Stability of the antimalarial drug dihydroartemisinin in under physiologically-relevant conditions: implications for clinical treatment, pharmacokinetic and *in vitro* assays

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Running Head: Dihydroartemisinin stability

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Abstract

Artemisinins are peroxidic antimalarial drugs known to be very potent but chemically highly unstable; they degrade in the presence of ferrous iron, Fe(II)-heme or biological reductants. Less documented is how this translates into chemical stability and antimalarial activity across a range of conditions applying to *in vitro* testing and clinical situations. Dihydroartemisinin (DHA) is studied here because it is both an antimalarial drug on its own and the main metabolite of other artemisinins. The behavior of DHA in PBS, plasma or erythrocytes lysate at different temperatures and pH ranges was examined. The antimalarial activity of the residual drug was evaluated using the chemosensitivity assay on *P. falciparum*, and the extent of decomposition of DHA was established through use of HPLC-ECD analysis. The role of the Fe(II)-heme was investigated by blocking its reactivity using carbon monoxide. A significant reduction in the antimalarial activity of DHA was seen after incubation in plasma and to a lesser extent in erythrocytes lysate: activity was reduced by half after 3 hours and almost completely abolished after 24 hours. Serum-enriched media also affected DHA activity. Effects were temperature and pH-dependent and paralleled the increased rate of decomposition of DHA from pH 7 upwards and in plasma.

These results suggest that particular care should be taken in conducting and interpreting *in vitro* studies, prone as they are to experimental and drug storage conditions. Disorders such as fever, hemolysis or acidosis associated with malaria severity may contribute to artemisinins instability and reduce their clinical efficacy.

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Introduction

Artemisinin derivatives are the most potent antimalarials available to-date; they are highly active against all *Plasmodium* species and parasite stages, including young gametocytes, and constitute the backbone of malaria case management, both for severe and uncomplicated malaria (as a component of ACT, artemisinin combination therapy) (1). The emergence and spread of artemisinin resistance in Southeast Asia is therefore a serious threat to malaria control (2, 3).

All artemisinin derivatives share a distinctive 1,2,4-trioxane pharmacophore, which confers their antimalarial activity (the corresponding acyclic analogues lacking the endoperoxide bridge are inactive) (4, 5), but makes also these molecules particularly highly reactive and thus difficult to quantify in plasma or blood. Of the various artemisinin-type compounds in use, dihydroartemisinin (DHA), the reduced lactol derivative of artemisinin, is both an antimalarial compound on its own (currently co-formulated with piperaquine), and the main bioactive metabolite of artesunate and artemether. DHA itself is chemically fragile, and displays a marked propensity to undergo ring opening of the lactol and rearrangement under neutral conditions, leading to a new, biologically-active peroxide which, in turn, rapidly decays to the inert end product deoxyartemisinin (6). Under aqueous conditions, artemisinins tend to react with labile ferrous iron and heme-Fe(II) and in certain organic solvent, such as DMSO, they degrade very quickly (7). Moreover, under physiological conditions at pH 7.4, artemisinins are also reduced by biological reductants, such as flavin cofactors (8). Artemisinin-type compounds are short-lived (depending on the route of administration, elimination half-lives range 0.67-20.2 hours), but their antimalarial activity persists without measurable drug levels (9, 10).

While these characteristics are well-known, more incomplete is our understanding of how artemisinin derivatives behave in various conditions, and how this relates to their antimalarial activity. Here, we describe the effects on chemical decay and *in vitro* activity on cultures of exposing DHA to a range of conditions (pH, temperature, media, blood products) likely to be

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relevant to the interpretation of *in vitro* assays and clinical use of DHA, and which could affect its overall activity.

Methods

In vitro cultures of *P.falciparum*

The CQ-sensitive (D10) and the CQ-resistant (W2) strains of *P.falciparum* were maintained at 5% hematocrit (human type A-positive red blood cells) in RPMI 1640 (EuroClone) medium supplemented with 1% AlbuMaxII (lipid-rich bovine serum albumin) (Invitrogen), 0.01% hypoxantine (Sigma), 20mM Hepes (EuroClone), 2 mM glutamine (EuroClone). All the cultures were maintained at 37°C in a standard gas mixture consisting of 1% O_2, 5% CO_2, 94% N_2 (11).

Preparation of erythrocytes lysate

Blood samples from human donors kept in citrate–phosphate–dextrose solution were obtained from AVIS Milano. Blood was centrifuged to remove plasma and leukocytes, and RBCs were washed two times with RPMI medium.

One mL of 100% erythrocytes was diluted in 10 mL of PBS, pH 7.4. The suspension was then divided in two aliquots. In order to obtain carbon monoxide (CO) hemoglobin, 5 mL of PBS diluted RBCs were flushed with a gas mixture containing 2% CO, 5% CO_2 and 93% nitrogen for 5 minutes. Both normal and CO-flushed erythrocytes were then lysed by a cycle of freeze–thawing at -80°C; the stable complexation of Fe(II)-heme with CO was confirmed by UV–VIS spectrophotometry, which showed the shift in absorption from 414nm of oxy-hemoglobin to 420nm of carboxy-hemoglobin (HbCO) (12, 13). Erythrocytes lysates were further diluted 1:5 in PBS to perform the experiments described below.

