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Major pitfalls in the measurement of artemisinin derivatives in plasma in clinical studies

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ABSTRACT

A bioanalytical method for the analysis of artesunate (ARS) and its metabolite dihydroartemisinin (DHA) in human plasma using protein precipitation and liquid chromatography coupled to positive tandem mass spectroscopy was developed. The method was validated according to published US FDA-guidelines and showed excellent performance. However, when it was applied to clinical pharmacokinetic studies in malaria, variable degradation of the artemisinins introduced an unacceptable large source of error, rendering the assay useless. Haemolytic products related to sample collection and malaria infection degraded the compounds. Addition of organic solvents during sample processing and even low volume addition of the internal standard in an organic solvent caused degradation. A solid phase extraction method avoiding organic solvents eliminated problems arising from haemolysis induced degradation. Plasma esterases mediated only approximately 20% of *ex vivo* hydrolysis of ARS into DHA. There are multiple sources of major preventable error in measuring ARS and DHA in plasma samples from clinical trials. These various pitfalls have undoubtedly contributed to the large inter-subject variation in plasma concentration profiles and derived pharmacokinetic parameters for these important antimalarial drugs.

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1. Introduction

Artemisinin derivatives are the most potent antimalarial drugs available for clinical use and are the cornerstone of current antimalarial treatment of falciparum malaria [1–3]. The exact mechanism of antimalarial action of artemisinin and analogues is still a subject of intense debate. However there seems to be consensus that a cation-dependent reaction with the reactive peroxide bridge and the production of free radicals or other reactive intermediates is crucial for antimalarial activity [4–7]. The main cation implicated is thought to be ferrous iron (II). Iron chelators such as desferrioxamine and deferipone inhibit antimalarial activity *in vitro* [6,8–10]. *In vitro*, artemisinin derivatives undergo rapid degradation in the presence of ferrous iron (II) but are relatively stable in the presence of ferric iron (III) (i.e. in the absence of reducing agents) [8,9,11–13]. Artesunate (ARS), the most widely used of the artemisinin derivatives, acts as an ester prodrug and is rapidly hydrolysed *in vivo* to the active metabolite dihydroartemisinin (DHA). However, ARS can also form DHA *ex vivo* (i.e. in clinical plasma samples after collection) both through chemical hydrolysis and through plasma esterase mediated hydrolysis [4,14]. Anticoagulants containing fluoride inhibit *ex vivo* plasma esterase mediated hydrolysis but do not inhibit chemical hydrolysis. Some assays have specified the use of fluoride–oxalate to circumvent esterase mediated conversion [14] while others have not used fluoride, and blood has been anticoagulated with heparin [15–17], EDTA [18] or an unspecified anticoagulant [19]. Thus inherent properties of these drugs necessary for their potent antimalarial activity also create serious problems for quantification of the individual compounds in biological samples.

We recently developed a sensitive robust high throughput LC–MS/MS method for quantification of ARS and DHA in human fluoride–oxalate plasma [20]. We initially developed the LC–MS/MS assay based on protein precipitation as this would facilitate a rapid turnaround time and enable high sample throughput. The

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method was validated successfully according to published US FDA-guidelines [21,22] and showed good performance. However, it proved useless for clinical pharmacokinetic studies. We identified the reasons for the failure of the method, and on the basis of these pitfalls, developed and validated a new and robust assay based on solid phase extraction [20]. Here we present the investigation of the method failure, and its solution. We highlight and discuss pitfalls encountered when measuring the concentrations of artemisinin derivatives in plasma from clinical studies.

2. Experimental

The instrumentation, chemicals and materials were identical to those described in the final LC–MS/MS method. Stock solutions and plasma standards were prepared in the same manner as previously described [20].

2.1. Analytical procedure—original protein precipitation assay

Seventy-five microliter of ammonium acetate buffer 10 mM pH 3.5 was added to 150 μL plasma in a 96-wellplate. An eppendorf stream multistepper was used to add 450 μL acetonitrile (containing internal standards SIL-ARS/SIL-DHA) to the samples. The 96-wellplate was covered with a Nunc seal mat and mixed on a MixmateTM for 5 min at 800 rpm. The plate was thereafter centrifuged at about 1100 × *g* for 5 min and stored at 4 °C for 15 h to enable α/β epimer equilibration before analysis. With proper needle offset it was possible to inject 5 μL directly from the precipitated plate into the LC–MS/MS system.

