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FERRIPROTOPORPHYRIN CATALYSED DECOMPOSITION OF ARTEMETHER: ANALYTICAL AND PHARMACOLOGICAL IMPLICATIONS

D. K. MUHIA,* C. G. THOMAS,† S. A. WARD,† G. EDWARDS,†‡§ E. K. MBERU* and W. M. WATKINS*†‖

*Wellcome Trust Research Laboratories, P.O. Box 43640, Nairobi, Kenya;
†Department of Pharmacology and Therapeutics, The University of Liverpool, Liverpool L69 3BX, U.K.; ‡Division of Biomedical Science, Liverpool School of Tropical Medicine;
‖Kenya Medical Research Institute, Nairobi, Kenya

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Abstract—The ability of the products of erythrocytic haemolysis and ferriprotoporphyrin (FP-IX)-containing substances to facilitate the decomposition of the antimalarial drug artemether has been evaluated *in vitro*. The products of haemolysis accelerate the degradation of artemether to unidentified compounds which are undetectable by currently available HPLC methods. This decomposition is temperature dependent and occurs after relatively brief artemether (ARM)-catalyst contact. Using radiolabelled [16-^{14}C]ARM, we have demonstrated that the extractability of total radioactivity into the organic phase diminishes with increasing FP-IX concentration with an appreciable reduction in the organic extractability as ARM in the presence of FP-IX and associated increase in water solubility of radioactivity when the concentration of FP-IX increases from 0 to 7.7 mM. This effect appears to be temperature dependent for incubations with haematin. Characterisation of the organically extractable radioactivity from incubations of [16-^{14}C]ARM with FP-IX has shown a loss of ARM and the formation of two radioactive products which occurs in proportion to the concentration of FP-IX. We believe these findings are of particular relevance to the determination of plasma concentrations of ARM and dihydroartemisinin (DHA) in malaria patients where extensive haemolysis and the presence of breakdown products of haemoglobin may contribute to a reduction in the circulating concentration of these substances. In these circumstances, measurement of ARM and DHA by conventional methods may be meaningless.

Key words: ferriprotoporphyrin IX; artemether, artemisinin; malaria

Resistance of *Plasmodium falciparum* to chloroquine and mefloquine has rapidly increased both in degree and prevalence and, as a result, the search for and development of new, more effective chemotherapeutic agents has been intensified. Among the more promising new antimalarials is artemisinin, the antimalarial principle of *Artemisia annua L.* and its analogues, notably ARM¶ and ART. These are derivatives of DHA a reduction product of artemisinin with greater antimalarial activity than the parent drug [1]. Aside from the Chinese literature, there is little information on the clinical pharmacology of these drugs, and the development of analytical methodologies that would permit the measurement of drug concentrations in biological fluids poses challenging problems. For example, ARM is thermally labile, lacks UV or fluorescent chromophores and possesses no functional group for derivitisation. Two analytical approaches have been adopted: acid catalysed decomposition to UV absorbing compounds [2, 3] followed by HPLC of the decomposition products and HPLC with reductive electrochemical detection [4].

Whatever method is employed, the assumption made in the design of any dosage regimen for ARM is that the concentration measured is related directly to the pharmacological response. Recently, while attempting to adapt one particular analytical method for the determination of ARM (and ART) in whole blood it became clear that there were significant losses of this analyte after storage at room temperature, 20°. This loss of material was not apparent when experiments were conducted with drug stored in water, plasma, or serum. We hypothesised [5] these observations to be the result of drug decomposition or sequestration of ARM/ART within the blood or binding to a component of the red cell membrane. Several lines of evidence suggest that the peroxide linkage, a common feature of the molecular structures of artemisinin and its derivatives, may play an important chemical role in the observed phenomenon. This functional moiety is also essential for antiparasitic activity. It has been suggested that these drugs interact with intraparasitic haem iron present in haemozoin, a storage form of FP-IX unique to the malarial parasite [6, 7]. This interaction results in the reductive decomposition of both ARM and its principal metabolite DHA *in vitro*. The observations that antimalarial activity can be reduced by iron chelators and antioxidants suggests that this interaction between the endoperoxide linkage and haem iron results in the

§ Corresponding author: Department of Pharmacology and Therapeutics, The University of Liverpool, Liverpool L69 3BX, U.K.