DHA preparation and incubation conditions

DHA (a gift from Prof R. Haynes) was weighed and dissolved in ethanol at a concentration of 40 mM. The drug was then diluted at 2 μM in PBS, plasma (inactivated at 56°C for 40 min), serum, plasma diluted in PBS, erythrocytes lysate or CO-flushed erythrocytes lysate diluted 1:5 in PBS.

Samples were then incubated at 37°C for different lengths of time (3, 6, 18 hours) in a CO_2 incubator. Some experiments were performed at 4°C, room temperature or 40°C. DHA was also incubated in erythrocyte lysate or in plasma in the presence of ascorbic acid (Sigma-Aldrich) (0.5-1 mM) dissolved in PBS at 100 mM and then diluted to the desired concentrations directly in the incubation mixtures. Artesunate (a gift from Prof R. Haynes) and chloroquine (Sigma-Aldrich) were dissolved at 40 mM in DMSO or water, respectively, and then diluted to 2 µM in the conditions described above.

For the experiments performed at different pHs, phosphate buffer at pHs 7.2, 7.4 and 7.6 was prepared using different ratio of NaH_2PO_4 and Na_2HPO_4 and pHs were measured using a pHmether. The activity of each DHA preparation was then determined by the semi-automated pLDH assay (Freedom Evo 75, Tecan Italia, Srl). Briefly, samples containing DHA were diluted in complete medium at 100 nM DHA concentration, placed in 96 wells flat-bottom microplates (Costar) and serially diluted. Control (fresh DHA, artesunate, and chloroquine –CQ) were dissolved in DMSO or water at 10 mg/ml and then diluted to the desired concentration in complete medium. Asynchronous cultures with parasitemia of 1–1.5% and 1% final hematocrit were aliquoted into the plates and incubated for 48 h at 37°C. Parasites growth was determined spectrophotometrically (OD_{650}) by measuring the activity of the parasite lactate dehydrogenase (pLDH), according to a modified version of Makler's method in control and drug-treated cultures (14). Antimalarial activity was determined as concentration of drugs inducing 50% of growth inhibition (IC_{50}). Three independent experiments were performed and each one in duplicate.

Measurement of DHA half-life at different pH and in plasma

Chromatographic determinations were performed by high-performance liquid chromatography (HPLC) grade acetonitrile by electrochemical detection operating in the reductive mode at -1000 mV in an oxygen free environment (HPLC-ECD BAS 200A, Bioanalytical System, Inc., West Lafayette, IN, USA). Chromatographic separations were obtained on a Nucleosil C_{18} stainless steel column 250 x 4.6mm I.D., 5 µ particle size (Phenomenex, Torrance, USA).

Buffer solutions of varying species with pH ranges of 1.0 to 8.6 (pH <2 hydrochloric acid buffer, pH 2.0 – 4.5 phthalate buffer, pH 6.0 – 7.4 phosphate buffer, pH 8.6 borate buffer) were prepared at a constant total buffer species concentration of 0.2M and ionic strength, $\mu = 0.5$ by adding sodium chloride. Sample solutions of DHA at 20, 30, 40 and 50 mg/L were prepared by using a specific volume of a 1.0 mg/mL DHA stock solution (in ethanol) and make up to 100mL with buffer (preheated to 37^{o}C). The DHA stock solution was prepared in ethanol because of the poor solubility of DHA in buffer solutions. The resulting mixture was sealed to prevent water evaporation and maintained at the appropriate temperature in a thermostat water bath (Memmert water bath model W270, Schwabach, Germany). The temperature was kept at 37 ± 0.1ºC. At appropriate time intervals, 500 µL aliquots of the reaction mixture were removed from the reaction vessel, pipetted into test tubes, sealed and immediately stored in a -70ºC freezer and analysed as quickly as possible. At the time of analysis, samples were removed from the freezer and thawed. 100µL of the internal standard stock solution (40mg/L) prepared in ethanol:water (50:50 v/v) and 400µL of ethanol:water (50:50 v/v) was added into the sample test-tube containing 500 µL sample and vortex mixed. A fixed volume (20 µL) of these samples was assayed chromatographically to determine the concentrations of DHA and artemisinin. This procedure was used for all pH values.

To determine the kinetics of decomposition of DHA in plasma, 40 microlitres of DHA (50ng/µL) solution in ethanol-water (50:50 v/v) was spiked in a salinized glass culture tube containing 500 µL plasma and 460 µL of phosphate buffer (pH 7.4) to obtain a final concentration of 2000ng/0.5mL plasma. The total reaction volume was 1mL and the incubation was carried out at 37 ± 0.1^{o}C. At appropriate time intervals (0, 0.5, 1, 1.5, 2, 2.5 and 3 hours) the samples were withdrawn and immediately stored in -70^{o}C freezer until analysis by HPLC-ECD. At the time of analysis, samples were removed from the freezer and thawed at 4^{o}C. 20µL of the internal standard stock solution (40mg/L) was added and extracted, according to the method of Navaratnam et al(15), and subsequently analyzed by HPLC-ECD.

Statistical analysis

Data were expressed as mean ± SD and analyzed using two-tailed Student's t test with a level of significance of $p < 0.05$.