2.2. Validation

The developed method was validated thoroughly according to published FDA-guidelines [21,22]. Back-calculated concentrations for the calibration standards during 4 days were used to verify linearity and calibration model. Precision and accuracy throughout the calibration range was evaluated by analysis of 5 replicates at three different concentrations daily for 4 days. Selectivity and matrix effects were thoroughly evaluated using blank plasma from six different donors. A quantitative estimation of the matrix effects was obtained by comparing the peak area for samples spiked in precipitation solution with precipitated blank matrix spiked with the same nominal concentration of the compounds. A qualitative visualization of the matrix effects was obtained through post-column infusion experiments. A continuous post-column infusion of ARS/DHA solution at 10 μL/min by a Harvard infusion pump through a T-connector was introduced to the mass spectrometer while samples to be tested were injected.

2.3. Clinical samples—a validated method proves useless

The validated method was applied to two pharmacokinetic studies. The first was a cross-over study of ARS in the treatment of uncomplicated *P. falciparum* infections during pregnancy. Twenty pregnant women with uncomplicated *P. falciparum* infections were studied in the second or third trimester of gestation and re-studied again 3 months after delivery. The second was an efficacy study of different doses of ARS for uncomplicated falciparum in Western Cambodia. The samples were taken at varying time points after oral administration of ARS, and before the partner drug mefloquine had been given.

2.4. Re-developed internal standard solution

Stock solutions of all compounds (250–1000 μg/mL) were prepared in ethanol. Combined working solutions of DHA (ranging from 0.0978 to 125 μg/mL) and ARS (ranging from 0.0593 to 36.4 μg/mL) were prepared by serial dilution of the stock solutions in ethanol–water (50–50, v/v). A combined working solution of SIL-DHA/SIL-ARS (800/5000 ng/mL) was prepared in ethanol and stored in 200 μL aliquots at −86 °C until use. On the day of assay this working solution was diluted with a plasma–water solution (50–50, v/v containing sodium fluoride/potassium oxalate 2/3 mg/mL) down to 3.27/20.4 ng/mL. This solution was prepared and kept on ice during use. The stock solutions of SIL-DHA and SIL-ARS were stored at −86 °C until use.

3. Results and discussion

The initial LC–MS/MS method was based on protein precipitation to minimize cost and to enable a high daily sample throughput. Mass spectrometry has unsurpassed selectivity and sensitivity but is vulnerable because of the possibility of matrix effects. Ion suppression (or enhancement) occurs when endogenous constituents co-elute with the analyte of interest and interfere with the ionization process in the interface [23]. In these investigations stable isotope labeled internal standards were available for both ARS and DHA and are generally considered to compensate for any unforeseen matrix effects. However, partial separation between the stable isotope labeled internal standard and the analyte does occur and can occasionally lead to differences in suppression/enhancement between the analyte and internal standard of up to 50%, differences so large that the results become invalid. Two recent reports showed that under certain circumstances stable isotope labeled internal standards were unable to compensate for matrix effects [24,25]. In the present paper the stable isotope labeled internal standards co-eluted approximately with the unlabeled ARS and DHA, which minimized the likelihood of differential matrix effects. Nevertheless, in line with international recommendations and our internal validation practices, matrix effects were thoroughly evaluated during the validation process [20,21]. Phospholipids are a common cause of matrix effects occurring during LC–MS analysis of biological samples with little or no sample pre-treatment (e.g. protein precipitation methods) [26]. Little et al. suggested an approach where the most common phospholipids could be monitored by in source fragmentation and selected reaction monitoring of the transitions 184/184 and 104/104. Fig. 1 shows a chromatogram after injection of protein precipitated blank human plasma (with internal standards) during post-column infusion 10 μL/min of ARS, DHA, SIL-ARS and SIL-DHA 5/13/2/13 ng/mL, demonstrating no regions with severe matrix effects (i.e. no sharp drops or increases in the response) at the retention times of ARS and DHA. It can also be seen that the problematic phospholipids came out early (104/104) or in the wash out gradient (184/184). Precision and accuracy for the validated protein precipitation LC–MS/MS assay clearly met the acceptance criteria (Table 1), and significant ion suppression/enhancement was excluded.

