DOI:10.1111/j.1365-2125.2004.02045.x

Protein binding and α:β anomer ratio of dihydroartemisinin *in vivo*

Kevin T. Batty,[1,2,3] Kenneth F. Ilett[1] & Timothy M. E. Davis[2]

[1]*Pharmacology Unit, School of Medicine and Pharmacology, University of Western Australia, Crawley, Western Australia,* [2]*Fremantle Medicine Unit, School of Medicine and Pharmacology, University of Western Australia, Crawley, Western Australia and* [3]*School of Pharmacy, Curtin University of Technology, Bentley, Western Australia*

Correspondence
Dr Kevin Batty, School of Pharmacy, Curtin University of Technology, GPO Box U1987, Perth, Western Australia, 6845.
Tel: + 61 8 9266 7369
Fax: + 61 8 9266 2769
E-mail: Kevin.Batty@curtin.edu.au

Keywords
anomer, dihydroartemisinin, protein binding

Received
3 July 2003
Accepted
27 October 2003

Aims
To determine the ratio of α:β anomers and the protein binding of dihydroartemisinin (DHA) *in vivo*.

Methods
10-[^{3}H]-DHA was synthesized by reduction of artemisinin with sodium boro-[^{3}H]-hydride and purified with preparative thin layer chromatography. A solution of ^{3}H-DHA (2000 ng in 20 μl) was added to 2 ml whole blood from 15 healthy volunteers and 22 Vietnamese patients with falciparum or vivax malaria. The blood was centrifuged and the plasma stored at −25 °C until analysed by HPLC with radiochromatographic detection. Protein-free ultrafiltrate of the plasma was assayed to determine the free fraction of DHA and the *in vivo* ratio of α-DHA : β-DHA.

Results
The DHA fraction unbound (mean ± SD) was 0.068 ± 0.032 in Vietnamese patients with falciparum malaria (n = 17), 0.065 ± 0.009 in Vietnamese patients with vivax malaria (n = 5), 0.117 ± 0.015 in Vietnamese volunteers (n = 7) and 0.092 ± 0.020 in Caucasian volunteers (n = 8). The ratios of α-DHA : β-DHA for the four groups were 6.3 ± 0.9, 6.9 ± 0.8, 6.9 ± 0.6 and 5.4 ± 0.8, respectively.

Conclusions
DHA is approximately 93% protein-bound in patients with malaria infection and there is a preferential existence *in vivo* of the α-DHA anomer. Knowledge of this stereochemistry may be valuable in elucidation of the mechanisms of DHA action and/or toxicity, and in the synthesis of new trioxane antimalarials.

Introduction

The artemisinin drugs have become an integral component of antimalarial treatment regimens in south-east Asia and their use is expanding to many tropical countries, including Africa. Dihydroartemisinin (DHA) is one of the semisynthetic derivatives in clinical use and it is the principal metabolite of other derivatives such as artesunate and artemether. The conventional pharmacokinetic parameters for DHA are well documented, mostly from studies in which artesunate or another artemisinin derivative has been administered [1, 2]. DHA has an elimination half-life of 40–60 min in healthy Asian and Caucasian volunteers and Asian patients with uncomplicated and severe malaria [3–9]. It has a high hepatic extraction ratio [10] and is metabolized to the inactive α-DHA-β-glucuronide by UGT1A9 and UGT2B7 enzymes [11, 12].

There are limited published data relating to parameters such as erythrocyte binding or uptake, blood : plasma ratio and protein binding of DHA. Gu *et al.* [13]

investigated the uptake of DHA by *Plasmodium falciparum* infected erythrocytes at 8–10% parasitaemia and 10% haematocrit, and found that the concentration within parasitized erythrocytes was more than 300 times that in the culture medium. By contrast, intact erythrocytes concentrated DHA only two-fold. Li *et al.* [14] found that protein binding of DHA, artemisinin and artemether was 44%, 64% and 77%, respectively, in human plasma. However, recent studies in healthy volunteers and patients with uncomplicated falciparum malaria have shown that the protein binding of artemisinin was 85–88% [15, 16]. Detailed investigations have shown that the protein binding of arteether [17] and artemether [18] in human volunteer plasma was 79% and 95%, respectively, and was independent of drug concentration. Binding of arteether and artemether to human serum albumin (essentially fatty acid free; 40 g l^{-1}) was 71% and 88%, respectively, and binding to α_1-acid glycoprotein (AAG) 0.75 and 1 g l^{-1} was 61% and 90%, respectively [17, 18]. There are no reports of DHA protein binding in patients with malaria. Thus, we sought to determine the ratio of α:β anomers and the protein binding of DHA *in vivo*.