Results

Blood components reduced DHA effectiveness against *P.falciparum*

To investigate the effects of blood components on DHA activity against *P. falciparum*, DHA (2 µM) was incubated for 18 hours at 37°C in PBS, erythrocyte lysate or plasma. The role of Fe(II)heme in DHA activity was investigated by flushing erythrocytes with CO, which firmly binds Fe(II)heme (16), thus inhibiting its reactivity. Fresh DHA, weighed and dissolved immediately before the assay, was used as control. As shown in figure 1A, DHA activity was reduced after incubation in all the conditions used. In particular, DHA activity was almost completely lost after incubation in plasma and to a lesser extent in erythrocytes lysate; the presence of CO partially restored activity. When human serum was used instead of human plasma, the results were comparable, indicating that the reduction in drug activity could not be ascribed to anticoagulants. When DHA was incubated in plasma serially diluted in PBS, the reduction in DHA activity was inversely related to the dilution (Figure 1B).

To verify if the loss in drug activity was related to the peroxide moiety, artesunate (extensively converted to DHA(17)) and chloroquine (a 4-aminoquinoline) were incubated in PBS or in the blood components for 18 hours before testing their activity on parasite. Table 1 shows the mean IC_{50} of the drugs and the ratio between the IC_{50} of the fresh drug and the IC_{50} of the drug after incubation in blood components. Chloroquine was only marginally affected (the 7-20 % drop in activity across the different conditions was not statistically significant). The effects of PBS and HbCO on artesunate were mild, but marked reductions were observed in plasma, though proportionally less than for DHA.

DHA activity was also reduced after 18h incubation in the serum-enriched medium usually used for *in vitro* cell or parasite cultures, namely RPMI containing 10% fetal calf serum or 1% AlbuMax

(Table 2). DHA activity was partially preserved at 4°C or at room temperature. These temperatures were chosen to reproduce common conditions for handling drugs in *in vitro* screening assays.

DHA stability at different pH and in plasma

Table 3 provides the values of k_{obs} and $t_{1/2}$ at the various pH values and at 37°C. The pH rate profile curve for DHA given in Figure 2 is U shaped, which is typical of reactions that are susceptible to specific acid-base catalysis. Assuming there is no appreciable catalytic effect of the buffer systems used in the study, the observed rate constant generally obeys the equation $k_{obs} = k_{H+}[H+]^m$ when the catalysis is largely by hydrogen ions, and $k_{obs} = k_{OH-}[OH]^n$ when catalysis is mainly by hydroxyl ions, where k_{H+} is the rate constant for hydrogen-ion-catalyzed reaction, k_{OH-} is the rate constant for the hydroxyl-ion-catalyzed reaction and m and n are constants. The values of k_{H+}, m, k_{OH-} and n were obtained by plotting log k_{obs} versus pH in the low (pH < 2.2) and high (pH >7) regions and were 1.62×10^{-3} Lmol $^{-1}s^{-1}$, –1.19, 1.43×10^{-11} Lmol^{-1} s^{-1} and 0.86, respectively.

In plasma, the rate constant, k for degradation was higher than that of the corresponding buffer solution at pH 7.4: $8.55 \times 10^{-5}s^{-1}$ vs. $3.48 \times 10^{-5}s^{-1}$, respectively. The corresponding $t_{1/2}$ were 5.5 hours (332 min) and 2.3 hours (135 min).

DHA decay followed pseudo first-order kinetics at constant temperature and ionic strength over the pH range 1.0 – 8.6 (beyond which the reaction was rapid and the procedure unsuitable for determining the rate constant). This pseudo first-order dependence is illustrated in Figure 4, which shows that the log concentration versus time plots are linear and is confirmed by the absence of a significant influence of drug concentration on k_{obs} or $t_{1/2}$ in the range 20 mg/L ($k = 6.22 \times 10^{-5} \pm 2.08 \times 10^{-7}$ sec^{-1}; t ½ 11138± 37 sec) – 50 mg/L ($6.74 \times 10^{-5} \pm 2.08 \times 10^{-6}$ sec^{-1}; t ½ 10290 ± 312 sec).

Antiplasmodial DHA activity depends on temperature and pH

DHA was incubated in plasma at 37°C or 40°C (to mimic increased body temperature during a malaria attack) for 3 and 6 hours. Longer incubation was not considered since DHA activity was completely lost after 18 hours in plasma (see Fig1A). Table 4 shows a time-dependent loss of DHA

activity from 3 to 6 hours incubation in plasma at 37°C and 40°C; the residual activity was <50% and decreased to 15% after 3 or 6 hours of incubation, respectively (Figure 3a), in agreement with DHA half-life calculated in plasma (2.3 hours). The loss of activity was increased by higher temperature (40°C).

To better investigate small variations of pH around the physiological 7.4, DHA was incubated in phosphate buffer at pH 7.2, 7.4 and 7.6 at 37°C for 6 hours, showing a progressive reduction of activity from pH 7.2 to 7.6 (Table 5). A high degree of correlation was found between IC_{50} and K_{obs} in the pH 7.2 - 7.6 range, clearly indicating that the loss in DHA activity is related to increased degradation (Figure 3b).