3.1. Clinical samples—a validated method proves useless

When the protein precipitation method was implemented for analysis of the first clinical pharmacokinetic study, the performance for the method was monitored continuously by analysis in triplicate of QC samples at low, medium, and high concentrations, as outlined in the FDA-guidelines for routine analysis [21]. All except one of the QC samples ($n = 270$ in total for ARS and DHA over

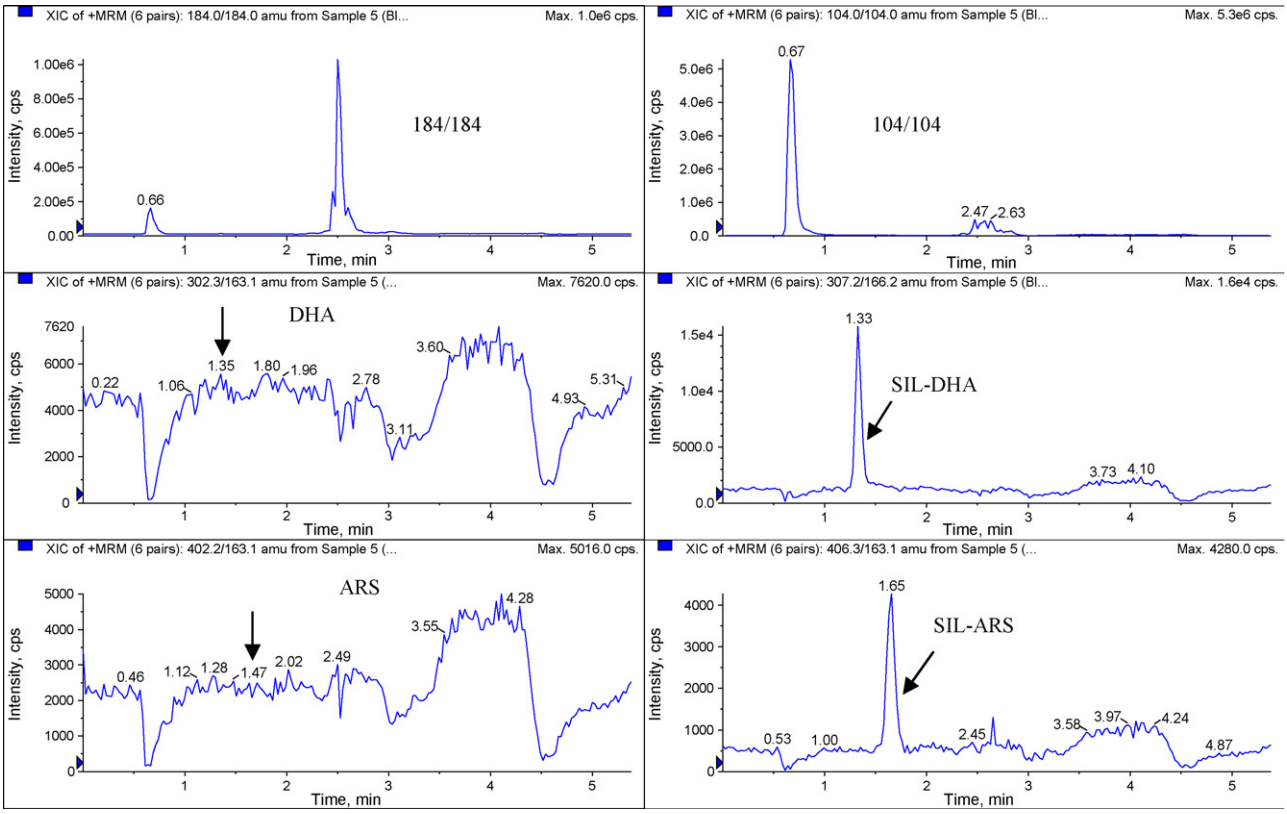

Fig. 1. Injection of protein precipitated blank human plasma (with internal standards) during post-column infusion 10 μL/min of ARS, DHA, SIL-ARS and SIL-DHA 5/13/2/13 ng/mL. Isocratic elution using mobile phase containing acetonitrile–ammonium acetate 10 mM pH 3.5 (50:50, v/v) with a wash out gradient using acetonitrile–methanol (25:75, v/v).

Table 1
Inter-, intra- and total-assay precision (ANOVA) for DHA and ARS in human plasma using protein precipitation method.

DHA	Inter-assay (%)	Intra-assay (%)	Total-assay (%)	ARS	Inter-assay (%)	Intra-assay (%)	Total-assay (%)
QC 1, 7.32 ng/mL	10.51	5.27	6.39	QC 1, 4.90 ng/mL	8.04	6.05	6.41
QC 2, 117 ng/mL	3.11	5.49	5.19	QC 2, 51.7 ng/mL	2.43	4.96	4.65
QC 3, 1880 ng/mL	8.38	5.99	6.42	QC 3, 546 ng/mL	3.70	5.51	5.26

$n = 5$ and $h = 4$.

