄生虫学的効果，臨床検査値異常，副作用を検討した。

結果

1）患者背景（表 1）

推定感染地は，軽症例，重症例ともに全例が薬剤耐性熱帯熱マラリアが推定されるサハラ以南熱帯アフリカであった。抗マラリア薬予防内服は，軽症例 3 例，重症例 1 例に行われていたが全て薬剤選択または内服期間が不適切で，適正な予防内服を行っていた症例はいなかった。軽症例における本剤開始時の体温は 38.4 ～ 40.3℃，末梢血原虫寄生率は 0.01% 未満～ 2.4% であった。重症例の合併症は 2 例とも脳症であった。

2）合併症のない熱帯熱マラリア患者（軽症例）

a）臨床効果

本剤投与終了翌日（投与 3 日目）の解熱患者は 4 例（26.7%）であったが，開始 1 週間後には遷延性の溶血性貧血を呈したために発熱が持続した 1 例を除く 14 例（93.3%）が解熱を認めた。発熱消失時間（FCT）の平均は 85.0 時間（12 ～ 204），転帰は全例治癒であった。

b）寄生虫学的効果

本剤初回投与翌日に 8 例（53.3%）の患者に一過性の原虫寄生率上昇がみられたが，投与終了翌日（投与 3 日目）に 7 例（46.7%），投与 5 日目には全例の患者に原虫消失を認め，以後再燃はみられなかった。原虫消失時間 (PCT) の平均は 73.1 時間 (20 ～ 108) であった。

c）臨床検査値異常（表 2）

文献[4)5)] を参考に，臨床検査値異常のクライテリアを設定し，投与開始時，開始 2 週間後，開始 4 週間後，全経過中にこのクライテリアを満たした症例数を調べた。白血球数減少，血小板数減少，総ビリルビン値上昇は投与開始時および全経過中に，中等度以上のトランスアミナーゼ上昇は投与開始時，開始 2 週間後および全経過中にクライテリアを満たした症例がそれぞれ数例みられたが，開始 4 週間後には改善していた。

d）副作用

嘔吐が 4 例（26.7%）にみられたが，いずれも本剤内服 1 時間以降に出現し，高熱を呈していた時の嘔吐で，制吐剤の併用，解熱時には嘔吐は認められなかった。

表2　合併症のない熱帯熱マラリア（n＝15）におけるマラロン® 使用後の主な臨床検査値異常

パラメーター	クライテリア	クライテリアを満たした症例数 (%)			
		投与開始時	開始2週間後	開始4週間後	全経過中
WBC	＜3000/μL	1 (6.7%)	0	0	4 (26.7%)
Hb	＜7.5 g/dL	0	0	0	0
Plt	＜50x10³/μL	1 (6.7%)	0	0	4 (26.7%)
AST	＞100 IU/L	0	1 (6.7%)	0	2 (13.3%)
ALT	＞100 IU/L	1 (6.7%)	3 (20.0%)	0	5 (33.3%)
T-Bil	＞2 mg/dL	2 (13.3%)	0	0	5 (33.3%)

3) 重症熱帯熱マラリア（重症例）

キニーネ注射剤の持続点滴により意識状態の改善を待って本剤による経口療法へ変更した2例は，経過観察期間中に再燃，副作用を認めなかった。

考案

薬剤耐性熱帯熱マラリアが推定される合併症のない熱帯熱マラリア患者において，マラロン®による治療は全例が治癒し，再燃は認められなかった。海外の報告同様[4)~7)]，本剤は効果発現が遅く，初回投与翌日に約半数の患者に一過性の原虫寄生率上昇を認めたが，開始1週間後には93%の患者に解熱を，全例に原虫消失を認めた。マラリア感染または薬剤性，溶血等が原因と思われる臨床検査値異常が本剤開始時および経過中にみられたが，開始4週間後には全て改善した。副作用では嘔吐が26.7%に認められたが，いずれも投薬1時間以降に出現し，高熱を呈していた時の嘔吐で，本剤との関連性は低いと考えられた。以上より，薬剤耐性熱帯熱マラリアが推定される合併症のない熱帯熱マラリア患者の急性期治療において，マラロン®は効果発現が遅いが，治癒率および忍容性が高く，内服方法も簡便で有用性が高いと考えられた。

重症熱帯熱マラリア患者2例は，キニーネ注射剤投与により病状改善した後に経口療法として本剤に切り換えたが，再燃，副作用を認めず治癒に至った。

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Plasmodium falciparum: selenium-induced cytotoxicity to *P. falciparum*

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Abstract

The in vitro antimalarial activity of sodium selenite (NaSe) was investigated and the mechanism of its action was studied. NaSe had antimalarial activity against both the chloroquine-susceptible strain FCR-3 and chloroquine-resistant strain K-1 of *Plasmodium falciparum*. The shrunken cytoplasm of the parasite was observed in a smear 12 h after treatment with NaSe. Co-treatment with copper sulfate ($CuSO_4$) in culture did not affect the antimalarial activity of NaSe, but NaSe cytotoxicity against the mammalian cell line Alexander was decreased significantly. The intracellular reduced glutathione level of parasitized red blood cells was decreased significantly by treatment with NaSe, and the decrease was consistent with their mortality. Treatment with NaSe had a strong inhibitory effect on plasmodial development, and NaSe cytotoxicity to human cells was decreased by co-treatment with $CuSO_4$. These results suggest that co-treatment with NaSe and $CuSO_4$ may be useful as a new antimalarial therapy.
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Index Descriptors and Abbreviations: Plasmodium falciparum; antiplasmodial activity; sodium selenite; pRBCs, parasitized red blood cells; RBCs, red blood cells; GSH, reduced glutathione; NaSe, sodium selenite; $CuSO_4$, copper sulfate

1. Introduction

Drug resistance in malaria is an important public health concern. *Plasmodium falciparum* has developed resistance to most of the commonly used antimalarial drugs including chloroquine, sulfadoxine–pyrimethamine, and mefloquine (Wongsrichanalai et al., 2002). Therefore, a search for new antimalarial drugs is very important.

Selenium is an essential trace element in mammals. Numerous epidemiological and experimental studies have found an anticarcinogenic activity of selenium (Clark et al., 1996; Combs and Gray, 1998; Comstock et al., 1992; Siwek et al., 1994; Spallholz, 1994; Spyrou et al., 1996; Thompson et al., 1994). Recently, it has been argued that the anticarcinogenic activity of selenium depends on its ability to generate superoxide, which is known for its DNA-damaging activity and induction of apoptosis (Lu et al., 1994).

There are some preliminary experiments suggesting that oxidative stress is the cause of selenium cytotoxicity and growth inhibition. Some investigators reported that the superoxide anion was generated by the reaction of sodium selenite (NaSe) with reduced glutathione (GSH) in a cell-free system (Davis and Spallholz, 1996). Other investigators reported that the cytotoxicity of selenium was decreased by co-treatment with copper sulfate ($CuSO_4$) (Jensen, 1975). But the mechanisms of these beneficial reactions are not known.

Since the reactive oxygen species is known to have the toxic effects for the malaria parasite and NaSe generate the superoxide and consume the intracellular GSH, we have evaluated the antimalarial effect of NaSe and investigated its interaction with intracellular GSH for development of new antimalarial drug to resolve drug resistance problem.

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2. Materials and methods

2.1. Parasites and cells

Chloroquine-susceptible *P. falciparum* strain FCR-3 and chloroquine-resistant *P. falciparum* strain K-1 were grown asynchronously, following the modified method of Trager and Jensen (1976), in RPMI 1640 medium supplemented with 10% human B serum, 25 mM Hepes, 25 μg/ml gentamicin (Sigma–Aldrich, St. Louis, MO), sodium bicarbonate, and human type O red blood cells (RBCs) in disposable sterile dishes under a controlled atmosphere of 5% CO_2 at 37 °C.

The human hepatocellular carcinoma cell line Alexander (Alex cells) was a kind gift of Dr. Takeaki Nagamine, Gunma University School of Health Sciences. Alex cells were grown continuously in complete Dulbecco's modified Eagle's medium (DMEM; BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin (100 U/ml, 10 ml/liter; BRL, Grand Island, NY) under a 5% CO_2 atmosphere at 37 °C.