Reductants promote DHA degradation

The presence of the antioxidant ascorbic acid (or N-acetylcysteine, data not shown) in the RBC lysate strongly reduced DHA activity in the presence of HbO (Table 6). CO binding to Fe(II)-heme conferred partial protection against this loss in activity. Instead, adding ascorbic acid to plasma had no effects on DHA activity. Three hours incubation times were used in these experiments as DHA activity dropped rapidly in the presence of ascorbic acid.

Discussion

The implications of the findings of this paper cover the use of DHA in the clinics, the measurement of levels in biological fluids, and *in vitro* sensitivity assays. We detected a drop in DHA activity upon *P. falciparum in vitro* after incubation in different conditions which broadly mimic physiological and pathological situations occurring during malaria infection. We also identified conditions of *in vitro* assays which are likely to influence the interpretation of DHA *in vitro* data. The dose of DHA used in the experiments (2 µM) is biologically relevant since it is in the range of maximal concentration (C_{max}) detected in the plasma of adults patients after oral or parenteral administration of artesunate (18).

Consistent results were obtained in this paper between experiments measuring chemical degradation and biological activity, confirming that loss in activity and decomposition of DHA are related. Both the chemical degradation and the corresponding reduction in biological activity are pH, time and temperature dependent. Of all the conditions tested, plasma (and serum, data not shown) had the largest effect on drug activity.

Changes in DHA activity on *P. falciparum* were consistent with *in vitro* degradation rates which were tested over pH values ranging from highly acidic to slightly basic as well as in human plasma. DHA appears to be more prone to decomposition than artesunate around neutral pH and in plasma. Artesunate itself is rapidly and extensively transformed into DHA *in vivo* (17). In experimental conditions, artesunate half-life (t ½) at pH 7.4 is 10.8 hours and in plasma 7.3 hours (Olliaro and Navaratnam, unpublished observation), compared to 5.5 and 2.3 hours for DHA. This means that after 18 hours' incubation of artesunate, assuming a linear decay throughout, only about 31% and 18% of the drug will still be artesunate itself (the rest being DHA and its further degradation products (6)). This translates in 89% of the activity of the parent drug remaining after incubation in PBS for 18 hours for artesunate vs. 38% for DHA; the respective values in plasma are 17% for artesunate and 0% for DHA.

Here we show that DHA is stable within the pH range 2 to 6 (conditions which apply to the stomach and small intestine where the drug is absorbed), while pH lower than 2 and higher than 6 promote DHA degradation. This U-shaped pH profile is typical of reactions that are susceptible to specific acid-base catalysis. The reaction follows pseudo-first order kinetics with a log-linear decay over time up to pH 8.6.

A possible limitation of this study is that it measured the disappearance of DHA but did not characterize the formation and disappearance of other species, notably the rearranged bio-active peroxyhemiacetal metabolite (6). The parallel between chemical decay and biological activity on *P. falciparum* should therefore allow for all the bio-active species.

248 To mimic conditions that may occur *in vivo*, we incubated DHA in phosphate buffer around the
249 physiological pH (7.2, 7.4, 7.6) and found a high correlation between the speed at which
250 degradation occurs and loss in activity. DHA was relatively more stable and more active at pH 7.2,
251 in conditions such as those encountered in malaria-related acidosis; for instance, compared to pH
252 7.4, at pH 7.2 the DHA t ½ in buffer would be ca. one-third longer (8.1 hours instead of 5.5 hours),
253 and potency ca. one-fourth higher (IC_{50} 7.8 vs. 10.6). This difference between the t ½ and IC_{50} ratios
254 is likely accounted for by the presence of other bioactive species, such as the above-mentioned
255 peroxyhemiacetal metabolite. However, if acidosis might drive the equilibrium one way,
256 intravascular hemolysis could compromise DHA activity by liberating Fe(II)-heme. Hemolysis,
257 another condition related to severe manifestations of malaria, was reproduced in the present work
258 by incubating the drug in erythrocytes lysate. Previous studies show that Fe(II), but not Fe(III) can
259 decompose artemisinin (19, 20), and that peroxidic antimalarials are stable in the presence of
260 hemoglobin but react with free heme (21). In this work, further evidence of the role of Fe(II)-heme
261 is that flushing RBC lysate with CO, which tightly binds Fe(II)-heme inhibiting its reactivity,
262 moderated the effects on DHA activity. These data are in agreement with a previous study, wherein
263 it was observed that the activity of artemisinin antimalarials is increased when parasites were grown
264 in the presence of CO, and that artemisinins are unaffected by carboxy-hemoglobin or CO-heme,
265 but are decomposed by Fe(II)-hemoglobin or Fe(II)-heme (13, 22). Thus, we can expect DHA to be
266 degraded faster and therefore be less active, when intravascular hemolysis occurs. Also supporting
267 the role of Fe(II)-heme is the finding that the introduction of ascorbic acid, a reducing agent, during
268 DHA incubation in normal erythrocytes lysate (but not in CO-erythrocytes lysate or plasma in the
269 absence of hemolysis), further reduces DHA activity. Ascorbic acid would reduce the pool of
270 Fe(III) to Fe(II), which then in turn would degrade DHA.
271 The results of this study have also practical methodological implications for pharmacokinetic and in
272 vitro drug assays. They support the idea of using methods for artemisinin determination in blood
273 that employ oxidizing agents (i.e., potassium dichromate or hydrogen peroxide) to stabilize blood

and prevent degradation (23, 24). In addition, it is important to consider factors that could favor hemolysis and exposure to heme products. These results are also important for the conduct and interpretation of *in vitro* assays; DHA activity dropped after incubation in the media containing fetal calf serum or AlbuMax, which are routinely used for *in vitro* culture studies. It is therefore important to use the drug immediately after preparation or keep the drug in conditions that prevent loss of activity (e.g. stock solution in ethanol at 4°C (25)).