17 days) were within ±15% from their nominal values. However, after the first batches erratic observations for the internal standards were noted. For some of the samples (i.e. 5–15) in each batch ($n = 72$ per batch) the response for the internal standards was significantly reduced and for some samples was almost absent. Both ARS and DHA internal standards were affected in the same way so that these samples also showed very low or absent peak areas for ARS/DHA. We first investigated if haemolysis was causing this effect. Previous reports indicated that haemolysis accelerates the degradation of artemisinin compounds and that degradation occurs rapidly after contact with the haemolysate [27]. Haemolysis was evident for a few samples where a reduction in internal standard could be seen, but in the majority there was no evident haemolysis. Since this was a cross-over study comparing treatment of malaria in pregnancy in the post-partum period, the same subjects were re-studied again after delivery when fully recovered from malaria. Surprisingly, compared with the problems encountered in the acute malaria samples only two post-partum samples showed a marked reduction in the internal standard response and in both cases there was evident visible haemolysis in the samples. Fig. 2 shows the response for the stable isotope labeled DHA for all samples in the study. We concluded that this phenomenon was associated with malaria, and could not be explained by *ex vivo* haemolysis in all cases. The first hypothesis tested was that the reduction in internal standard response for the non-haemolysed samples was caused by disease specific matrix effects (i.e. ion suppression in the mass spectrometer). However, post-column infusion experiments

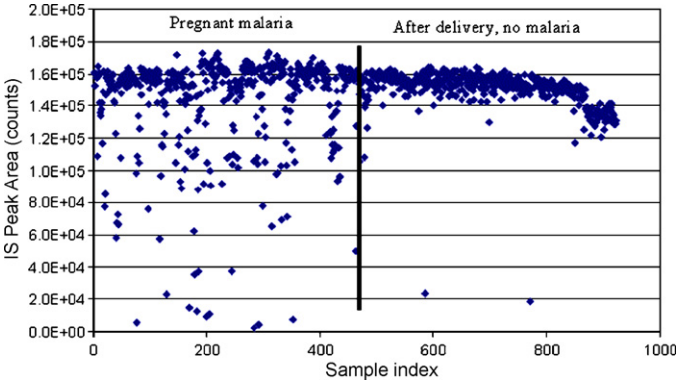

Fig. 2. Response of SIL-DHA for the cross-over study of ARS in the treatment of uncomplicated *P. falciparum* infections during pregnancy using the protein precipitation method.

did not indicate any region with ion suppression around the retention times for ARS and DHA. The second hypothesis was that the compounds decomposed in the presence of disease products. *P. falciparum* parasites consume hemoglobin within red blood cells utilizing the products of hemoglobin digestion. During this process iron (II)-heme is released which is toxic to *P. falciparum*. To detoxify the heme *P. falciparum* polymerizes toxic iron (II)-heme to a non-toxic iron (III) polymer (or stacked dimer), called hemozoin or malaria pigment [28]. *In vitro*, artemisinin derivatives decompose in the presence of iron (II) but are relatively stable in the presence of iron (III) [12,29] so it is likely that breakdown products of the hemoglobin digestion process (i.e. iron (II) products) rather than hemozoin (iron (III)) caused the degradation of the internal standards and the compounds in the assays investigated here. Although several samples had significant reductions in both internal standard responses and ARS/DHA responses, the two fell in parallel such that predicted concentrations seemed to be unaffected (i.e. did not deviate from the log linear concentration–time profile). This meant that the degradation happened after or at the same time as the internal standards were added to the samples (i.e. in the protein precipitation step) and that the internal standards compensated during this process. This excluded significant *ex vivo* decomposition before the assay. However, before implementation of the method for the next pharmacokinetic study steps were taken to minimize the problem. Experiments with samples from the first study indicated that there was less reduction of the responses if all steps were conducted on ice with ice cold solutions and centrifugation was performed at 4 °C. This was in agreement with a previous report indicating that the degradation rate of artemether after contact with haemolysate was reduced at lower temperatures [27]. The modified method was implemented for the second study, an efficacy study of ARS at different doses in Cambodia. However, assessment of samples from this study showed even more pronounced problems of degradation probably because parasitaemias were higher. Despite the method modifications many samples had very low or absent responses for both the internal standards and ARS/DHA. All samples from this study were assessed using the protein precipitation method, whereas a second aliquot of each sample was stored for reassessment once all problems in the assay had been resolved. Fig. 3 shows the response for the stable labeled DHA for all samples in the study. In line with the results for the first study the predicted concentration seemed to be unaffected for samples in which the internal standard response was at least 5% of original response. However, too many samples did not show any peaks for the internal standard (about 15%) and therefore could not be quantified. The method therefore had to be abandoned and a new method developed to circumvent these problems. The main conclusions from the analyses of the two studies were that the degradation was initiated during the protein precipitation step and that the catalyst came from two sources: haemolytic plasma and haemolysed products in samples with no visible haemolysis apparent.