2.2. Investigation of in vitro plasmocidal effect of NaSe

Since several studies have shown effective anticarcinogenic activity of NaSe between 10 and 100 μM (Batist et al., 1986; Caffrey and Frenkel, 1992; Davis et al., 1998), RPMI 1640 medium was supplemented with either a 10 or 100 μM NaSe solution that was sterilized by filtration through a 0.22-μm filter. The NaSe-supplemented medium was changed every 24 h. Five hundred microliters of a parasitized-RBCs (pRBCs) suspension was placed in each well of a 24-well flat bottom culture plate (Corning Costar, New York, NY) at a hematocrit of 5% and an initial parasitemia of 0.1%. Thin-smeared specimens stained with Giemsa solution were made every 24 h and parasitemia was evaluated by counting the parasites in 3000 RBCs. Morphological changes of the parasites were monitored by observing Giemsa-stained thin-smeared specimens 2, 4, 12, and 24 h after treatment.

2.3. Determination of IC_{50} of NaSe against parasite

The pRBCs were synchronized with 5% D-sorbitol for 15 min at room temperature as described by Lambros and Vanderberg (1979) and then were washed three times with RPMI 1640 medium by centrifugation at 400*g* for 5 min. After washing, the pRBCs were resuspended in RPMI 1640 medium supplemented with 10% human B serum, 25 mM Hepes, 25 mM gentamicin, and sodium bicarbonate. Synchronous pRBCs having about 2% parasitemia were placed in 24-well cell culture plates as described above. Twenty microliters of NaSe solution was added to each well to give a series of concentrations from 10 to 100 μM. Six wells per plate served as NaSe-free controls to monitor parasite growth. After 24 h of incubation under a 5% CO_2 atmosphere at 37 °C, the control wells were checked for parasite growth. When the schizonts were fully grown in the control wells, the culture plate was removed from the incubator. Thin-smear specimens stained with Giemsa solution were made from the contents of each well. We first counted RBCs in the control smears until we encountered a total of 50 schizonts. The effect of NaSe on parasite growth was evaluated by comparing the number of schizonts in the same number of RBCs as was counted in the control cultures. The growth inhibition effect (%) was calculated as follows: (test well schizont count/control well schizont count) × 100. The NaSe concentration inhibiting parasite growth by 50% (IC_{50}) was calculated by the probit method (Inaba et al., 2001).

2.4. Detection of hemolysis caused by treatment with NaSe and $CuSO_4$

Hemolytic level was determined by measuring the hemoglobin that eluted into the medium with the SLS-hemoglobin method (Hemoglobin B test Wako, Wako Pharmaceutical, Osaka, Japan). Briefly, after exposure of pRBCs or RBCs to 40 μM NaSe that completely inhibited parasite growth and 2.5–40 μM $CuSO_4$, as described above, samples were centrifuged at 1000*g* for 5 min at 20 °C, and the supernatant was collected and analyzed. These Se:Cu ratios were shown to decrease NaSe toxicity against carcinoma cells (Shen et al., 2001).

2.5. Cytotoxicity of NaSe and $CuSO_4$ to *P. falciparum* and Alex cells

Synchronized FCR-3 parasitized erythrocytes having about 2% parasitemia were cultured in 24-well cell culture dishes. Each well, containing 500 μl of a 5%—hematocrit RBC suspension was supplemented with a 100% growth inhibition (IC_{100})—concentration of NaSe and various concentrations of $CuSO_4$ (2.5–40 μM). The remaining procedures were the same as described above. The growth inhibition effect was calculated as shown above. Morphological changes of the parasites in these conditions were monitored by observing Giemsa-stained thin-smeared specimens at 2, 4, 12, and 24 h after treatment.

To assess NaSe and $CuSO_4$ cytotoxicity to a mammalian cell line, the same experiment was carried out with Alex cells. Alex cells were seeded at 0.5×10^6 cells/ml in 24-well culture plates and incubated (37 °C, 5% CO_2) 24 h in 1 ml of DMEM supplemented with NaSe (40 μM) and $CuSO_4$. After the incubation period, detached cells were collected; attached cells were rinsed in the wells once with phosphate-buffered saline (PBS, pH 7.4) and then trypsinized to remove them from the solid

matrix. Cells were then collected by centrifugation (10 min, 830*g*, 25 °C). Cell viability was assessed by trypan blue exclusion.

2.6. GSH measurement in pRBCs, normal RBCs, and Alex cells

Intracellular GSH measurement was done according to Hissin and Hilf's method (Hissin and Hilf, 1976). Briefly, after treatment with NaSe and $CuSO_4$, cells were counted with a hemocytometer. After washing with PBS, homogenization buffer (4:1 mixture of 0.1 M sodium phosphate, 0.005 M EDTA buffer, pH 8, and 25% metaphosphoric acid) was added. All suspensions were homogenized on ice with a sonic wave homogenizer for 40 s. The homogenate was centrifuged (30 min, 21000*g*, 4 °C), and the supernatant was collected for analysis. After addition of fluorescent agent *o*-phthalaldehyde (0.1% in methanol), GSH level was determined with a multi-well plate reader (CytoFluor, Perspective Biosystem, Framingham, MA) at an excitation wavelength of 360 nm and an emission wavelength of 420 nm. Standards contained 0–2 μg GSH/ml.

2.7. Data analysis

Data are presented as means ± SEM from at least three sets of independent experiments. Student's *t* test was used for statistical analyses. A *P* value <0.05 was considered statistically significant.

3. Results

3.1. The plasmocidal activity of NaSe to chloroquine-susceptible and -resistant *P. falciparum*

To confirm the plasmocidal activity of NaSe, chloroquine-susceptible and -resistant *P. falciparum* were exposed to medium containing either 10 or 100 μM NaSe for 3 days and growth inhibition was monitored by determining the parasitemia (Fig. 1). In the presence of 10 μM NaSe, the growth of chloroquine-susceptible FCR-3 caused less parasitemia (1.14 ± 0.34%) than in the control culture (2.41 ± 0.30%, $P < 0.0005$, Fig. 1A). The growth of chloroquine-resistant K-1 also caused less parasitemia (0.20 ± 0.06%) than in the control culture (1.29 ± 0.01%, $P < 0.005$, Fig. 1B). In the presence of 100 μM NaSe, the growth of both strains was inhibited completely.

Results of the in vitro drug susceptibility tests using NaSe are shown in Fig. 1C. The IC_{50} values for NaSe were 21.26 ± 0.94 μM for FCR-3 and 15.50 ± 1.60 μM for K-1. There was no significant difference between IC_{50} values of chloroquine-susceptible FCR-3 and -resistant K-1 ($P > 0.05$). No intact parasites were observed after treatment with 40 μM NaSe. For this reason, 40 μM NaSe was applied in the following experiments.

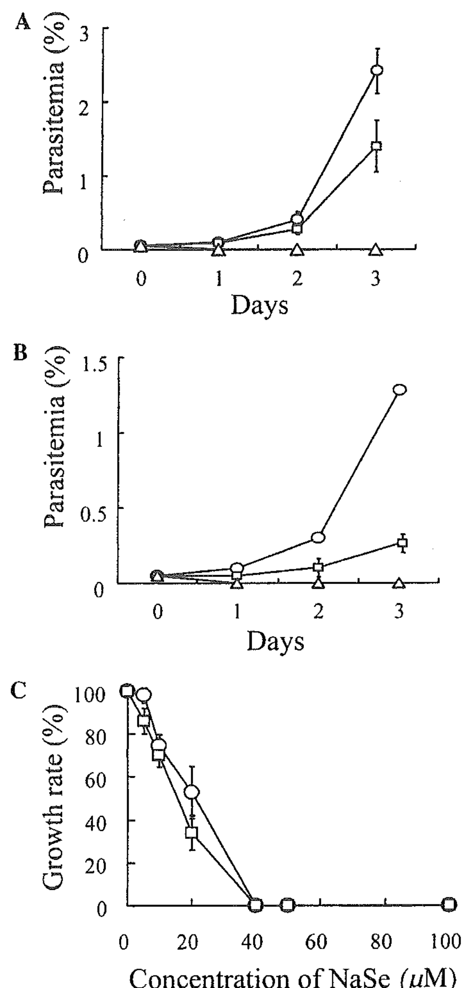

Fig. 1. Antimaralial effect of NaSe. NaSe was added to cultures of chloroquine-susceptible strain FCR-3 (A) and chloroquine-resistant strain K-1 (B) at concentrations of 100 μM (triangle), 10 μM (square), and 0 μM (circle). Parasitemia (%) was determined every 24 h. (C) Dose-dependent effect of NaSe on the inhibition of FCR-3 (circle) and K-1 (square) maturation in vitro. K-1 has less sensitivity than does FCR-3. Results are presented as means ± SEM. SE bars smaller than symbols are not shown.