¶ Abbreviations: FP-IX, ferriprotoporphyrin-IX; ARM, artemether; ART, arteether; DHA, dihydroartemisinin.

generation of free radicals which can then react with cellular macromolecules [8]. Indeed, this is the current view of the mechanism of action of these drugs with parasite selectivity resulting from the significant accumulation of haemozoin within the food vacuole of the malaria parasite [9]. This argument has been restricted to drug reactivity within the parasite, although it has been shown that these drugs can form both iron-dependent and iron-independent covalent adducts with human serum albumin and red blood cells (RBC) [10]. It would seem logical that the reactivity of these compounds, especially in the presence of FP-IX, should not be restricted to intraparasitic drug. This would pose major difficulties in relating measurements of free concentrations of artemisinin and its derivatives in blood to a pharmacological end point when a proportion of the drug remains either strongly or variably bound or subject to biological degradation *in situ* and/or *in vivo*. These problems would be exacerbated in malaria patients, owing to erythrocytic lysis, as a result of merogony of parasitised red blood cells and their destruction in the spleen together with the liberation of FP-IX into the host circulation.

We now report the results of a series of investigations which begin to define the nature of this disappearance and/or decomposition examining in particular the role of iron-containing FP-IX derivatives and red blood cell lysis products on the analytical recovery *in vitro* of ARM and DHA.

MATERIALS AND METHODS

Reagents

ARM, DHA and progesterone (internal standard) were obtained from SAPEC (Lugano, Switzerland) via the World Health Organisation in Geneva, Switzerland. ARM and DHA were stored as powders in a desiccator at 4°. Stock solutions were prepared in methanol and stored at −20°. Working solutions were prepared by diluting stock solutions in normal saline. Acetonitrile, hexane and methanol (all HPLC grade), hydrochloric acid and sodium hydroxide (both Analar) and ammonia (SG 0.91) were obtained from BDH (Poole, U.K.). Human haemoglobin-A (H-0267), haemin, haematin and methaemoglobin were obtained from the Sigma Chemical Co. (Poole, U.K.) and used without further purification, although spectrophotometric analysis was used to verify the presence of haemoglobin in the preparation.

[16-^{14}C]β-artemether (sp. act. 12.1 mCi/mmol) was synthesised and supplied by the Research Triangle Institute (Research Triangle Park, NC, U.S.A.).

Methods

Chromatography of ARM and DHA. Separation of ARM from DHA, decomposition and chromatography were carried out by an adaptation of the method of Thomas *et al.* [2] and are to be published elsewhere (Muhia DK *et al.*, in preparation). In brief, in the modified method, plasma containing drug is basified with ammonia prior to extraction with hexane, which extracts only ARM, leaving DHA in the aqueous phase (ARM assay). If plasma is not basified, both ARM and DHA are extracted (total assay). DHA is quantified by the difference between the two assays. Decomposition was achieved with HCl (5 M) at 53° for 45 min. Chromatography was performed using an Isochrom solvent delivery system (Thermo Separations, Stone, Staffs, U.K.). Separation was achieved using a Hypersil 5 ODS stainless steel column (25 cm × 4.6 mm) (HPLC Technology, Macclesfield, U.K.) at an operating pressure of *ca.* 110 bar. The mobile phase consisted of water : acetonitrile (40 : 60 v/v) flowing at 1.5 mL/min. Retention times were shorter than reported in the original method, being 5.5 min for ARM or DHA and 6.5 min for the internal standard.

Collection of plasma. Whole blood (20 mL) was drawn slowly from a volunteer, using a wide bore needle, into acid citrate dextrose anticoagulant with immediate mixing. The mixture was centrifuged (6000 *g*, 5 min) and the supernatant stored at −20°.

Preparation of RBC lysate. Whole blood was subjected to three freeze/thaw cycles and centrifuged (6000 *g*, 10 min). The supernatant was filtered (0.45 μm membrane filter) and the haemoglobin (Hb) concentration measured (Coulter Counter haemoglobinometer). The lysate was then diluted with distilled water to produce a working solution of 20 mg/mL.

Studies with haemolysate and haemoglobin

Aliquots (0.5 mL) of plasma were prepared containing ARM (0.5 μg/mL) and increasing concentrations (0, 0.3, 0.6, 1.2, 2.4 and 4.8 mg/mL) of RBC lysate or haemoglobin. Each sample was assayed in duplicate for ARM and total drug-related material as above. Using a larger reaction volume containing ARM (0.5 μg/mL), similar experiments were designed to study the effect of temperature (23° and 37°) on the effect of haemoglobin at a constant concentration (4.85 mg/mL). Samples (0.5 mL) were removed from the incubation mixture at zero time and at 1, 2, 3, 4, 5 and 24 hr, and were again assayed in duplicate for ARM and total drug-related material as described.