Methods

Synthesis of 10-[^{3}H]-dihydroartemisinin

The synthetic method used was a modification of that described by Lin *et al.* [19]. Artemisinin (300 mg; 1.06 mmol; Mediplantex Company, Hanoi, Vietnam) was dissolved in 40 ml of HPLC-grade methanol and cooled to 4 °C in an ice bath. Sodium boro-[^{3}H]-hydride (7.72 mg; 0.203 mmol; 100 mCi; Amersham Life Science, Buckinghamshire, UK) was added in two aliquots, with continuous stirring. The reaction mixture was maintained at 4 °C and stirred for 2 h before being neutralized with 0.2 mmol acetic acid (40 µl of 30% v/v glacial acetic acid in methanol). The mixture was evaporated to dryness at 40 °C under a stream of high-purity nitrogen. The residue was washed with 400 µl of 5% w/v sodium bicarbonate solution to neutralize any remaining acid, and extracted three times with 10 ml of ethyl acetate. The ethyl acetate solutions were combined and evaporated to dryness. Residue from the reaction mixture was dissolved in dichloromethane and spotted on to a preparative thin layer chromatography (TLC) plate (Silica Gel 60 F_{254}, 2 mm × 20 cm × 20 cm; E. Merck, Darmstadt, Germany). The TLC mobile phase was 75% v/v petroleum spirit (boiling range 100–120 °C), 22.5% v/v ethyl acetate and 2.5% v/v methanol. The plate was developed four times to a distance of 15 cm, with air-drying between each run. Artemisinin and ^{3}H-DHA bands were visualized with UV light (254 nm) and the ^{3}H-DHA band was scraped from the TLC plate. The ^{3}H-DHA was extracted with 15 ml of 20% v/v ethyl acetate in butyl chloride, evaporated to dryness under nitrogen and reconstituted with 1 ml of 96% v/v ethanol.

Radiochemical purity was determined by HPLC [20]. Eluent fractions were collected every 0.25 min using an LKB 2212 HeliRac Fraction Collector (LKB-Produkter AB, Bromma, Sweden) and 3 ml of Ecolite™ liquid scintillation cocktail (ICN Pharmaceuticals, Costa Mesa, CA, USA) was added to the scintillant vials. DHA was quantified from a standard curve and by liquid scintillation counting of both α- and β–^{3}H-DHA peaks (retention times approximately 7 and 9 min, respectively) using a Tri-Carb 1500 Liquid Scintillation Analyser (Packard Instrument Co., CT, USA). Specific activity was found to be 0.38 µCi μg^{-1} (106 µCi μmol^{-1}) and radiopurity was 90%. A contaminant comprising 6% of total radioactivity, which could be resolved by HPLC, was likely a degradation product of DHA and did not compromise the protein binding or anomer ratio studies.

Protein binding and α:β DHA ratio in vivo

Whole blood (2 ml) was obtained from healthy Vietnamese and Caucasian volunteers, and Vietnamese patients with uncomplicated falciparum or vivax malaria, all of whom had been recruited for clinical pharmacokinetic studies reported previously [3, 4, 6, 7]. These studies were approved by the Ministry of Health, Vietnam, the University of Western Australia Human Rights Committee or the QEII Medical Centre Clinical Drug Trials Committee. Blood used for the present study was taken prior to administration of any medication.

A solution of ^{3}H-DHA in 0.9% w/v sodium chloride solution (20 µl of 100 ng μl^{-1}; 38 nCi μl^{-1}) was added to the blood and allowed to equilibrate for 30 min [13] at room temperature (~ 30 °C in Vietnam and 23–25 °C in Australia), with gentle mixing. The DHA concentration in blood was approximately 1000 µg l^{-1} (3.5 µM). Based on reported stability data [21], less than 2% degradation of DHA would be expected to occur during the 30 min equilibration period. The blood was centrifuged at 1700 *g* and the plasma was transferred to microcentrifuge tubes and stored at −25 °C until analysed.