3.2. Effect of $CuSO_4$ on NaSe toxicity to Alex cells or FCR-3

The effect of $CuSO_4$ in combination with NaSe on parasite growth was investigated. Although the mortality of the Alex cells was 30.01 ± 10.00% upon exposure to medium containing 40 μM NaSe, the mortality of Alex cells was decreased by co-treatment with $CuSO_4$ (Fig. 2). In the presence of either 2.5 or 40 μM $CuSO_4$, the susceptibility of the parasites to NaSe was not affected. Mortality of parasites was higher (50.0 ± 15.9%) than that of Alex cells (0.13 ± 1.50%) upon exposure to 20 μM $CuSO_4$ only.

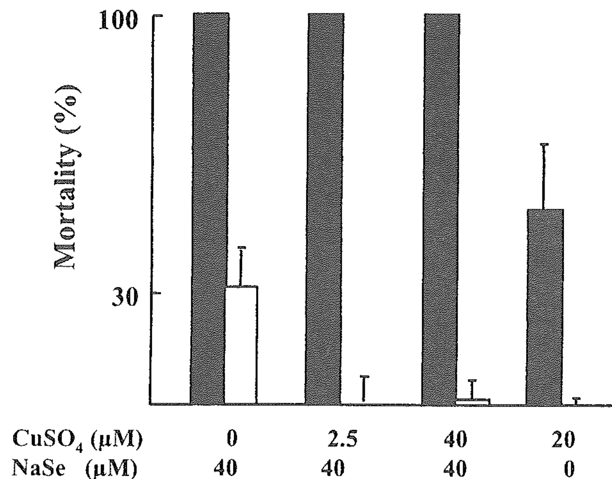

Fig. 2. Cell mortality after treatment with NaSe and $CuSO_4$ for 24 h. Trypan blue exclusion was used to assess mortality of Alexander cells (white bar). The effect of sodium selenite on *P. falciparum* growth was evaluated by comparing the number of schizonts in test erythrocytes with the number in the control cultures (black bar). The results are presented as means ± SEM.

3.3. Hemolysis caused by NaSe and $CuSO_4$

To detect hemolysis as an index of cytotoxicity, the concentration of hemoglobin in pRBCs and RBCs culture media was determined. No hemolysis was detected in the pRBCs culture medium in the presence of 40 μM NaSe only or of 20 μM $CuSO_4$ only. In the presence of 40 μM NaSe with 40 and 20 μM $CuSO_4$, the concentration of hemoglobin was significantly higher (6.53 ± 0.29 g/dl) than in the control culture (0.37 ± 0.05 g/dl, $P < 0.005$) (Table 1). At $CuSO_4$ concentrations less than 10 μM, the concentration of hemoglobin ranged from 0.53 ± 0.05 to 0.69 ± 0.22 g/dl, as compared to the control level of 0.37 ± 0.05 g/dl. These values were not significantly different ($P > 0.05$). Similar results were observed in RBCs culture.

Table 1
Lysis of pRBCs or RBCs induced by NaSe and $CuSO_4$

Concentration of NaSe/Cu (μM)	Hemoglobin (g/dl)[a]	
	pRBCs	RBCs
40/40	6.53 ± 0.29	12.08 ± 0.23
40/20	1.97 ± 0.15	4.16 ± 0.77
40/10	0.53 ± 0.05	0.63 ± 0.13
40/5	0.57 ± 0.08	0.56 ± 0.06
40/2.5	0.59 ± 0.13	0.73 ± 0.13
40/0	0.69 ± 0.22	0.59 ± 0.00
0/20	0.61 ± 0.19	0.50 ± 0.06
0/0	0.37 ± 0.05	0.49 ± 0.05

[a] Each value represents mean ± SEM.

3.4. Morphological changes of parasites exposed to NaSe and $CuSO_4$

Fig. 3 shows the morphological changes of the parasites when treated with 40 μM NaSe and 2.5 μM $CuSO_4$. In the control culture, only intact ring-form parasites were present after 4 h (Fig. 3A). Four hours after treatment with 40 μM NaSe and 2.5 μM $CuSO_4$, a small number of shrunken parasites were observed (Fig. 3B). In the presence of 40 μM NaSe and no $CuSO_4$, similar morphological changes of the parasites were observed (Fig. 3C). Twelve hours after treatment, most of the parasites were early and late trophozoites in culture with 0 μM NaSe and 0 μM $CuSO_4$ (Fig. 3D). Intact parasites were undetectable and almost all the parasites were shrunken after treatment with 40 μM NaSe and 2.5 μM $CuSO_4$ (Fig. 3E) and with 40 μM NaSe and 0 μM $CuSO_4$ (Fig. 3F).

3.5. Changes of intracellular GSH content after treatment with NaSe and $CuSO_4$

To evaluate the intracellular redox condition after treatment with NaSe and $CuSO_4$, intracellular GSH contents were determined. As shown in Fig. 4, intracellular GSH levels decreased significantly after a single treatment with 40 μM NaSe in pRBCs (33.2%), RBCs (27.5%), and Alex cells (36.2%), as compared with control GSH content ($P < 0.05$). $CuSO_4$ could prevent the depletion of intracellular GSH contents in Alex cells, even at 2.5 μM, but not in pRBCs and RBCs.

4. Discussion

Selenium is an essential trace element in humans. It exists in a number of forms with differing valence states, some of which have antineoplastic activity. The usual form of selenium used for supplementation is either selenite or selenomethionine; both have been given in doses up to 200 μg without toxicity. We studied the plasmocidal activity of NaSe against chloroquine-susceptible and -resistant *P. falciparum* strains. Our results showed that both *P. falciparum* strains were susceptible to the cytotoxic effects of NaSe and that the susceptibility to NaSe-induced cytotoxicity was unaffected by co-treatment with $CuSO_4$. In contrast, no cytotoxic effect of NaSe was found in Alex cells upon co-treatment with $CuSO_4$. These results suggest a selective inhibition of plasmodia by NaSe and $CuSO_4$. However, in considering the use of NaSe in antimalarial treatment, it will be a critical issue to select the appropriate dose to optimize both the plasmocidal effect and minimize adverse effects to host cells.

Growth inhibition by NaSe of all parasite developmental stages in vitro is obviously different from the