Radiochromatography

Sample preparation. ARM at a concentration of 2 μg/mL containing [16-^{14}C]β-ARM (0.05 μCi, sp. act. 12.1 mCi/mmol) was mixed with haemin, haematin methaemoglobin or haemoglobin (0 to 7.7 mmol/L) in phosphate buffer (0.01 M, pH 7.4 mL, 1.0 mL). Incubations were carried out over 30 min with continuous stirring at room temperature (23°) and, in addition, with haematin at 5.0 mg/mL at 0, 37 and 50°. After incubation each sample was extracted with dichloromethane (2 × 3 mL for 10 min). After centrifugation (3000 *g* for 5 min) the organic extracts were combined and evaporated to dryness under nitrogen at 37° and the residue was reconstituted in methanol (200 μL). The remaining aqueous phase was retained for further analysis. All incubations and extractions were carried out using glassware pretreated with dichlorodimethylsilane (5% v/v) in toluene to minimise adsorption to the glass. All incubations were carried out in replicates of at least six except for the temperature dependence evaluations which used the mean of three observations.

Table 1. The effect of added haemoglobin (as RBC lysate) on the assay result for ARM and total drug

Hb concentration (mg/mL)	ARM added (ng/mL)	Assay result (mean ± SD; N = 3)	
		ARM (ng/mL)	Total (ng/mL)
0	500	532 ± 3	498 ± 8
0.30	500	489 ± 4	496 ± 4
0.60	500	447 ± 2	503 ± 4
1.21	500	303 ± 1	492 ± 0
2.42	500	41 ± 10	515 ± 9
4.85	500	0	503 ± 3

Recovery of radioactivity. The aqueous phase was decolourised (100 μL, 30% hydrogen peroxide) and solubilised (Scintran®, 1.0 mL, BDM, Poole, U.K.) over 2 hr. Aliquots of the aqueous phase (350 μL) and the organic extract (50 μL) were removed and the radioactivity was determined by scintillation counting after the addition of scintillation fluid (20 and 3.0 mL) respectively.

HPLC separation. The HPLC system comprised an SP 8770 isocratic solvent delivery system coupled to an SP 8780 autosampler (Thermo Separations, Stone, Staffs, U.K.) and a Berthold LB506 radioactivity monitor and an Opus PC IV 286 microcomputer for data processing (Berthold Instruments, St Albans, U.K.). The separation was performed at ambient temperature on a Spherisorb 5 μm CN stainless steel column (25 cm × 0.4 mm O.D.; HPLC Technology, Macclesfield, U.K.) using a mobile phase of acetic acid (0.1 M, pH 5.0) acetonitrile (75:25 v/v) flowing at 1.0 mL/min.

RESULTS

Studies with haemoglobin and haemolysate using HPLC with UV detection

Following incubation with each of these materials, quantifiable plasma concentrations of ARM were compared to the control incubations in the absence of haemoglobin. The degree of reduction was proportional to the concentration of haemolysate or haemoglobin (Table 1). Analysis of extracts of these incubations pointed to the conversion of ARM into products which exhibited similar extraction characteristics to DHA. Using a larger incubation volume it could be demonstrated that there was an almost immediate loss of the chromatographic peak corresponding to ARM, and a slower, temperature-dependent, disappearance of the compound which was extracted under conditions developed for analysis of DHA. The concentration declined by 20 ng/mL/hr at 23° (data not shown), while at 37°