The plasma samples were thawed at room temperature (23 °C) and 100 µl was mixed with 900 µl of acetonitrile (−25 °C) in microcentrifuge tubes to precipitate the proteins. The tubes were centrifuged at 10 000 *g* for 1 min and 50 µl of the supernatant was assayed by HPLC with radiochromatographic detection. Further aliquots of 800 µl of plasma were transferred to Centri-

free Micropartition System MPS-1 units with YMT 30 000 MW membrane filters (Amicon, Beverly, MA, USA) and centrifuged at 1500 *g* for 30 min at 4 °C (Avanti J25I centrifuge with JA25.50 34° fixed angle rotor; Beckman Instruments Inc., CA, USA) to obtain protein-free ultrafiltrate of the plasma. The ultrafiltrate (50 µl) was assayed by HPLC. The free fraction of DHA was determined from the ratio of the corrected concentrations of DHA in protein-free ultrafiltrate to DHA in plasma. The *in vivo* ratio of α-DHA : β-DHA was determined from the radiochromatograms of protein-free ultrafiltrate.

Albumin binding in vitro

Solutions of ^{3}H-DHA in ethanol (50 µM, 100 µM, 250 µM, 500 µM and 2.5 mM; all with specific activity of 4 nCi µl^{-1}) were added to solutions of bovine serum albumin (Fraction V; Code A-3350; Sigma Chemical Company, St Louis, MO, USA) at concentrations of 25 g l^{-1}, 40 g l^{-1} and 50 g l^{-1} in 0.1 M phosphate buffer (pH 7.4) to give approximate final concentrations of DHA in each of the albumin solutions of 200 µg l^{-1} (0.7 µM), 390 µg l^{-1} (1.4 µM), 950 µg l^{-1} (3.4 µM), 1900 µg l^{-1} (6.7 µM) and 9500 µg l^{-1} (33 µM). These were incubated at 37 °C for 30 min and 400 µl aliquots were transferred to Centrisart C4 10 000 MW microcentrifuge filters (Sartorius AG, Göttingen, Germany) and centrifuged at 10 000 *g* for 15 min to obtain protein-free ultrafiltrate of the original solution. Aliquots of protein-free ultrafiltrate and the albumin solutions (100 µl) were added to 100 µl of acetonitrile (–25 °C) to precipitate the albumin and these solutions were centrifuged at 13 000 *g* for 2 min. The supernatant was assayed by HPLC and all experiments were done in duplicate. The free fraction of DHA was determined from the ratio of the concentration of DHA in protein-free ultrafiltrate to the concentration in the corresponding albumin solution.

Statistical analysis

Statistical analyses were performed using SigmaStat® Version 2.03 (SPSS Inc., Chicago, USA; 1997). Data are summarized as mean ± SD unless otherwise indicated. Analysis of variance (ANOVA) was used for comparison of groups.

Results

Demographic data for the patients and volunteers are given in Table 1. The four groups were comparable, except for a greater mean body weight in the Caucasian volunteers and a higher mean serum bilirubin in Vietnamese patients with falciparum malaria. In patients with malaria infection, DHA was shown to be approximately 93% protein bound and to have an α:β ratio of 6.5 : 1 (Table 1). DHA protein binding was 88% in Vietnamese volunteers and 91% in Caucasian volunteers. There was a weak positive correlation between the free

Table 1

Results from studies of patients with falciparum malaria (PF [6]), vivax malaria (PV [7]), healthy Vietnamese volunteers (VV [3]) and healthy Caucasian volunteers (CV [4]). Data are mean ± SD unless otherwise indicated