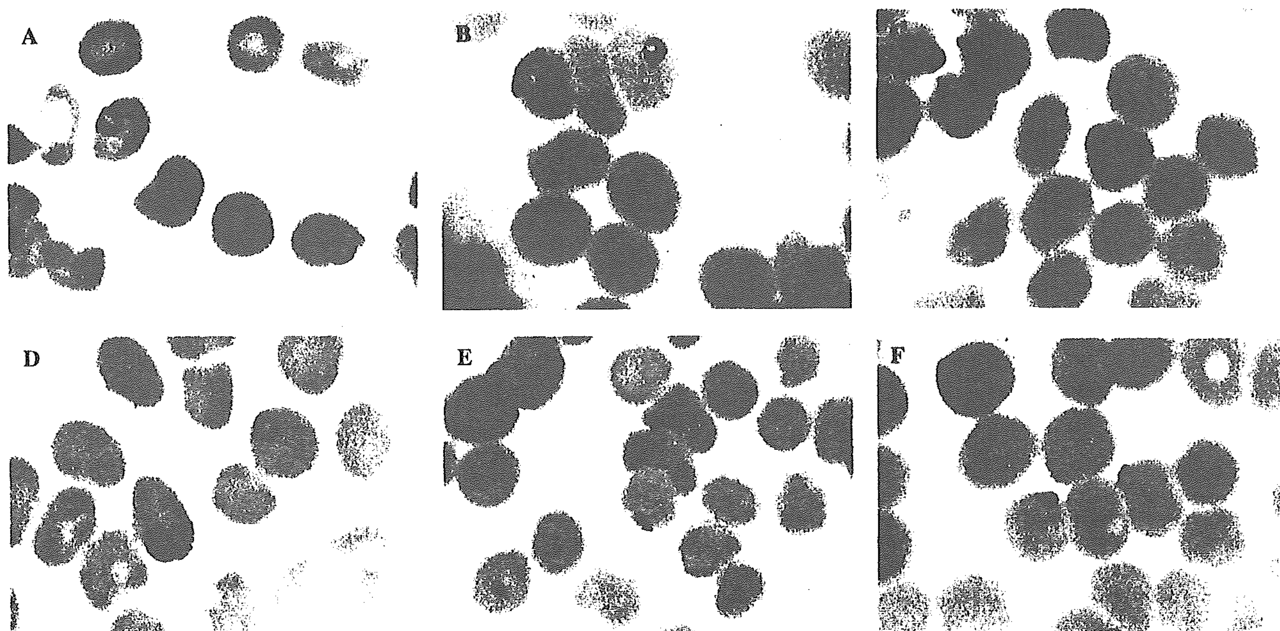

Fig. 3. Light micrographs of Giemsa-stained blood smears. (A) The parasite culture without NaSe or $CuSO_4$ for 4 h, (B) with 40 μM NaSe and 2.5 μM $CuSO_4$ for 4 h, (C) with 40 μM NaSe and 0 μM $CuSO_4$ for 4 h, (D) without NaSe or $CuSO_4$ for 12 h, (E) with 40 μM NaSe and 2.5 μM $CuSO_4$ for 12 h, and (F) with 40 μM NaSe and 0 μM $CuSO_4$ for 12 h (magnification, 1000×).

chloroquine effect on growth (data not shown). The shrunken and pyknotic appearance of parasites was observed by light microscopy (Fig. 3). Similar morphological changes of the parasites have been observed in owl monkeys infected with *P. falciparum* and treated with the Qhinghaosu derivative artemether (Kawai et al., 1993). Qhinghaosu derivatives, including artemether, are sesquiterpene lactone endoperoxide antimalarials and act through the generation of free radicals that alkylate parasite proteins (Meshnick et al., 1989). It is also well known that NaSe-induced cell death is induced by oxidative stress associated with selenite metabolism and that selenite metabolism is very sensitive to cell GSH (Davis and Spallholz, 1996; Seko and Imura, 1997; Shen et al., 2000). Morphological changes shown in this study and these facts indicate that the plasmocidal action induced by NaSe is mediated by mechanisms similar to those associated with Qhinghaosu derivatives.

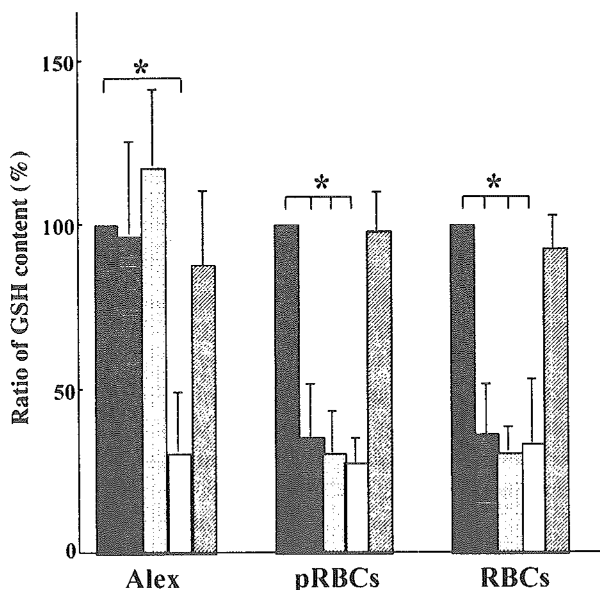

Fig. 4. The ratio of intracellular GSH content in Alexander (Alex) cells, pRBCs, and RBCs after treatment with NaSe and $CuSO_4$ to that in control cells. Results are presented as means ± SEM. The black filled column represents the control ratio for cells without NaSe and $CuSO_4$. The gray shaded column represents the ratio after treatment with 40 μM NaSe and 40 μM $CuSO_4$, the spotted column represents the ratio with 40 μM NaSe and 2.5 μM $CuSO_4$, the open column represents the ratio with 40 μM NaSe and 0 μM $CuSO_4$, and the hatched column represents the ratio with 0 μM NaSe and 20 μM $CuSO_4$. $^*P < 0.05$.

No different morphological changes of the parasites were observed between those treated with NaSe alone and with NaSe and $CuSO_4$. Treatment of human cells with NaSe and $CuSO_4$ is considered beneficial. The copper cation did not block selenite-induced oxidative stress in erythrocytes, as it does in other cells (Davis et al., 1998; Shen et al., 2001). Our data have also shown the prevention of GSH consumption by $CuSO_4$ in Alex cells. Several in vitro studies have reported that selenite treatments lead to a decline of intracellular GSH level, and co-treatment with copper prevents that decrease (Davis et al., 1998; Shen et al., 2000, 2001). However, in RBCs, GSH consumption by NaSe was not affected by $CuSO_4$. These results suggest that parasites might be killed in the same or a similar way as that seen after a single treatment with NaSe.

Detoxification of selenite toxicity in host cells by copper has been shown in in vitro (Davis et al., 1998; Shen et al., 2001) and in vivo (Jensen, 1975). It was suggested that the detoxification of selenite-induced cytotoxicity is caused by complexation with Cu^{2+}, forming GSSe–Cu^{2+}–SeSG or simply Cu^{2+}-selenide, that prevents the generation of oxygen-free radicals. It also was reported that Cu^{2+} acts as a superoxide dismutase mimic and can detoxify the selenite-induced cytotoxicity because inhibition of free radical generation was observed at a Cu:Se molar ratio of less than 1:1 (Davis and Spallholz, 1996). However, the treatment with NaSe and $CuSO_4$ caused marked cytotoxicity to *P. falciparum* but not to normal RBCs and Alex cells. This difference might be due to charge transfer from hemoglobin iron to copper in erythrocytes. Formation of methemoglobin prevented charge transfer to copper and took away its ability to complex with NaSe. Copper may act as a generator of free radical (Fernandes et al., 1988). Therefore, $CuSO_4$ may not work as a scavenger of superoxide or an inhibitor of the conversion of selenite to selenium by consumption of GSH in RBCs.

In conclusion, NaSe has a strong inhibitory effect on in vitro plasmodial development and is devoid of cytotoxicity towards human cells if there is co-treatment with $CuSO_4$. Co-treatment with NaSe and $CuSO_4$ may be a useful antimalarial regimen.

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THE PHARMACOKINETICS OF ORAL DIHYDROARTEMISININ AND ARTESUNATE IN HEALTHY THAI VOLUNTEERS

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Abstract. The pharmacokinetics of oral dihydroartemisinin (DHA) following the dose of 2 and 4 mg/kg body weight dihydroartemisinin (Twisinin™, T-2 Program, Thailand) and 4 mg/kg body weight oral artesunate (AS; Guilin Pharmaceutical Works, Guangxi, China) were investigated in 20 healthy Thai volunteers (10 males, 10 females). All formulations were generally well tolerated. Oral DHA was rapidly absorbed from gastrointestinal tract with marked inter-individual variation. The pharmacokinetics of DHA following the two dose levels were similar and linearity in its kinetics was observed. Based on the model-independent pharmacokinetic analysis, median (95% CI) values for C_{max} of 181 (120-306) and 360 (181-658) ng/ml were achieved at 1.5 hours following 2 and 4 mg/kg body weight dose, respectively. The corresponding values for $AUC_{0-\infty}$, $t_{1/2z}$, CL/f and V_z/f were 377 (199-1,128) *vs* 907 (324-2,289) ng.h/ml, 0.96 (0.70-1.81) *vs* 1.2 (0.75-1.44) hours, 7.7 (4.3-12.3) *vs* 6.6 (3.1-10.1) l/kg, and 90.5 (28.6-178.2) *vs* 6.6 (3.1-10.1) ml/min/kg, respectively (2 *vs* 4 mg/kg dose). Oral AS was rapidly biotransformed to DHA, which was detectable in plasma as early as 15 minutes of AS dosing. Following 4 mg/kg dose, median (95% CI) value for C_{max} of 519 (236-284) ng/ml was achieved at 0.7 (0.25-1.5) hours. $AUC_{0-\infty}$, and $t_{1/2z}$ were 657 (362-2,079) ng.h/ml, 0.74 (0.34-1.42) hours, respectively. C_{max} of DHA following oral AS were significantly higher, but total systemic exposure was greater following oral DHA at the same dose level (4 mg/kg body weight). There was no significant sex difference in pharmacokinetics of DHA.