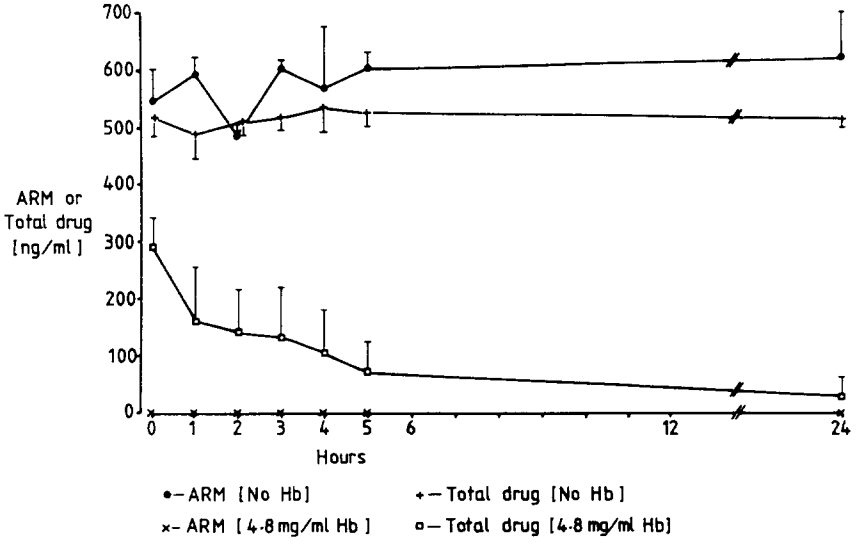

Fig. 1. Effect of time on the recovery of ARM and total drug following incubation of ARM (0.5 μg/mL) with haemoglobin at 37°. Values are means (N = 3) with SD shown as vertical bars. ●, ARM (-Hb); X, ARM (+4.8 g/L Hb); +, total drug (-Hb); □, total drug (4.8 g/L Hb).

Table 2. Effect of added FP-IX analogues on the organic (org.) and aqueous phase (aq.) recovery of ^{14}C radioactivity following their incubation with [16-^{14}C]β-ARM for 30 min at room temperature

Concn of FPP-IX (mmol/L)	Radioactivity recovered (dpm × 10^3) Haematin Aq.	Org.	Haemin Aq.	Org.	Haemoglobin Aq.	Org.	Methaemoglobin Aq.	Org.
0	1.72	70.6	1.36	67.3	1.31	70.6	0.65	68.7
	(0.62)	(7.7)	(0.21)	(7.1)	(0.60)	(6.27)	(0.12)	(23.8)
0.42	14.30	42.8	7.07	43.1	2.10	65.6	1.20	51.6
	(8.1)	(6.1)	(1.7)	(8.5)	(0.72)	(3.9)	(0.31)	(7.7)
0.95	19.4	49.1	13.3	36.3	2.28	65.8	1.24	55.2
	(2.8)	(8.2)	(5.7)	(3.7)	(1.14)	(7.29)	(0.29)	(3.13)
1.9	23.8	40.2	19.2	42.8	2.53	60.5	3.79	56.8
	(4.1)	(3.5)	(4.0)	(2.4)	(0.94)	(6.8)	(2.37)	(14.4)
3.86	16.6	59.7	26.6	34.5	4.52	62.2	3.6	54.9
	(2.8)	(6.0)	(4.4)	(2.1)	(1.16)	(13.7)	(1.2)	(8.3)
7.7	14.2	54.7	23.2	47.2	5.27	70.3	4.13	59.8
	(8.4)	(7.3)	(1.4)	(3.8)	(1.7)	(18.7)	(1.3)	(12.2)

Values are dpm × 10^3 (SD).

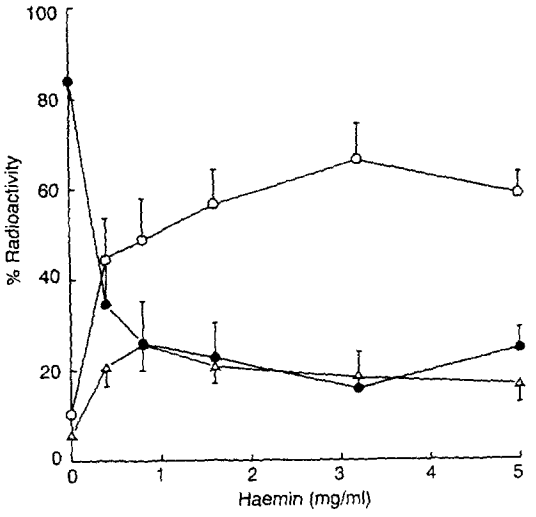

Fig. 2. Effect of increasing concentration of haematin (0–5 mg/mL, 0–7.7 mM) on the concentrations of ARM (●) and the two products 1 (○) and 2 (△) after incubation for 30 min at room temperature. Values are means (N = 6) with SD shown as vertical bars.