	Study group [Reference]			
	PF [6]	PV [7]	VV [3]	CV [4]
Number[a]	17	5	7	8
Gender (M/F)	16/1	5/0	7/0	4/4
Age (years)	25 ± 6	28 ± 11	29 ± 7	20 ± 1
Body weight (kg)	50 ± 4	50 ± 4	52 ± 6	61 ± 7[b]
Venous haematocrit (%)	37 ± 7	40 ± 5	42 ± 3	42 ± 5
Serum creatinine (µmol l^{-1})	92 ± 17	100 ± 18	82 ± 10	83 ± 15
Serum bilirubin (µmol l^{-1})	19 ± 12[c]	9 ± 7	8 ± 4	9 ± 3
Serum alanine aminotransferase (units l^{-1})	36 ± 18	31 ± 9	52 ± 42	15 ± 5
Serum albumin (g l^{-1})	39 ± 4[d]	44 ± 8	49 ± 7	48 ± 2
Parasitaemia (per µl)	17 419 (1 050–333 300)[e]	3 782 (460–14 500)[e]	–	–
DHA fraction unbound (f_u)	0.068 ± 0.032	0.065 ± 0.009	0.117 ± 0.015[f]	0.092 ± 0.020
DHA α:β anomer ratio	6.3 ± 0.9	6.9 ± 0.8	6.9 ± 0.6	5.4 ± 0.8[b]

[a]Total patients in original studies were: PF = 26; PV = 12; VV = 10; CV = 8; [b]P < 0.05 compared with all other groups (ANOVA), [c]P < 0.05 compared with volunteer groups (VV and CV; ANOVA), [d]n = 5 (mean ± SD albumin in original study was 43 ± 7 g l^{-1}), [e]Geometric mean (range), [f]P < 0.05 compared with patient groups (PF and PV; ANOVA).

fraction of DHA and the albumin concentration in the Vietnamese patients and volunteers ($r = 0.54$; $P = 0.03$; power = 0.58). The DHA α:β ratio in Caucasian volunteers was significantly lower than all other groups (Table 1; ANOVA).

In the albumin binding studies, the free fraction of DHA was not concentration-dependent at each of the three albumin concentrations (data not shown). Hence, the data at each albumin concentration were pooled for analysis. The free fraction of DHA was 0.25 ± 0.02 at 50 g l^{-1} albumin concentration, 0.27 ± 0.01 at 40 g l^{-1} and 0.35 ± 0.03 at 25 g l^{-1} ($P < 0.001$ for 25 g l^{-1} compared with 40 g l^{-1} and 50 g l^{-1}). There was a negative correlation between the free fraction of DHA and the albumin concentration in the *in vitro* studies ($r = 0.9$; $P < 0.001$; power = 0.99).

Discussion

This study provides data that are important for interpretation of pharmacological studies involving artemisinin derivatives. The novel finding of a preferential existence *in vivo* of the α-DHA anomer complements our recent report that α-DHA-β-glucuronide is the principal metabolite of DHA [12]. Knowledge of this stereochemistry increases our understanding of DHA metabolism and may be valuable in elucidation of the mechanisms of DHA action and/or toxicity and in the synthesis of new trioxane antimalarials.

For *in vitro* studies involving DHA, it could be important to conduct experiments with the α:β ratio close to the physiological value. However, consideration should be given to reports of analytical methods for DHA which indicate that the anomer ratio is time and solvent-dependent. Navaratnam *et al.* [22] noted that equilibration of α:β DHA anomers (to a ratio of 4.5 : 1) took at least 18 h after reconstitution in 50% v/v ethanol in water. We have found that a stable α:β DHA anomer ratio (3.3 : 1) is achieved 10–12 h after reconstitution in HPLC mobile phase comprising 50% v/v acetonitrile in 0.1 M acetate buffer pH 4.8 [20]. By comparison, in an earlier stability study, we observed an α:β DHA anomer ratio of approximately 6 : 1 in HPLC mobile phase comprising 45% v/v acetonitrile in 0.067 M Sörenson's phosphate buffer pH 5.6 [21]. We would suggest that if a physiological α:β DHA ratio for *in vitro* studies is desirable, determination of the α:β DHA anomer ratio should be conducted and simple HPLC methods for DHA can be developed if there are no interfering compounds in the matrix [21].