INTRODUCTION

Malaria is a leading cause of mortality and morbidity in developing areas of the world, and remains a major public health problem in endemic regions (Berman *et al,* 2001). Resistance to available drugs is increasing, and therefore creating a need for new drugs that are well tolerated and simple to use. In the face of this ominous situation, artemisinin and derivatives (artesunate, arteether, and dihydroartemisinin) have lately become a renewed hope for combating the emerging generations of resistant malaria (Hein and White, 1993; Harinasuta and Karbwang, 1994; McIntosh and Olliaro, 1998). These artemisinin drugs have different physicochemical properties and are available in a variety of formulations that influence their routes of administration and dosage regimens (de Vries and Dien, 1996; van Agtmael *et al,* 1999; Navaratnam *et al,* 2000).

Artesunate (AS) is a water-soluble hemisuccinate derivative of artemisinin that is widely used in the treatment of both uncomplicated (oral formulation) and severe falciparum malaria (intravenous or suppository formulation) (White, 1994; Barradell and Fitton, 1995; de Vries and Dien, 1996; Looareesuwan *et al,* 1996; Newton *et al,* 2003). Dihydroartemisinin (DHA), a reduced lactol derivative, is the main acting blood schizontocidal metabolite of the semisynthetic artemisinin derivatives, with activity 2-5 fold that of the parent drugs (Basco and Le Bras, 1993). DHA is the chemical intermediate in the production of AS and other semisynthetic artemisinin derivatives (Lin *et al,* 1987), as well as their principal active metabolite (Lee and Hufford, 1990). Although DHA is not sufficiently water-soluble

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to be formulated as an intravenous injection, it is cheaper to produce than other aremisinin derivatives. The production of this drug is simple with the high yield. Since AS is rapidly deesterified to DHA (Yang *et al*, 1985; Batty *et al*, 1998a; b; Zhao *et al*, 1988), it may be equally acceptable to administer DHA itself. DHA is currently in clinical use as formulated tablets/capsules or suppositories. Pharmacokinetic and bioavailability data for DHA, when given as an oral or suppository formulation, have been reported (Yang *et al*, 1985; Zhao *et al*, 1988; Na-Bangchang *et al*, 1997; 1998a, b; 1999; Batty *et al*, 1998a,b; Hung *et al*, 1999; Binh *et al*, 2001; Ilett *et al*, 2002). The objective of the present study was to describe the pharmacokinetics and tolerability of the two oral doses of a new oral formulation of DHA (Twisinin™, T-2 Program, Thailand) in healthy Thai volunteers. This was performed in comparison with oral formulation of AS (Guilin Pharmaceutical Works, Guangxi, China) which has been registered for clinical treatment of uncomplicated falciparum in Thailand.

MATERIALS AND METHODS

Subjects

Twenty healthy male and female Thai volunteers, aged between 20 and 35 years, weighing 46.7 to 59 kg, who were residents of the Bangkok area, participated in the study. Inclusion criteria included: non-lactating and non-pregnant (females), no significant abnormal findings on history or examination, particularly liver, kidney, cardiovascular diseases or peripheral neuropathy, no history of antimalarial drug ingestion in the preceding three months, and no other drugs or medications ingested in the preceding week. None was a smoker or alcohol drinker nor was on regular medication. Written informed consent for participation was obtained from all the volunteers before initiation of the study. The study was approved by the Ethics Committees of the Faculty of Tropical Medicine, Mahidol University and the Ministry of Public Health, Thailand.

At enrollment, a medical history was taken, including a full physical examination; each volunteer had a thorough physical examination, routine laboratory investigations, plain chest x-ray, urinalysis, and a 12-lead electrocardiogram (ECG).

Drug administration and study design

The trial design was a single randomized three-phases cross-over model. Study participants received, in random order, the following three study sessions: (i) a single oral dose of 2 mg/kg body weight DHA (Twisinin™: 50 or 100 mg per capsule, the T-2 Program, Thailand); (ii) a single oral dose of 4 mg/kg body weight DHA (Twisinin™: 50 or 100 mg per capsule; and (iii) a single oral dose of 4 mg/kg artesunate (AS: 50 mg per tablet; Guilin Pharmaceutical Works, Guangxi, China).

Compliance with all drug intake was under investigators' supervision. No food was allowed until 2 hours after drug intake. The washed-out period after each occasion was at least 2 days. Volunteers were hospitalized in the Bangkok Hospital for Tropical Diseases one day prior to, and on the day of pharmacokinetic study. No other concurrent drugs or alcohol were taken two weeks prior to, and during the study period.

Blood sample collection

Blood samples (5 ml each) were collected through an indwelling intravenous Teflon™ catheter, inserted into a forearm vein of the subject; the patency was maintained with sodium-heparinized saline. Samples for the assay of DHA, and/or AS were collected pre-dose, and at 15, 30, 45, 60, 90, and 120 minutes, and 3, 4, 6, and 8 hours after drug administration. Plasma samples were obtained through centrifugation within 10 minutes (1,500*g*, 15 minutes), and stored at -80°C until analysis.

Adverse reaction monitoring

The volunteers were physically examined and adverse reactions during the study were recorded with the date and time at which they appeared and disappeared. Adverse effects were assessed on the basis of non-suggestive questioning by the study investigators. These included gastrointestinal, central nervous, cardiovascular, and dermatological effects, as well as other changes possibly attributable to the study drugs. Routine blood investigations (hematology and biochemistry), and urinalysis were performed prior to and at the end of (2 days after last drug administration) the study.

Drug analysis

Concentrations of DHA and/or AS in the

plasma were determined by reductive mode high-performance liquid chromatography (HPLC-EC), according to the method of Na-Bangchang *et al* (1998b). The procedure involved the extraction of AS, DHA, and the internal standard - artemisinin (AN) with the mixture of dichloromethane and *tert*-methyl-butyl-ether (8:2, v/v). Chromatographic separation consisted of the mobile phase (acetonitrile: water containing 0.1 M acetic acid pH 4.8 = 45: 55%) running through the column (Nova-Pak™ C_{18}, 3.9 mm i.d. x 150 cm, 5 mm particle size). The average recoveries of AS, DHA-(α-anomer) 2 and 4 mg, and AN at the concentration range of 10-800 ng/ml were 81.9, 88.2, 101.1 and 84.3 %, respectively. The coefficients of variation (precision and repeatability) were below 10% for all three compounds at concentrations of 800, 400, 200, 500, and below 20% at concentration of 10 ng/ml. The limits of quantification for both AS and α-DHA in spiked plasma samples were 5 and 3 ng/ml, respectively.

Pharmacokinetic analysis

Pharmacokinetic parameters of DHA following the administration of a single oral dose of DHA or AS were calculated by model-independent (oral DHA, DHA as an active plasma metabolite of AS) and model-dependent (oral DHA) methods from plasma concentration-time data (Gibaldi, 1991).

Model-independent method. The time at which maximum plasma concentration occurred (t_{max}), and the maximum concentration (C_{max}) were obtained directly from concentration-time data. The area under the curve from zero time to the last observed time (AUC_{0-t}) was calculated by the linear trapezoidal rule for ascending data points and by the log-linear trapezoidal rule for descending data points. The area under the curve extrapolated from the last data point to infinity ($AUC_{t-\infty}$) was estimated by dividing the estimated concentration at the last data point with the elimination rate constant (λ_z). The total area under the curve ($AUC_{0-\infty}$) was calculated as AUC_{0-t} + $AUC_{t-\infty}$. The terminal elimination rate constant (λ_z) and half-life ($t_{1/2z}$) were estimated by log-linear regression of at least four last concentration-time data. The apparent total body clearance (CL/f) and apparent volume of distribution associated with terminal phase (V_z/f) were calculated as CL/f = dose/AUC and V_z/f = (Cl/f)/λ_z, respectively.