3.2. Method re-development

Since degradation appeared to be initiated during the protein precipitation process the goal was to develop a method where ARS/DHA and the internal standards would be extracted from the plasma samples without addition of organic solvents. Solid phase extraction was the primary candidate technique since this could enable a 'solvent free' extraction procedure. The Oasis μ-elution HLB SPE 96-wellplate was chosen as it facilitates low elution volumes and is particular suitable for working with low sample volumes. A method was developed in which the plasma samples were loaded directly onto the SPE bed (without any prior buffering) and followed by a wash step consisting of pure water. Optimum elution was achieved with an elution solution containing methanol–acetonitrile (90–10, v/v). A second elution step with pure water was subsequently drawn through the SPE wells to dilute the organic solvent content in the samples and allow direct injection into the LC–MS/MS system. This optimized SPE method was prevalidated and implemented for a subset of patient samples from the second study. However large drops in internal standard response could still be seen in some samples despite a 'solvent free' extraction. The explanation was found in the sample preparation step prior to SPE. The internal standards had to be added to the plasma samples prior to SPE. SIL-DHA has very limited solubility in water and was therefore added in organic solvent. Initially, both internal standards were added in 10 μL acetonitrile. Despite the low volume this was still enough to cause some protein precipitation and significant degradation of the internal standards and ARS/DHA. When the internal standards were dissolved in ethanol–water (50–50, v/v) instead, the degradation was less prominent but still clearly a problem. We therefore planned to dissolve the internal standards in a plasma–water mixture as the plasma proteins would facilitate dissolution of the lipophilic SIL-DHA. Some fluoride–oxalate was also added to prevent esterase mediated hydrolysis in the solution. These steps improved the assay remarkably and the internal standard response was now similar between the samples in the subset. The internal standard response for the subset of patient samples with internal standards dissolved in acetonitrile, ethanol–water and plasma–water can be seen in Fig. 4.

For the final method a combined working solution of SIL-DHA/SIL-ARS (800/5000 ng/mL) was prepared in ethanol and stored in 200 μL aliquots at −86 °C until use. On the day of assay

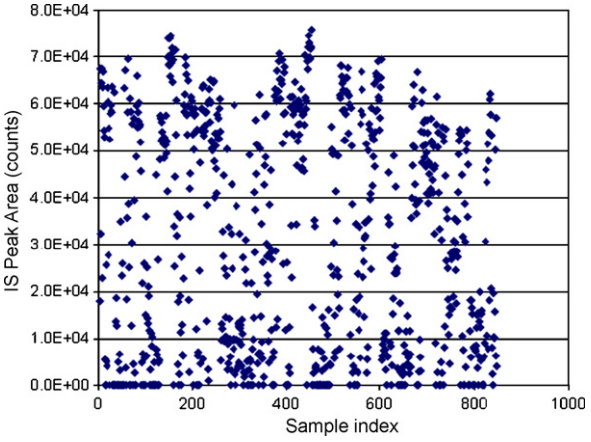

Fig. 3. Response of SIL-DHA for the efficacy study in Cambodia using the protein precipitation method.

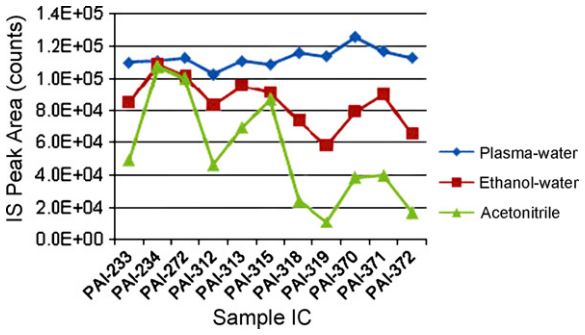

Fig. 4. Response of SIL-DHA for a subset of patient samples when added in acetonitrile, ethanol–water or plasma–water.

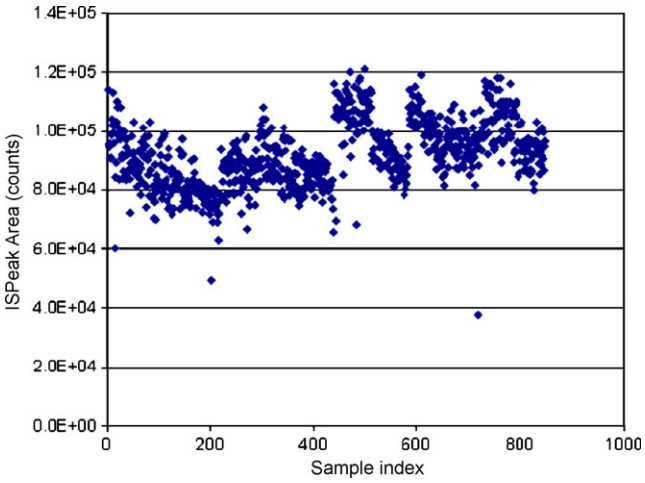

Fig. 5. Response of SIL-DHA for the efficacy study in Cambodia using the SPE method.