Finally, DHA decomposition may bear on the rise of artemisinin resistance; chemical decomposition, with consequent decay in antimalarial activity and parasites being exposed to suboptimal DHA concentrations, could select for drug-resistant parasites in patients treated with DHA itself or artemisinin derivatives metabolized to DHA.

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Figure legends

Figure 1. DHA (2μM) was incubated for 18 hours in the presence of PBS, normal (HbO) and CO-flushed (HbCO) RBC lysate and plasma (a) or in the presence of plasma diluted with PBS at different plasma: PBS ratios (b). *P. falciparum* (W2 strain) cultures were then exposed to the drug for 48 hours. Fresh DHA dissolved immediately before the assay was used as control. A representative experiment is shown.

Figure 2. pH degradation rate profile of DHA. K is the rate constant per second.

Figure 3. Residual activity of DHA (IC_{50} of fresh DHA/ IC_{50} of DHA after incubation) in PBS or in plasma at different temperatures and times (a). Correlation-regression of K_{obs} (degradation rate constant per second) and biological activity (IC_{50} in nM) in the pH range 7.2 – 7.6 (b)

Figure 4. Observed pseudo first-order plots for the hydrolysis at 37^{o}C. Log DHA concentration over time (sec)

382 Table1. Antiplasmodial activity of DHA, artesunate or chloroquine following exposure to different

383 conditions

	DHA[b] IC_{50} (nM)	**Ratio IC_{50} fresh DHA/ IC_{50} DHA after incubation**	**Artesunate[b] IC_{50} (nM)**	**Ratio IC_{50} fresh Artesunate/ IC_{50} Artesunate after incubation**	**Chloroquine[b] IC_{50} (nM)**	**Ratio IC_{50} fresh Chloroquine/ IC_{50} Chloroquine after incubation**
Fresh drug[a]	2.6 ± 1.1		4.7 ± 1.2		436.2 ± 97	
PBS	7.6 ± 4*	0.34	5,3 ± 1.5	0.87	548.8 ± 55	0.79
HbO	19.4 ± 8.5*	0.13	15.4 ± 6.4*	0.3	551.7 ± 31	0.79
HbCO	11.9 ± 5.3*	0.22	7.8 ± 0,9*	0.59	544.2 ± 35	0.8
Plasma	>200		34.9 ± 1.7**	0.13	464.5 ± 107	0.93

384 [a] Drugs (2 µM) were used as fresh preparations or incubated for 18 hours at 37°C in PBS, normal or

385 CO flushed RBC lysate (HbO or HbCO) and plasma. *P. falciparum* (W2 strain) cultures were then

386 exposed to the drugs for 48 hours and the parasite viability was assessed by the pLDH method.

387 [b]Data are the mean IC_{50} (nM) ± SD of three different experiments in duplicate

388 * $p<0.05$ vs fresh drug; ** $p<0.01$ vs fresh drug

Table 2. DHA activity after incubation in commonly-used serum-enriched cell culture media

	IC_{50} (nM) [b]		
	4°C	room temperature	37°C
Fresh DHA[a]	2.73 ± 1.5		
RPMI-AlbuMax	7.04 ± 1.7	9.73 ± 0.8*	118.1± 49*
RPMI-FBS	6.23 ± 1.7	10.6 ± 1.4*	108.6 ± 10**

[a]DHA (2µM) was used as fresh preparation or incubated for 18 hours in a medium containing 10% FBS or 1% AlbuMax at three different temperatures. *P. falciparum* (W2 strain) cultures were then exposed to the drugs for 48 hours and the parasite viability was assessed by the pLDH method.

[b]Data are the mean IC_{50} (nM) ± SD of three different experiments in duplicate

* $p<0.05$ vs fresh DHA; ** $p<0.01$ vs fresh DHA

395 Table 3. Observed degradation rate constants (k_{obs}) in sec^{-1} and half-lives ($t_{1/2}$ in minutes and hours)

396 of DHA at various pH at 37 ± 0.1°C

	pH	k(s-1)	$t_{1/2}$(min)	$t_{1/2}$ (hrs)
DHA	1	1.02x10-4	113	1.9
	1.2	6.22x10-5	186	3.1
	1.5	2.52x10-5	458	7.6
	2.2	3.85x10-6	3001	50.0
	3	7.76x10-7	14887	248.1
	6	1.32x10-6	8752	145.9
	7	1.62x10-5	713	11.9
	7.4	3.48x10-5	332	5.5
	8	1.04x10-4	111	1.9
	8.6	3.99x10-4	29	0.5
	Plasma	8.55x10-5	135	2.3

397

Table 4. Effect of short incubation times and higher temperature on DHA activity

	IC_{50} (nM) [b]			
	3 hours		**6 hours**	
	37°C	40°C	37°C	40°C
Fresh DHA[a]	2.4 ± 0.5			
PBS	3.31 ± 1.3	7 ± 1.9	4.3 ± 2.1	ND
Plasma	6.13 ± 1.9*	12.8 ± 1.8*	10.08 ± 4.1*	38.2 ± 3.5*

[a]DHA (2µM) was used as fresh preparation or incubated for 3 or 6 hours in PBS or in plasma at 37°C or 40°C. *P. falciparum* (W2 strain) cultures were then exposed to the drugs for 48 hours and the parasite viability was assessed by the pLDH method.