Our finding that DHA is 93% protein-bound in patients with malaria infection, and 88–91% bound in healthy volunteers, contrasts with the original study by Li *et al.* [14] in which it was reported that DHA protein binding was 44%. Whilst the reasons for this discrepancy are unclear, it is notable that our results are consistent with recent reports that the protein binding of artemisinin in healthy volunteers and patients is in the range of 85–88% [15, 16], rather than 64% as previously reported [14]. The clinical relevance of the high protein binding of DHA may be exemplified by our recent report demonstrating that the concentration of DHA in the CSF was very low (approximately 100 nM) following intravenous administration of 120 mg artesunate [23].

Factors that may affect the plasma protein binding of DHA include plasma pH and the concentration of α_1-acid glycoprotein (AAG) in acute malaria infection. In the present *in vivo* study, the impact of pH and AAG could not be investigated because resources were not available in the rural health facility in Vietnam where the clinical pharmacokinetic studies were conducted. Previous studies have shown that arteether [17], artemether [18] and possibly artemisinin [16], bind to AAG. It has been reported that the plasma AAG concentration is substantially higher in severe malaria infection (1.93 g l^{-1} [24]) than in patients with uncomplicated malaria (1.55 g l^{-1} [24]; 1.23 g l^{-1} [16]) and healthy Thai volunteers (0.71 g l^{-1} [24]). Two observations from the present study indicate that DHA could be subject to AAG binding. Firstly, our *in vitro* studies showed that the free fraction of DHA in albumin (0.25 at 50 g l^{-1} and 0.27 at 40 g l^{-1}) was at least two-fold higher than the free fraction in plasma (0.07 in malaria infection; 0.12 in volunteers). Secondly, the free fraction of DHA was inversely related to albumin concentration (but independent of DHA concentration) *in vitro*, but positively correlated with the albumin concentration in Vietnamese patients and volunteers (Caucasian data were excluded to preclude ethnic bias). Hence, the increased binding of DHA in patients with malaria may be related to AAG or other physiological changes associated with the infection.

As DHA has a high hepatic extraction ratio [10], the variability in protein binding between Vietnamese patients, healthy Vietnamese volunteers and Caucasian volunteers will not usually affect interpretation of clinical pharmacokinetic data from different ethnic groups. However, our data are important when considering issues such as the prediction of *in vivo* hepatic clearance from *in vitro* drug metabolism studies and investigating the mechanisms of action and toxicity for DHA.

This work was supported by a Project Grant from the National Health and Medical Research Council

(NHMRC) of Australia (Timothy M. E. Davis and Kenneth F. Ilett). Kevin T. Batty was a recipient of an NHMRC Dora Lush (Biomedical) Scholarship.

We gratefully acknowledge the advice and assistance of Dr Simone Vonwiller (University of Sydney), Mr Michael Rafferty (Radiation Safety Office, University of WA), Dr Le Thi Anh Thu, Dr Truong Xuan Mai and Dr Tran Quang Binh (Cho Ray Hospital, Vietnam) and Dr Nguyen Canh Hung, Dr Huynh Van Thien, Dr Vu Nam Bien and Mrs Dang Thi Vinh Thuan (Bao Loc Hospital, Vietnam).

References

1 de Vries PJ, Dien TK. Clinical pharmacology and therapeutic potential of artemisinin and its derivatives in the treatment of malaria. Drugs 1996; 52: 818–36.

2 Navaratnam V, Mansor SM, Sit N, Grace JM, Li Q, Olliaro P. Pharmacokinetics of artemisinin-type compounds. Clin Pharmacokinet 2000; 39: 255–70.

3 Binh TQ, Ilett KF, Batty KT, et al. Oral bioavailability of dihydroartemisinin in Vietnamese volunteers and in patients with falciparum malaria. Br J Clin Pharmacol 2001; 51: 541–6.

4 Batty KT, Ilett KF, Powell SM, Martin J, Davis TM. Relative bioavailability of artesunate and dihydroartemisinin: investigations in the isolated perfused rat liver and in healthy Caucasian volunteers. Am J Trop Med Hyg 2002; 66: 130–6.

5 Benakis A, Paris M, Loutan L, Plessas CT, Plessas ST. Pharmacokinetics of artemisinin and artesunate after oral administration in healthy volunteers. Am J Trop Med Hyg 1997; 56: 17–23.