this working solution was diluted with a plasma–water solution (50–50, v/v containing sodium fluoride/potassium oxalate 2/3 mg/mL) down to 3.27/20.4 ng/mL. This solution was prepared and kept on ice during use. The final method was validated and once again implemented for analysis of the efficacy study of ARS at different doses in Cambodia using the second aliquot. This time the response for the stable labeled DHA for all samples in the study was high and consistent (Fig. 5). Only a few samples showed any reduction in response and in each case this was associated with clearly evident haemolysis of the sample. The benefits and improvement of the method can clearly be seen when comparing the initial response using protein precipitation (Fig. 3) with the new 'solvent free' method (Fig. 5).

3.3. The influence of haemolysis on sample integrity

As analytical responses for the artemisinin derivative in haemolysed samples are reduced significantly, or absent, or deviate significantly below non-haemolysed samples in the concentration–time profile, it has been assumed that artemisinin derivatives are degraded rapidly in contact with products of haemolysis [27]. While this might be true these conclusions are influenced by the nature of all previous analytical methods, all of which involve organic solvent in one or more steps. As a consequence haemolysed samples have been considered unsuitable for assay and the result for these samples often rejected in the data analysis.

Our investigations suggested that the protein precipitation step initiated the degradation, so experiments were conducted to evaluate stability and integrity in haemolysed samples and malaria patient samples.

Haemolysate was prepared from whole blood through repeated cycles of freeze/thaw (+vigorous mixing) followed by centrifugation. Haemolysate was diluted with blank plasma to produce haemolysed plasma test standards containing 20%, 4%, 0.8% or 0% haemolysate (and the rest blank plasma). Twenty-five microliter of haemolysed plasma standard was added to 25 μL of the highest QC sample (i.e. DHA/ARS 1880/546 ng/mL) in a 96-wellplate on ice to produce samples with DHA/ARS 940/273 ng/mL containing 10%, 2%, 0.4% or 0% haemolysate (and the rest blank plasma). These samples were analysed in triplicate with both the protein precipitation method and the new SPE method. It can be seen clearly both in Fig. 6a and b that the protein precipitation method fails to produce any response for the two most haemolysed samples. The response

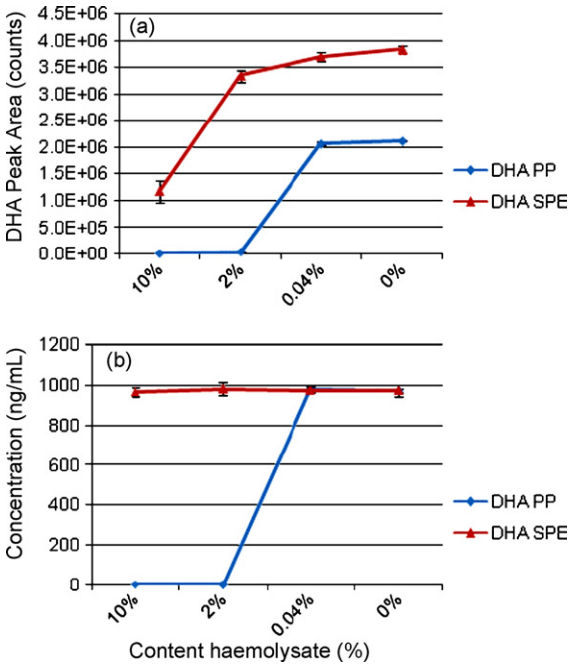

Fig. 6. Response for haemolysed samples when using protein precipitation method or SPE method area (a) and predicted concentration (b).