[b]Data are the mean IC_{50} (nM) ± SD of three different experiments in duplicate

*p< 0.05 vs fresh DHA

404 Table 5: Influence of pH in the range 7.2 – 7.6 on antiplasmodial DHA activity

	IC_{50}(nM) [b]	**Ratio IC_{50} fresh DHA/IC_{50} DHA after incubation**
Fresh DHA[a]	2.8 ± 0.7	
pH 7.2	7.79 ± 1**	0.36
pH 7.4	10.6 ± 1.1**	0.26
pH 7.6	14.3 ± 3.5*	0.20

405 [a]DHA (2µM) was used as fresh preparation or incubated for 6 hours at pH values 7.2 – 7.6. *P.*

406 *falciparum* (W2 strain) cultures were then exposed to the drug for 48 hours and the parasite viability

407 was assessed by the pLDH method.

408 [b]Data are the mean IC_{50} (nM) ± SD of three different experiments in duplicate

409 * $p<0.05$ vs fresh DHA; ** $p<0.01$ vs fresh DHA

410

411 Table 6: Effect of ascorbic acid on DHA activity

	control		+ Ascorbic acid 0.5mM		+ Ascorbic acid 1mM	
	IC_{50} (nM) [b]	**Ratio IC_{50} fresh DHA/IC_{50} DHA after incubation**	**IC_{50} (nM)**	**Ratio IC_{50} fresh DHA/IC_{50} DHA after incubation**	**IC_{50} (nM)**	**Ratio IC_{50} fresh DHA/IC_{50} DHA after incubation**
Fresh DHA[a]	2.25 ± 0.3					
HbO	4.74 ± 0.2**	0,47	18.91 ± 6.1	0,12	>100	
HBCO	5.01 ± 0.2**	0,44	8.97 ± 0.6	0,25	17.79 ± 2.3	
Plasma	6.73 ± 2,3*	0,33	5.35 ± 2.3	0.42	5.57 ± 2	0.4

412 [a]DHA (2µM) was used as fresh preparation or incubated for 3 hours in blood components in the

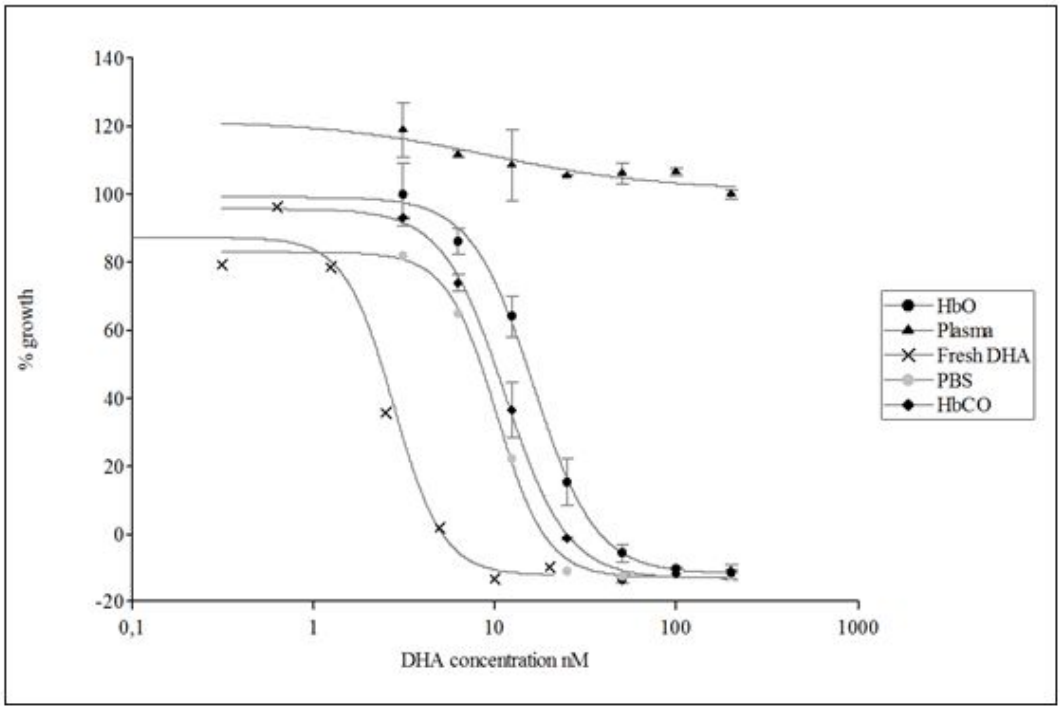
413 presence of ascorbic acid. *P. falciparum* (W2 strain) cultures were then exposed to the drug for 48

414 hours and the parasite viability was assessed by the pLDH method.