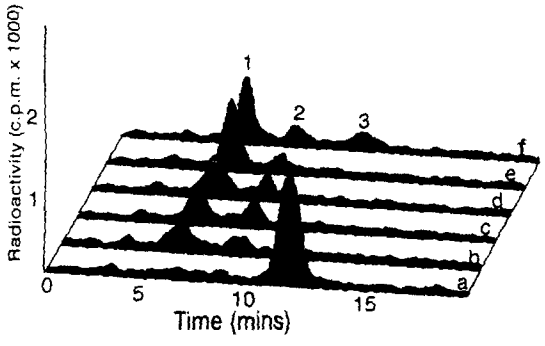

Fig. 3. Percentage recovery in the organic phase as radioactive products 1 and 2 after incubation of [16-^{14}C]β-ARM (2 μg/mL; 0.4 μCi) with haemin [0–7.7 mM; (0–5 g/L); a–f] for 30 min at room temperature. The vertical axis represents the radioactivity (cpm × 10^3) associated with each peak in the chromatograms, and the horizontal axis represents the retention times under the conditions described in the text.

(Fig. 1) the rate of decline in concentration was 36 ng/mL/hr. At both temperatures, ARM was stable for 24 hr in haemoglobin-free plasma.

Experiments with [16-^{14}C]*β-ARM*

Recovery. Increasing concentrations of haemin, haematin, haemoglobin or methaemoglobin produced an increase in the quantity of ^{14}C radioactivity that was recoverable in the aqueous phase in the rank order haematin > haemin > haemoglobin > methaemoglobin. This effect was mirrored by a decrease in concentration of organically recoverable radioactivity (Table 2).

HPLC. In the absence of haemin, all of the recoverable radioactivity was accounted for as a single peak which co-eluted with authentic standard for the parent drug [3: retention time (R_t) = 12 min 12 ± 4 sec]. As the concentration of haemin was increased, two product peaks appeared on the chromatogram (1: R_t = 5 min 56 ± 5 sec; and 2: R_t = 8 min 44 ± 6 sec; Fig. 2). These peaks were not representative of DHA which, based on chromatography of a radiolabelled standard, has a retention time of 6 min 36 sec under these chromatographic conditions. The conversion of ARM into each of these products at room temperature over a 30 min period of incubation relative to the concentration of haemin, haematin, haemoglobin and methaemoglobin is described in Fig. 3 (haemin) and Table 3. The temperature dependence of the reaction between ARM and haematin (0.5 mg/mL) is shown in Table 4. Clearly each compound has the propensity

Table 3. Conversion of ARM to products 1 and 2 (as % radioactivity in the organic phase) in a 30 min incubation as a function of catalyst concentration

	% Radioactivity, organic phase					
	Catalyst = 0 mg/mL			Catalyst = 5.0 mg/mL		
Catalyst	ARM	Prod. 1	Prod. 2	ARM	Prod. 1	Prod. 2
Haemin	84	10	6	25	60	15
Haematin	82	11	7	58	30	12
Haemoglobin	89	7	4	84	11	5
Methaemoglobin	90	8	2	85	10	5

Table 4. The effect of temperature on the recovery of gross radioactivity from aqueous and organic phases following incubation of [^{14}C]ARM with haematin (5.0 mg/mL) (mean ± SD for three expts)

Temperature (°)	Organic		Aqueous	
	dpm × 10^4	(% Total)	dpm × 10^4	(% Total)
0	3.01 ± 0.66	82	0.60 ± 0.08	16
23	2.76 ± 0.39	65	1.46 ± 0.10	35
37	2.26 ± 0.16	27	6.00 ± 0.10	73
50	1.6 ± 0.22	21	6.00 ± 0.31	79

to catalyse the formation of each of the products when compared with controls, although there is a rank order of haemin > haematin > haemoglobin or methaemoglobin ($P \leq 0.05$; Kruskal–Wallis test). Comparison between the latter two compounds was made difficult by the presence of methaemoglobin in the Sigma preparation (spectra not shown). However, neither compound was a particularly effective catalyst in this series of experiments (Table 3). Organic phase recovery of total ^{14}C radioactivity is increased with increasing temperature with a corresponding decrease in the amount remaining in the aqueous phase.