for DHA is also reduced significantly with the SPE method but since the SIL-DHA is reduced proportionally the predicted concentration is still accurate (Fig. 6b). These experiments support the theory that with haemolysed samples the sample preparation method influences the results. We hypothesize that artemisinin compounds in the native plasma sample (either haemolysed or malaria patient sample) are not significantly degraded by the iron (II) products until they come into contact with organic solvents. The reason is probably that the iron (II) products and the drugs initially are 'shielded from each other' due to the protein binding of the drugs and more importantly extensive binding of the active products themselves. Protein precipitation methods release these active products. The reason for the small analyte decomposition using the SPE method for the most haemolysed sample is therefore probably due to the low quantities of protein–iron (II) complexes retained on the column or the inlet filter. In the elution step the SPE bed is soaked with organic solvent and the iron (II) products are released to degrade the analytes. To support this theory stability experiments in haemolysed samples were conducted. Two haemolysed samples and a non-haemolysed sample (i.e. samples containing 10%, 2% or 0% haemolysate).) were used to assess the stability freshly, on ice and at ambient temperature for 2 h. The results confirmed that the stability in haemolysed plasma was indeed not different from the stability in normal plasma (Table 2). All samples for both ARS and DHA were stable when kept on ice for 2 h while none were stable at ambient temperature for 2 h. However, there was no significant difference between the mean values for normal plasma or haemolysed plasma; the haemolysed products did not increase degradation.

3.4. The influence of anticoagulant

ARS can form DHA *ex vivo* both through chemical hydrolysis and through plasma esterase mediated hydrolysis [4,14]. The SPE method was validated using the anticoagulant fluoride–oxalate to inhibit *ex vivo* plasma esterase mediated hydrolysis [20]. An experiment was conducted to assess the relative contributions of esterase mediated and chemical hydrolysis. Heparin plasma

Table 2
Stability of DHA and ARS in haemolysed plasma.

	Fresh (ng/mL) (sd)	2 h ice (ng/mL) (sd)	Recovery (% nominal)	2 h ambient (ng/mL) (sd)	Recovery (% nominal)
ARS 243 ng/mL (n = 3)					
Haemolysate 10%	251 (16.8)	255 (8.54)	105	182 (8.39)	75.0
Haemolysate 2%	264 (4.73)	262 (1.41)[a]	108	178 (2.65)	73.3
Haemolysate 0%	253 (12.7)	267 (10.0)	110	190 (7.02)	78.3
DHA 940 ng/mL (n = 3)					
Haemolysate 10%	889 (47.4)	945 (13.0)	101	548 (31.3)	58.3
Haemolysate 2%	963 (15.0)	949 (26.9)[a]	101	542 (20.5)	57.7
Haemolysate 0%	938 (35.5)	978 (3.61)	104	577 (3.51)	61.4

[a] Only two replicates due to blocked SPE well.

Table 3
Hydrolysis of ARS in heparin and fluoride–oxalate (F–Ox) plasma at 25 °C.

	1 h heparin (ng/mL) (sd)	1 h F–Ox (ng/mL) (sd)	2 h heparin (ng/mL) (sd)	2 h F–Ox (ng/mL) (sd)	3 h heparin (ng/mL) (sd)	3 h F–Ox (ng/mL) (sd)
ARS 426 ng/mL (n = 3)						
Source 1	398 (4.93)	413 (10.0)	382 (5.51)	395 (8.96)	356 (5.77)	364 (7.02)
Source 2	391 (9.50)	410 (9.61)	369 (9.54)	386 (6.43)	338 (6.08)	367 (3.61)
Source 3	381 (8.72)	412 (5.51)	352 (5.77)	386 (2.31)	311 (14.7)	357 (6.03)
Source 4	385 (2.31)	418 (3.51)	351 (3.21)	381 (7.02)	303 (8.54)	347 (6.08)
Formation of DHA						
Source 1	22.6 (0.569)	17.3 (0.321)	38.8 (0.458)	30.2 (0.800)	56.6 (2.09)	44.3 (1.30)
Source 2	23.7 (0.569)	17.9 (0.321)	41.0 (0.379)	32.0 (1.40)	59.2 (1.42)	46.8 (1.31)
Source 3	25.6 (0.400)	18.7 (0.173)	42.9 (0.907)	32.1 (0.416)	62.3 (4.18)	48.2 (1.76)
Source 4	30.6 (0.839)	25.3 (1.03)	52.4 (1.16)	43.7 (1.13)	76.9 (3.86)	63.4 (0.404)