415 [b]Data are the mean IC_{50} (nM) ± SD of three different experiments in duplicate

416 * $p<0.05$ vs fresh DHA; ** $p<0.01$ vs fresh DHA

(a)



(b)

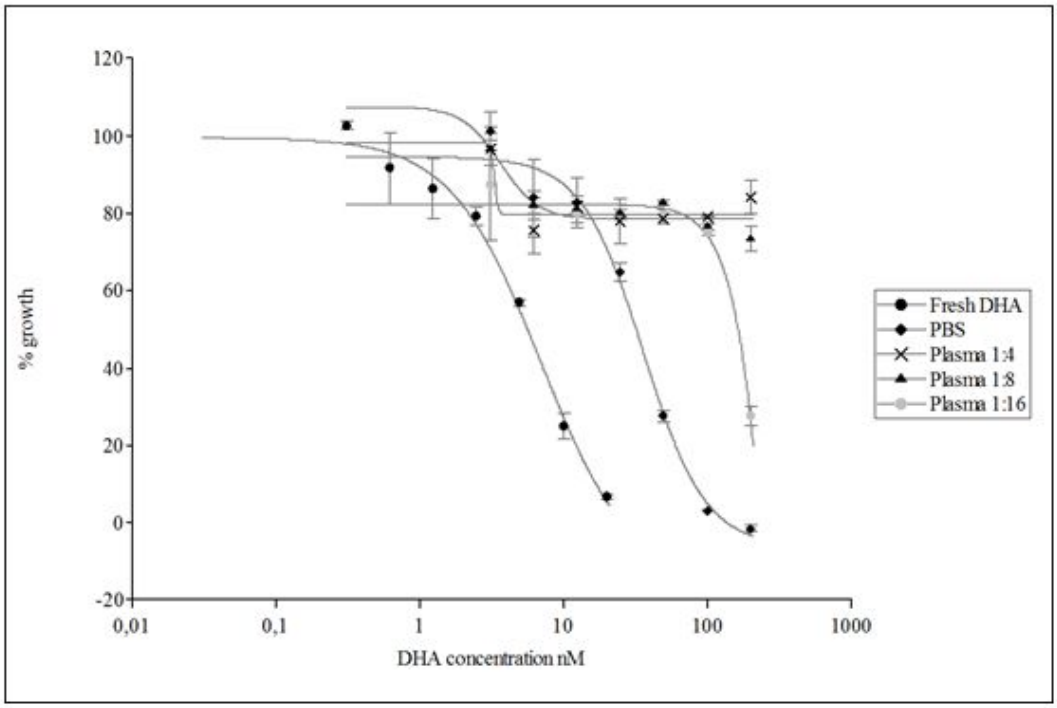


Figure 1. DHA (2μM) was incubated for 18 hours in the presence of PBS, normal (HbO) and CO-flushed (HbCO) RBC lysate and plasma (a) or in the presence of plasma diluted with PBS at different plasma: PBS ratios (b). *P. falciparum* (W2 strain) cultures were then exposed to the drug for 48 hours. Fresh DHA dissolved immediately before the assay was used as control. A representative experiment is shown.

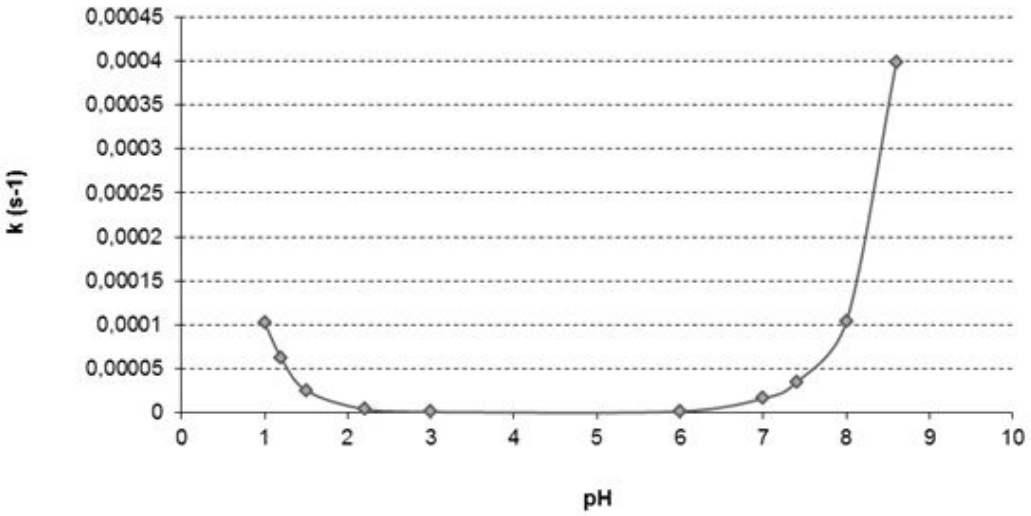


Figure 2. pH degradation rate profile of DHA. K is the rate constant per second.