Model-dependent. To better characterize the absorption phase, a one-compartment open model with first order input and first-order elimination was fitted to the data by an iterative least squares curve fitting Program TopFit™. The observed concentrations were weighted as the reciprocal of the analytical variance. The adequacy of the pharmacokinetic models chosen was based on statistical methods to assess the validity of the models for describing the experimental data, *ie* *F*-ratio test, Akaike's information, Schwartz and Imbimbo criteria.

Statistical analysis

Statistical analysis of the data was performed with SPSS for Windows (SPSS Software, Gorichem, The Netherlands). The distribution of data was assessed for normality using the Schapiro-Wilks test. Data were expressed as medians with 95% CIs values.

The pharmacokinetics of DHA in healthy Thai volunteers following the administration of 2, 4 mg/kg body weight DHA, or 4 mg/kg body weight AS were compared using Kruskal Wallis test and Wilcoxon signed ranks test for non-normally distributed data. Comparison of pharmacokinetic parameters between sex (male, female) in each drug regimen was performed by Mann-Whitney *U*-test. Categorical data (adverse reactions) were analyzed by calculating chi-square with Yate's correction or by Fisher's exact test. Significance level for all tests was set at α <0.05.

RESULTS

Tolerability

All volunteers were healthy, verified by laboratory results, physical examination, and vital sign monitoring. Table 1 presents demographic and baseline laboratory (hematology/ biochemistry) data of the volunteers. Significant laboratory changes in some hematological or biochemical tests were noted at the end of the study (2 days after drug administration). Parameters which decreased at the end of the study included hemoglobin, hematocrit, total protein, albumin,

Table 1
Demographic and baseline laboratory data of 20 healthy Thai volunteers (10 males, 10 females); data are presented as median (95% CI) values.

	Male	Female
Age (y)	21 (20, 33)	23 (21, 35)
Body weight (kg)	53.7 (47.8, 59)	49.3 (46.7, 51.6)
Hematology		
Hemoglobin (mg/dl)	13.85 (11.8, 15.1)	12.7 (11.4, 13.8)
Hematocrit (%)	43 (36, 46)	39.5 (36, 42)
Red cells (x 10^{12} /l)	1.44 (4.57, 6.78)	4.67 (4.01, 6.41)
Platelets (x 10^9 /l)	233 (203, 310)	309 (192, 328)
White cells (x 10^9 /l)	7.7 (5.1, 9.8)	7.4 (4.5, 9.3)
PMN (%)	49 (37, 61)	49.5 (42, 62)
Lymphocyte (%)	36 (26, 55)	41 (27, 49)
Monocyte (%)	6 (0, 11)	6 (2, 7)
Eosinophil (%)	5 (2, 12)	3 (1, 13)
Biochemistry		
Direct bilirubin (mg/dl)	0.15 (0.1, 0.6)	0.165 (0.006, 0.25)
Total bilirubin (mg/dl)	0.685 (0.36, 2.4)	0.595 (0.32, 1.3)
Alkaline phosphatase (U/l)	64.5 (44, 112)	67.5 (44, 79)
SGOT (U/l)	22 (15, 50)	18 (16, 24)
SGPT (U/l)	20 (12, 98)	11 (7, 19)
Total protein (g/dl)	7.35 (6.3,7.9)	7.65 (7.2,8.2)
Albumin (g/dl)	4.75 (4.3, 4.9)	4.5 (4.2, 4.6)
Globulin (g/dl)	2.6 (2, 3.1)	3.0 (2.6, 3.4)
Creatinine (mg/dl)	0.9 (0.78, 1.2)	0.75 (0.65, 0.9)
BUN (mg/dl)	11.5 (8.3, 17.7)	9.95 (7, 13.8)
Glucose (mg/dl)	93 (80, 113)	86 (81, 115)
Sodium (mmol/l)	141.5 (139, 145)	142 (139, 144)
Potassium (mmol/l)	3.8 (3.6, 4.8)	4.15 (3.7, 4.6)
Chloride (mmol/l)	106 (104, 108)	106.5 (105, 108)
Bicarbonate (mmol/l)	24.5 (24, 26)	24.5 (24, 26)

globumin, while parameter which increased at the end of the study was platelet count. However, these values returned to normal within 2 weeks after the termination of the drugs. None of the volunteers complained of adverse reaction or drug-related effect during the study.

Pharmacokinetics

Median plots of plasma concentration-time profiles of DHA and/or AS following the administration of a single oral dose of 2 or 4 mg/kg body weight DHA, or 4 mg/kg body weight AS in 20 healthy Thai volunteers are shown in Figs 1a and 1b. Oral DHA was rapidly absorbed from gastrointestinal tract with marked inter-individual variation. In most cases, the drug was detectable in plasma within 15 minutes of dosing; it disappeared thereafter from systemic circulation within 3-8 hours. Oral AS was rapidly biotransformed to DHA, which was detectable in plasma as early as 15 minutes of AS dosing. Considerable inter-individual variation in plasma DHA concentrations following both oral formulations of DHA and AS was observed. Systemic exposure of AS itself was seen only during 15 minutes to 1 hour but with markedly low concentrations.

The pharmacokinetics of DHA (median and 95% CI) following a single oral dose administration of 2 or 4 mg/kg body weight DHA, or 4 mg/kg body weight AS in 20 healthy Thai volunteers, calculated based on model-independent and

Table 2

Pharmacokinetics of DHA (model-independent) following a single oral dose of 2 or 4 mg/kg body weight DHA (Twisinin™), or 4 mg/kg body weight AS (Guilin Pharmaceutical Works) in healthy Thai males (n=10) and females (n=10) data are presented as median (95% CI) values.

Pharmacokinetic Parameters	2 mg/kg DHA	4 mg/kg DHA	4 mg/kg AS
C_{max} (ng/ml)[a]	181 (120-306)	360 (181-658)	519 (236-284)
$AUC_{0-\infty}$ (ng.h/ml)[b]	377 (199-1,128)	907 (324-2,289)	657 (362-2,079)
t_{max} (h)[c]	1.5 (0.75-2.0)	1.5 (0.75-3.0)	0.7 (0.25-1.5)
λ_z (/h)[d]	1.009 (0.532-1.375)	0.802 (0.668-1.284)	1.301 (0.678-2.832)
$t_{1/2z}$ (h)[e]	0.96 (0.70-1.81)	1.2 (0.75-1.44)	0.74 (0.34-1.42)
V_z/f (l/kg)	7.7 (4.33-12.3)	6.6 (3.1-10.1)	-
CL/f (ml/min/kg)	90.5 (28.6-178.2)	72.2 (31.9-113.6)	-

[a]Significant difference between 2 and 4 mg/kg body weight DHA with p = 0.0001 (95% CI = 151-246); and between 2 mg/kg body weight DHA and 4 mg/kg body weight AS with p = 0.006 (95% CI = 202-430); and between 4 mg/kg body weight DHA and AS with p = 0.02 (95% CI = 9-235).
[b]Significant difference between 2 and 4 mg/kg body weight DHA with p = 0.00001 (95% CI = 425-689); and between 2 mg/kg body weight DHA and 4 mg/kg body weight AS with p = 0.0005 (95% CI = 98-420); and between 4 mg/kg body weight DHA and AS with p = 0.02 (95% CI = -481to -48).
[c]Significant difference between 2 mg/kg body weight DHA and 4 mg/kg body weight AS with p = 0.0002 (95% CI = -1 to -0.5); and between 4 mg/kg body weight DHA and AS with p = 0.00005 (95% CI = -14 to -0.5).
[d]No statistical test was performed.
[e]Significant difference between 2 mg/kg body weight DHA and 4 mg/kg body weight AS with p = 0.0097 (95% CI = -0.044 to -0.0009); and between 4 mg/kg body weight DHA and AS with p = 0.0005 (95% CI = -0.56 to -0.21).

Table 3

Pharmacokinetics of DHA (model-dependent) following a single oral dose of 2 or 4 mg/kg body weight DHA (Twisinin™) in healthy Thai males (n=10) and females (n=10); data are presented as median (95% CI) values.