6 Batty KT, Thu LT, Davis TM, et al. A pharmacokinetic and pharmacodynamic study of intravenous vs oral artesunate in uncomplicated falciparum malaria. Br J Clin Pharmacol 1998; 45: 123–9.

7 Batty KT, Thu LT, Ilett KF, et al. A pharmacokinetic and pharmacodynamic study of artesunate for vivax malaria. Am J Trop Med Hyg 1998; 59: 823–7.

8 Newton P, Suputtamongkol Y, Teja-Isavadharm P, et al. Antimalarial bioavailability and disposition of artesunate in acute falciparum malaria. Antimicrob Agents Chemother 2000; 44: 972–7.

9 Davis TM, Phuong HL, Ilett KF, et al. Pharmacokinetics and pharmacodynamics of intravenous artesunate in severe falciparum malaria. Antimicrob Agents Chemother 2001; 45: 181–6.

10 Batty KT, Ilett KF, Edwards G, et al. Assessment of the effect of malaria infection on hepatic clearance of dihydroartemisinin using rat liver perfusions and microsomes. Br J Pharmacol 1998; 125: 159–67.

11 Maggs JL, Madden S, Bishop LP, O'Neill PM, Park BK. The rat biliary metabolites of dihydroartemisinin, an antimalarial endoperoxide. Drug Metab Dipos 1997; 25: 1200–4.

12 Ilett KF, Ethell BT, Maggs JL, et al. Glucuronidation of dihydroartemisinin in vivo and by human liver microsomes and expressed UDP-glucuronosyltransferases. Drug Metab Dipos 2002; 30: 1005–12.

13 Gu HM, Warhurst DC, Peters W. Uptake of [^{3}H] dihydroartemisinine by erythrocytes infected with Plasmodium falciparum in vitro. Trans R Soc Trop Med Hyg 1984; 78: 265–70.

14 Li WH, Shu HL, Xu GY, Zeng YL. The binding of qinghaosu (artemisinine) and its derivatives to plasma protein [Chinese]. Acta Pharm Sin 1982; 17: 783–6.

15 Sidhu JS, Ashton M. Single-dose, comparative study of venous, capillary and salivary artemisinin concentrations in healthy, male adults. Am J Trop Med Hyg 1997; 56: 13–6.

16 Ashton M, Nguyen DS, Nguyen VH, et al. Artemisinin kinetics and dynamics during oral and rectal treatment of uncomplicated malaria. Clin Pharmacol Ther 1998; 63: 482–93.

17 Wanwimolruk S, Edwards G, Ward SA, Breckenridge AM. The binding of the antimalarial arteether to human plasma proteins in vitro. J Pharm Pharmacol 1992; 44: 940–2.

18 Colussi D, Parisot C, Legay F, Lefevre G. Binding of artemether and lumefantrine to plasma proteins and erythrocytes. Eur J Pharm Sci 1999; 9: 9–16.

19 Lin AJ, Klayman DL, Milhous WK. Antimalarial activity of new water-soluble dihydroartemisinin derivatives. J Med Chem 1987; 30: 2147–50.

20 Batty KT, Davis TM, Thu LT, Binh TQ, Anh TK, Ilett KF. Selective high-performance liquid chromatographic determination of artesunate and alpha- and beta-dihydroartemisinin in patients with falciparum malaria. J Chromatogr B 1996; 677: 345–50.

21 Batty KT, Ilett KF, Davis TM. Chemical stability of artesunate injection and proposal for its administration by intravenous infusion. J Pharm Pharmacol 1996; 48: 22–6.

22 Navaratnam V, Mordi MN, Mansor SM. Simultaneous determination of artesunic acid and dihydroartemisinin in blood plasma by high-performance liquid chromatography for application in clinical pharmacological studies. J Chromatogr B 1997; 692: 157–62.

23 Davis TM, Binh TQ, Ilett KF, et al. Penetration of dihydroartemisinin into cerebrospinal fluid after administration of intravenous artesunate in severe falciparum malaria. Antimicrob Agents Chemother 2003; 47: 368–70.

24 Silamut K, Molunto P, Ho M, Davis TM, White NJ. Alpha-1 acid glycoprotein (orosomucoid) and plasma protein binding of quinine in falciparum malaria. Br J Clin Pharmacol 1991; 32: 311–5.