DISCUSSION

The observation that the recovery of ARM and ART from whole blood is markedly reduced following storage or incubations [5] led us to hypothesise that there might be either drug decomposition or sequestration of ARM and ART within components of the blood. It also suggested that we should be cautious about attempts to relate measured concentrations of these compounds to an observed biological response. We believe that the results of the current investigations further strengthen the importance of these warnings. Early studies using HPLC with measurement of UV absorbing post-column decomposition products for the determination of artesunate [1] reported that analytical recovery from haemolysed plasma was poor, and this was assumed to be due to the formation of a complex between haemoglobin and artesunate. The work of Zhang *et al.* [8] indicates that in addition to forming adducts with artemisinin, haemin catalyses the reductive decomposition of artemisinin and DHA, with the latter exhibiting a maximum peak current at 1×10^{-5} M (or *ca.* 0.06 mg/mL) haemin. It is likely that the reduced recovery of artesunate reported by Edlund and co-workers [11] was in part due to the decomposition of artesunate in the presence of the products of haemolysis. The results of our studies have demonstrated that in the presence of moderate concentrations (<5 mg/mL) of haemoglobin, an HPLC assay which is dependent upon the partitioning of ARM into the organic phase cannot detect drug at an added concentration of 500 ng/mL. Moreover, there appears to be a two-stage process in operation, with other drug-related products showing a more gradual rate of disappearance from the incubation medium. Our studies with radiolabelled ARM show that the effect is temperature dependent and catalysed more effectively by haemin and haematin, but not by hepatoporphyrin or protoporphyrin-IX Zn^{2+} which lack the central haem iron (data not included). We have shown that ARM decomposes to at least two products. Although these products are awaiting definitive chemical characterisation, they have retention characteristics on HPLC which are distinct from the parent drug and the principal *in vivo* metabolite, DHA. Our experimental data suggest that these compounds may represent steps in a general decomposition cascade rather than independent drug products. The enhanced retention of radioactivity within the aqueous phase may indicate binding of drug to haem or may infer the presence of additional breakdown products which are not extractable under the conditions used here.

While the nature of the decomposition products remains unknown, they appear to retain antiparasitic action; preliminary experiments using a bioassay to measure drug as DHA equivalents (data not shown) in plasma containing up to 50 mg/mL Hb indicate that disappearance of drug when measured by HPLC is *not* accompanied by loss of activity against *Plasmodium falciparum* which for this class of compounds is in the low nanomolar range [12]. However, our findings are of particular relevance to the measurement by chemical methods of plasma drug concentrations in malaria patients treated with artemisinin and its derivatives. Falciparum malaria is invariably accompanied by haemolysis, since each infected erythrocyte will be destroyed either at merogony, or when removed by the reticulo-endothelial system, principally the spleen [13]. Increased destruction of non-parasitised RBCs is also a characteristic feature of the disease [14]. The outcome is anaemia [15] and haemoglobinaemia, which may be acute [14], and in renal failure there may be extensive intravascular haemolysis [16]. All of these factors will contribute to a reduction in circulating concentration of artemisinin and its derivatives.

There are no reports of the changes in plasma haemoglobin, haem and haematin which occur during clinical malaria. In health, the normal range for plasma haemoglobin is 0.0016–0.0058 mg/mL [17]. Since the plasma threshold for haemoglobinuria is 0.5–1.4 mg/mL [18, 19], it is likely that plasma haemoglobin exceeds 1.4 mg/mL in malaria, and may be much higher. Recent information on the plasma concentrations of haemoglobin breakdown products in malaria is lacking; although early reports suggested that solutions of haematin cause chills and fever and indicate that this pigment may be at least partially responsible for the symptoms of malaria [20, 21].

From the results of this study and our previous reports, we conclude that RBC haemolysis products in plasma accelerate the decomposition of ARM and the formation of as yet unknown compounds which cannot be measured by current HPLC methods. In plasma samples from patients with malaria, haemoglobin and haemoglobin breakdown products are likely to be present in concentrations sufficient to catalyse decomposition. Finally, catalysed ARM decomposition is temperature dependent; measurable decomposition occurs after brief ARM-catalyst contact in plasma at room temperature.

In terms of drug analysis, it would appear that accurate quantification in whole blood is impossible using available HPLC methods unless drug is extracted into organic solvent immediately on removal of the sample from the patient. Alternatively, if haem-free plasma can be prepared immediately (e.g. by the use of an iron chelator) subsequent analysis may provide a true measure of drug concentration at the time of sampling. However, we would conclude that the current HPLC assays for ARM, and perhaps for all artemisinin derivatives, must be regarded as being of little value in terms of their capacity to link pharmacokinetic variables with pharmacological action, and more appropriate methodology will become available only when the chemical nature of the products formed by the interaction of ARM and haemin is fully established.

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