Pharmacokinetic parameters	2 mg/kg DHA	4 mg/kg DHA
C_{max} (ng/ml)	144 (91-260)	283 (105-632)
$AUC_{0-\infty}$ (ng.h/ml)	415 (211-1,280)	919 (408-2,480)
t_{max} (h)	1.45 (0.81-2.2)	1.46 (0.81-2.2)
t_{lag} (h)	0.25 (0.11-0.25)	0.24 (0.19-0.25)
k_a (/h)	0.83 (0.51-2.59)	0.83 (0.42-3.84)
$t_{1/2a}$ (h)	0.83 (0.27-1.35)	0.84 (0.18-1.65)
k_e (/h)	0.82 (0.51-1.24)	0.81 (0.41-1.21)
$t_{1/2}$ (h)	0.83 (0.2-1.35)	0.86 (0.39-10.1)
V_c/f (l/kg)	4.8 (2.5-10.6)	5.2 (2.2-7.6)
CL/f (ml/min/kg)	82.4 (25.3-171.9)	68.6 (24.5-103.6)

[a]Significant difference between 2 and 4 mg/kg body weight DHA with p = 0.00001 (95% CI = 107 to 208).
[b]Significant difference between 2 and 4 mg/kg body weight DHA with p = 0.00001 (95% CI = 411 to 749).

model-dependent methods, are summarized in Tables 2 and 3, respectively. The fitting of the concentration-time curves of DHA either when given as oral DHA to a one-compartment model with first-order input and output yielded satisfactory results in all volunteers. Pharmacokinetics of DHA calculated using both methods were generally in good agreement. No significant absorption lag-time was observed from the time of drug administration until it was first detectable in the plasma. Large inter-individual variation among the pharmacokinetic parameters was noted, particularly with $AUC_{0-\infty}$ and CL/f as reflected by the values of coefficients of variation for both parameters (40-45%).

No significant difference was found in any of DHA pharmacokinetic parameters between male and female volunteers following the administration of either dose of DHA (2 or 4 mg/kg body weight), or as a single oral dose of 4 mg/kg body weight AS.

Model-independent analysis. Marked differ-

ences in the pharmacokinetic parameters of DHA were observed following the oral dose regimens of DHA and AS. Oral DHA at the dose level of 2 mg/kg body weight resulted in a significantly lower C_{max} and $AUC_{0-\infty}$ of DHA compared with 4 mg/kg body weight dose of DHA or AS. With respect to DHA, C_{max} increased proportionally with the dose with a median ratio of 2.3. C_{max} of DHA following the same dose of AS (4 mg/kg body weight) was significantly higher than that following DHA, but greater $AUC_{0-\infty}$ was achieved following DHA. In addition, t_{max} of DHA following AS was found to be significantly shorter than that following DHA at either dose level.

Model-dependent analysis. Pharmacokinetics of DHA following the two dose levels of DHA (2 and 4 mg/kg body weight) were generally comparable and consistent with the values calculated using model-independent method. Only two significant differences in dose-dependent pharmacokinetic parameters were noted; C_{max} and $AUC_{0-\infty}$ following the higher dose level were approximately double that seen in the model-independent analysis.

DISCUSSION

The pharmacokinetics of AS and DHA have been addressed in a few studies, with varying routes of administration, ethnicity of the subjects and disease states (Yang *et al*, 1985; Zhao *et al*, 1988; Na-Bangchang *et al*, 1997; 1998a; 1999; Batty *et al*, 1998a,b; Hung *et al*, 1999; Binh *et al*, 2001; Ilett *et al*, 2002). The concentration-time profiles of DHA following the administration of the oral doses of both DHA and AS observed in the present study were generally in accord with those previously reported. No marked sex diffrences in DHA pharmacokinetics was observed following either oral DHA or AS, which supports a previous report in healthy Vietnamese volunteers (Hung *et al*, 1999). The current formulation of oral DHA (Twisinin™) was rapidly absorbed from the gastrointestinal tract; C_{max} was attained at approximately 1-2 hours of dosing. The pharmacokinetic profile was generally well described by a one-compartment open model with first- order input and output, characterising the rapid absorption, distribution and elimination phase. Elimination half-life was estimated to be in the range of 0.8-1.5 hours. Little is known about the ultimate phase of dihydroartemisinin in human body. The *in vitro* studies using rat isolated perfused liver (IPRL) and microsomes have identified glucuronide conjugate as a sole and principal metablite of DHA (Maggs *et al*, 1997). The extent of the hepatic extraction of oral DHA is unknown. Oral AS (Guilin Pharmaceutical Works, China) was almost immediately biotransformed to the active metabolite, DHA. Systemic exposure to AS itself was very low (C_{max} of less than 200 ng/ml in most cases) and was observed only during a short period, the first hour, after drug intake. In contrast, its plasma metabolite, DHA, attained a relatively high C_{max} within 0.25-1.5 hours of AS dosing. Distribution/elimination of this metabolite was also rapid. The apparent elimination half-life was estimated to be in the range of 0.4-1.4 hours.

The pharmacokinetics of DHA following a single oral dose of 2 or 4 mg/kg body weight DHA were generally similar. This was indicated by the comparable values of dose-independent pharmacokinetic parameters. Linearity of DHA kinetics was seen at these two dose levels, which was ascribed by the proportional increase in $AUC_{0-\infty}$ with the dose (mean $AUC_{0-\infty}$ ratio of 2.4). It appears that the bioavailability of the current formulation of oral DHA (Twisinin™) is markedly low (approximately 50%) when compared with the formulation produced by Guilin Pharmaceticals, China (Cotexin™: tablet) or Arenco nv, Belgium (Dihydroartemisinin™: capsule) (Na-Bangchang *et al*, 1997; Hung *et al*, 1999). In a previous study in healthy Thai volunteers following 300 mg of Dihydroartemisinin™ (Arenco-nv, Belgium), median (range) $AUC_{0-\infty}$ and C_{max} of 2,010 (636-4,079) ng.h/ml and 679 (307-1,000) ng/ml were achieved, respectively (Na-Bangchang *et al*, 1997). Furthermore, in a study in healthy Vietnamese volunteers following 240 mg of Cotexin™, median (range) $AUC_{0-\infty}$ and C_{max} of 1,867 (420-3,535) ng.h/ml and 466 (128-787) ng/ml were attained, respectively (Hung *et al*, 1999).

It was noted that the pharmacokinetics of DHA following oral DHA and AS doses showed noticeable differences in pharmacokinetic. The

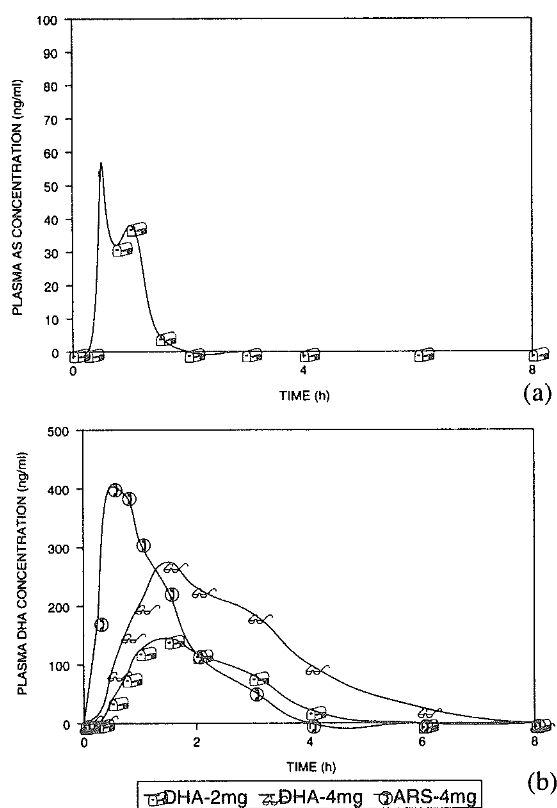

Fig 1–Median plasma concentration-time profiles of (a) artesunate (AS) following the dose of oral 4 mg/kg body weight oral AS, and (b) dihydroartemisin (DHA) following the doses of oral 2 and 4 mg/kg body weight oral DHA.