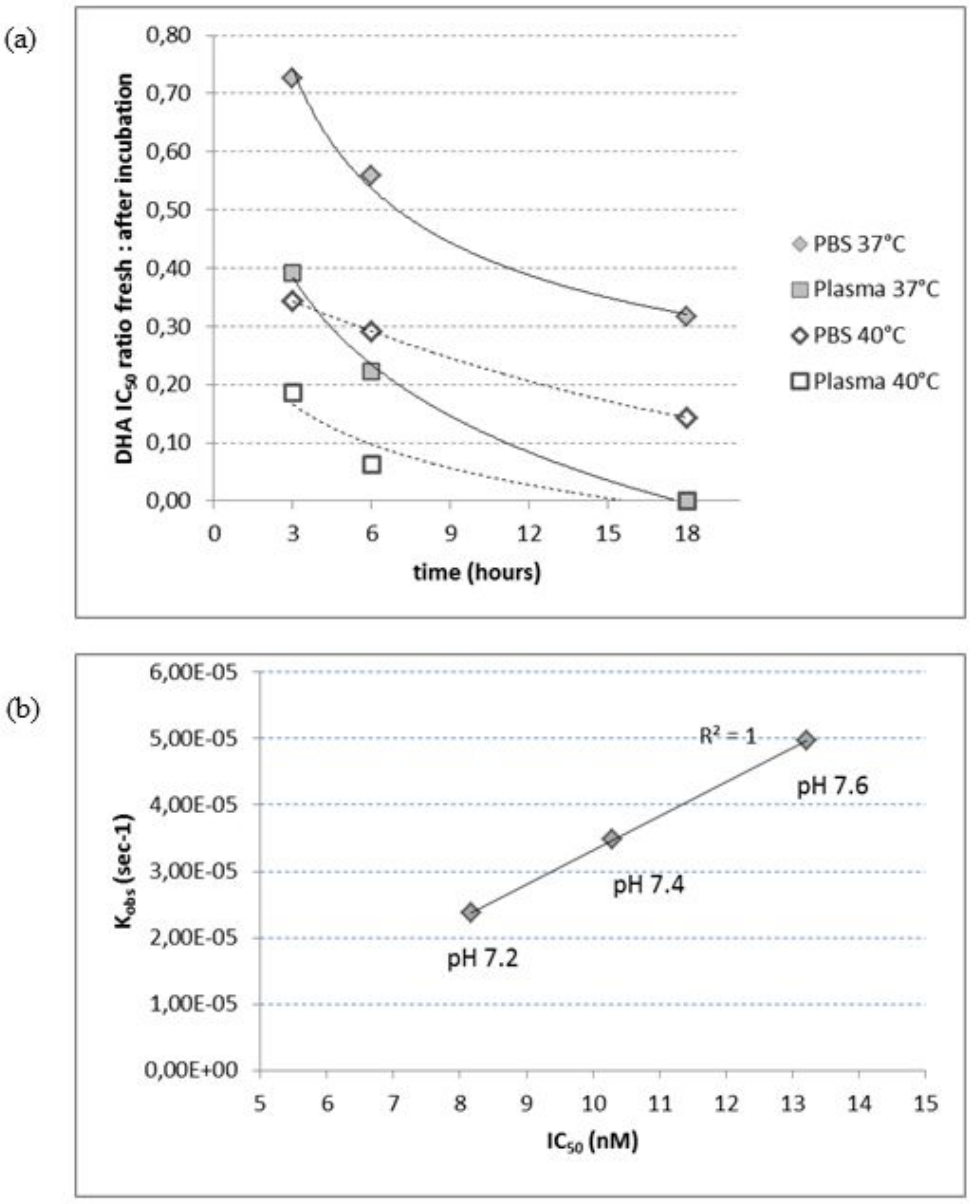


Figure 3. Residual activity of DHA (IC_{50} of fresh DHA/ IC50 of DHA after incubation) in PBS or in plasma at different temperatures and times (a). Correlation-regression of K_{obs} (degradation rate constant per second) and biological activity (IC_{50} in nM) in the pH range 7.2 – 7.6 (b)

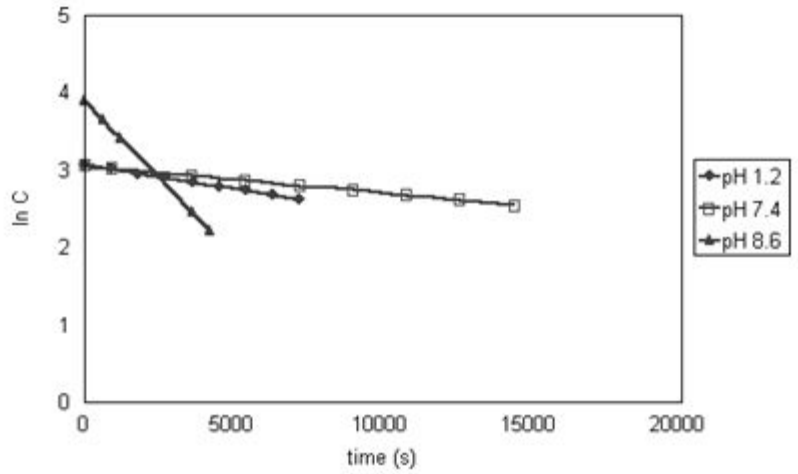


Figure 4. Observed pseudo first-order plots for the hydrolysis at 37°C. Log DHA concentration over time (sec)