was prepared on ice at an ARS concentration of 10,000 ng/mL. Twenty microliter of the plasma standard was added to four different sources containing either fluoride–oxalate or heparin as anticoagulants to produce samples at an ARS concentration of 426 ng/mL. These were stored at 25 °C and three replicates from each source and anticoagulant were analysed after 1, 2 and 3 h. Table 3 shows that there is large variability in stability between different sources of plasma. This is a very important finding since different sources can lead to different conclusions regarding stability. It also shows that the major contribution to hydrolysis comes from chemical hydrolysis rather than esterase mediated hydrolysis. The ratio between the fluoride–oxalate and heparin results gives an approximation of the contribution from chemical hydrolysis as fluoride–oxalate inhibits esterase mediated hydrolysis. Chemical hydrolysis accounts on average for approximately 80% and esterase mediated the remaining 20% of hydrolysis at 3 h. These figures were similar at all time points. During blood processing it was surprising to note that fluoride–oxalate seemed to produce significantly more plasma than heparin. The question arose if this would have an effect on the concentrations in clinical samples. Samples from two patients in a pharmacokinetic study were therefore taken in both heparin and fluoride–oxalate tubes. Twenty paired samples were used for the comparison and a paired t-test confirmed (p = 0.0003) that indeed fluoride–oxalate plasma gave lower concentrations than heparin plasma (i.e. due to the dilution). On average concentrations were 9.5% and 8.2% lower in fluoride–oxalate plasma than heparin plasma for ARS and DHA respectively. This is another variable important to consider when assessing results from studies using different anticoagulants.

3.5. The influence of temperature during sample processing

The poor stability of these drugs, in particularly DHA, at ambient temperature in plasma became evident during the development of the method. In contrast the drugs showed good stability when stored on ice. Standards and QC samples were prepared on ice and clinical samples were thawed and aliquoted on ice. A stability investigation for clinical samples was performed where 12 samples from a clinical study were analysed at time zero, after 1 h at ambient temperature, and after 24 h on ice (Table 4).

Table 4
Stability of ARS and DHA in clinical samples (fluoride–oxalate plasma).

Sample ID	0 h (ng/mL)		Ice 24 h (ng/mL)		Deviation from 0 h (%)		Ambient 1 h (ng/mL)		Deviation from 0 h (%)	
	DHA	ARS	DHA	ARS	DHA	ARS	DHA	ARS	DHA	ARS
PAI-003	1160	51.7	1130	51.4	−2.59	−0.58	932	42.7	−19.7	−17.4
PAI-081	69.0	214	72	212	4.35	−0.93	76.4	172	10.7	−19.6
PAI-083	750	254	688	246	−8.27	−3.15	657	211	−12.4	−16.9
PAI-110	49.8	40.6	47.5	37.9	−4.62	−6.65	44.6	34.1	−10.4	−16.0
PAI-112	462	216	454	206	−1.73	−4.63	404	179	−12.6	−17.1
PAI-160	29.8	102	25.6	85.9	−14.1	−15.8	36.7	82.1	23.2	−19.5
PAI-164	869	155	952	159	9.55	2.58	843	142	−2.99	−8.4
PAI-166	465	11.9	446	11.6	−4.09	−2.52	377	9.77	−18.9	−17.9
PAI-167	271	7.18	269	7.25	−0.74	0.97	226	6.05	−16.6	−15.7
PAI-168	225	24.2	210	22.1	−6.67	−8.68	187	19	−16.9	−21.5
PAI-169	87.4	4.77	90.2	4.72	3.20	−1.05	68.8	3.64	−21.3	−23.7
PAI-184	2290	256	2420	257	5.68	0.39	1950	214	−14.9	−16.4
Average	561	111	567	108	−1.67	−3.34	484	93	−9.39	−17.5

All samples (except ARS for one sample) were stable for 24 h on ice (i.e. within ±15% of the initial concentration determined at time zero) while many of the samples were unstable at ambient temperature for 1 h. It is clear that sample integrity for clinical samples can be improved greatly if the sample processing is conducted on ice. Many published studies, however, have performed the assay at ambient temperatures [14–19].

4. Conclusion

The findings in this investigation illustrate several potentially major pitfalls when analyzing ARS and DHA in clinical plasma samples. In contrast to previous suggestions breakdown products of haemoglobin do not increase degradation of artemisinin compounds in plasma directly. However, significant degradation of the compounds may occur during sample processing especially if organic solvents are added to the plasma samples. Not only visible haemolysis from sample collection but also malaria related haemolytic products can degrade the compounds rapidly and extensively. A 'soft' extraction method such as SPE where the compounds can be extracted from the plasma without disturbance of the protein complexes will eliminate problems arising from haemolytic degradation. It is therefore essential that the internal standard is added without disturbing the protein complexes i.e. dissolved in a plasma mixture rather than in organic solvent. Both ARS and DHA are thermo-labile compounds but stability and sample integrity can be maintained if the analytical process is conducted on ice. The anticoagulant fluoride–oxalate minimizes *ex vivo* hydrolysis of ARS into DHA but also increases plasma–water through red cell shrinkage. These various pitfalls have undoubtedly contributed to the large inter-subject variation in plasma concentration profiles and derived pharmacokinetic parameters for these important antimalarial drugs.

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