Disposition of DHA, when the drug was given as oral AS, was greatly influenced by the kinetics of the parent compound itself, *ie*, formation of DHA was rate-limited by kinetics (absorption, distribution, and metabolism) of AS. Higher C_{max} of DHA was attained at faster time following the oral AS, but total systemic exposure was higher following oral DHA given at the same dose level (4 mg/kg body weight). This may suggest that absorption of DHA from oral formulation may be more erratic but relatively complete compared with oral AS. DHA is poor water solubility, which means that it can only be administered orally or rectally. Previous data, however, showed that the bioavailability of orally administered DHA was only 45% relative to the DHA from intravenous AS (Binh *et al*, 2001).

In conclusion, the oral formulation of both AS and DHA were well tolerated. No clinically adverse reaction or drug-related effect was observed during the study. Nevertheless, adequate therapeutic plasma concentrations following the administration of the current formulation of oral DHA (Twisinin™) may not be guaranteed. This is of concern, especially in patients with malaria, whose absorption of the drug by the oral route may be erratic and incomplete.

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APPLICATION OF REAL-TIME POLYMERASE CHAIN REACTION (PCR) ANALYSIS FOR DETECTION AND DISCRIMINATION OF MALARIA PARASITE SPECIES IN THAI PATIENTS

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Abstract. A TaqMan real-time PCR system was used to detect and discriminate the 4 species of human malaria parasites in clinical blood samples. A 150-base pair (bp) region of the small subunit ribosomal RNA (SSU rRNA) gene of each malaria parasite, including species-specific sequences to be detected by TaqMan probe, was used as a target for PCR analysis. The PCR method used universal primers and species-specific TaqMan probes for *Plasmodium falciparum, P. vivax, P. ovale,* and *P. malariae.* The detection threshold for the method, as determined with serial dilution of cultured *P. falciparum*-infected erythrocytes, was 5 parasite-infected erythrocytes per reaction. Fifty blood samples of falciparum malaria and a second set of 50 samples of vivax malaria, diagnosed by microscopic examination at the Hospital for Tropical Diseases, Mahidol University, Thailand, were analyzed by real-time PCR. In the 50 samples of microscopically-diagnosed falciparum malaria, 40 were regarded as *P. falciparum* single infection, 7 were *P. falciparum* and *P. vivax* mixed infections, and 3 were *P. vivax* single infection by real-time PCR. In the second set of 50 samples of microscopically diagnosed vivax malaria, all were considered *P. vivax* single infection by PCR. Neither *P. ovale* nor *P. malariae* infection was identified in the 100 blood samples. Real-time PCR analysis was shown to be more sensitive and accurate than routine diagnostic methods. Application and extension of the PCR method reported here will provide a powerful tool for further studies of malaria.

INTRODUCTION

Accurate diagnosis of malaria and identification of the *Plasmodium* species responsible are essential for optimal patient management and for understanding the epidemiology of malaria. Microscopic examination of blood smears is the standard method for diagnosis of malaria. Although this method is sensitive and specific, it is subjective and time-consuming, particularly for detecting a small number of parasites. In addition, it is often very difficult to identify mixed infections (Snounou *et al*, 1993a; Rubio *et al*, 2002).

Various alternative diagnostic methods have been developed. These include fluorescence microscopy (Kawamoto and Billingsley, 1992), concentration techniques, such as the quantitative buffy coat method (Petersen and Marbiah, 1994), and immunological antigen capture assays such as malaria rapid diagnostic tests (Tham *et al*, 1999; Wongsrichanalai *et al*, 2003). However, persistence of the antigens in the bloodstreams of patients might result in false positive results in an antigen capture assay. Thus, use of more than one test is recommended to confirm diagnosis.

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In this study, we applied real-time PCR with TaqMan technology (Bell and Ranford-Cartwright, 2002) to analyze malaria parasite infections in clinical blood samples.

MATERIALS AND METHODS

Parasite culture

The FCR-3 strain of *P. falciparum* was cultured by the method of Trager and Jensen (1976) followed by synchronization with sorbitol treatment (Lambros and Vanderberg, 1979). When the parasites reached the ring stage, the culture was diluted serially, and blood samples containing 5×10^5 to 5×10^1 parasite-infected erythrocytes were prepared. Genomic DNA was extracted from the samples, and of the amount, 1/10 was used as a template for PCR. The minimum number of parasite-infected erythrocytes per reaction was 5.

Preparation of plasmid DNA

Approximately 150-bp of the small subunit ribosomal RNA (SSU rRNA) gene sequence was amplified from the parasite DNA with the universal

primers shown in Table 1. Genomic DNA served as the PCR template and was isolated from the clinical blood samples that contained *P. vivax*, *P. ovale*, or *P. malariae*, or from cultured blood for *P. falciparum*. The DNA fragment was cloned into the plasmid vector pCR® 2.1-TOPO® (Invitrogen Corp, Carlsbad, CA, USA), and the sequence was confirmed by cycle sequencing (Applied Biosystems, Foster City, CA, USA).

TaqMan PCR

The universal primers and TaqMan probes used are shown in Table 1. The TaqMan probes were labeled with a reporter dye, 5-carboxyfluorescein (FAM), and conjugated with minor groove binder (MGB) for increased sensitivity and decreased background fluorescence (Kutyavin *et al*, 2000). The probes were designed with Primer Express software (Applied Biosystems) based on the SSU rRNA gene sequence (Genbank accession numbers of the 4 parasite species are shown in Table 1). The primers and probes were synthesized and HPLC purified by Applied Biosystems Japan (Tokyo, Japan).

Real-time PCR was performed according to the manufacturer's instructions (Applied Biosystems). Briefly, 5 µl template DNA, 300 nM forward primer, 300 nM reverse primer, 100 nM TaqMan MGB probe and 1x Universal Master Mix in a total reaction volume of 25 µl were amplified on ABI PRISM 7700 or 7900 Sequence Detection Systems (SDS) (Applied Biosystems). The following PCR conditions were used: optimization of AmpErase uracil-N-glycosylase (Applied Biosystems) activity at 50°C for 2 minutes, initial hot start at 95°C for 10 minutes and 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. The fluorescence signal was detected and analyzed with ABI PRISM SDS software (Applied Biosystems). Plasmid DNA in the range of 0.001fg -10pg was used as a quantification standard for the SDS calibration curve.

Clinical blood samples

One hundred blood samples were obtained from patients at the Hospital for Tropical Diseases, Mahidol University, Thailand, in 1997. The patients provided written consent, and this study was approved by the Ethics Committee of Mahidol University. The blood was collected with EDTA, and thin blood smears were prepared. The blood smears were examined microscopically by the laboratory staff of the hospital. *P. falciparum* was confirmed in 50 samples, and *P. vivax* was confirmed in a second set of 50 samples. The blood samples were stored at -20°C until DNA extraction.

DNA extraction from blood samples

The frozen blood samples were thawed on ice, and 250 µl were taken for DNA extraction. Five hundred µl DNAzol® BD reagent (Invitrogen) were mixed in, 200 µl isopropanol were added, and the reaction was incubated for 5 minutes at room temperature. The samples were centrifuged at 6,000*g* for 6 minutes to sediment the precipitated DNA. The DNA pellets were washed with DNAzol® BD reagent and then washed with 75% ethanol. After removing the ethanol, the DNA pellets were dissolved in 50 µl 8 mM NaOH and frozen at -20°C until use.

Table 1
Sequence of primers and oligonucleotide probes used for the detection of SSU rRNA genes of the malaria parasites.

Primers/probes	Sequence (5'→3')	Position[a]	Origin	
Primer				
Forward	ACGATCAGATACCGTCGTAATCTT	1062-1085	M19172,	Kimura *et al*, 1997
Reverse	GAACCCAAAGACTTTGATTTCTCAT	1180-1204	M19172,	Kimura *et al*, 1997
Probe[b]				
Pf-probe	CATCTTTCGAGGTGACTT	1138-1155	M19172	
Pv-probe	TCTCTTCGGAGTTTAT	1471-1486	X13926	
Po-probe	TTTCCCCGAAAGGA	1152-1165	L48987	
Pm-probe	AGAGACATTCTTATATATGAGTG	1173-1195	M54897	

[a]Positions correspond to the 18S rRNA sequence.
[b]Pf = *P. falciparum;* Pv = *P. vivax;* Po = *P. ovale;* Pm = *P. malariae*