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High-performance liquid chromatographic method for determination of amodiaquine, chloroquine and their monodesethyl metabolites in biological samples

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Abstract

A high-performance liquid chromatographic method for determination of amodiaquine (AQ), desethylamodiaquine (DAQ), chloroquine (CQ) and desethylchloroquine (DCQ) in human whole blood, plasma and urine is reported. 4-(4-Dimethylamino-1-methylbutylamino)-7-chloroquinoline was used as internal standard. The drugs and the internal standard were extracted into di-isopropyl ether as bases and then re-extracted into an acidic aqueous phase with 0.1 *M* phosphate buffer at pH 4.0 for AQ samples and at pH 2.5 for CQ filter paper samples. A C_{18} column was used and the mobile phase consisted of methanol–phosphate buffer (0.1 *M*, pH 3)–perchloric acid (250: 747.5:2.5, v/v). The absorbance of the drugs was monitored at 333 nm and no endogenous compound interfered at this wavelength. The limit of quantification in whole blood, plasma and urine was 100 n*M* for AQ and DAQ (sample size 100 μl) as well as for CQ and DCQ in blood samples dried on filter paper. For 1000 μl AQ and DAQ samples, the limit of quantification was 10 n*M* in all three biological fluids. The within-assay and between-assay coefficients of variations were always <10% at the limits of quantification. Plasma should be preferred for the determination of AQ and DAQ since use of whole blood may be associated with stability problems.

Keywords: Amodiaquine; Chloroquine; Desethylamodiaquine; Desethylchloroquine

1. Introduction

Amodiaquine (AQ) and chloroquine (CQ) are 4-aminoquinoline antimalarial drugs.

AQ was formerly used as a prophylactic agent against malaria but lost its importance due to the risk of development of agranulocytosis and hepatic disorders [1]. For the same reason the usefulness of AQ for treatment of malaria remained limited. However, recently AQ has received renewed interest since its safety and efficacy have been demonstrated in several clinical trials [2,3].

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Optimal dosage regimens are needed that in turn require reliable data on interindividual variability in the pharmacokinetics of AQ. To be able to conduct such pharmacokinetic studies, methods for quantification of AQ and its active metabolites in biological fluids are necessary.

Since CQ is widely used in malaria endemic areas, this drug and its metabolites will be present in the blood of many of the individuals who will be included in AQ studies. A method which will enable a simultaneous determination of CQ and AQ samples is therefore important.

Methods for assay of AQ and CQ have been published [4–7,9], including a straight-phase CQ method developed in our laboratory [10], but none of these methods was completely satisfactory for our purpose.

During the preparation of this manuscript, Bergqvist et al. published an automated solid-phase extraction HPLC method for determination of AQ, CQ and metabolites in capillary blood [14]. We needed a simple, cost-effective and sensitive method in order to enable us to analyze small samples of blood and to quantify AQ and CQ and their active metabolites desethylamodiaquine (DAQ) and desethylchloroquine (DCQ) after single oral doses of these drugs using a reversed-phase chromatographic system with ultraviolet detection. The method we hereby describe is based partly on the previously published methods [6,8–10] and involves a simple two-step extraction procedure. It is simple and cost effective and can be applied in pharmacokinetic and epidemiological studies and for therapeutic monitoring of CQ and AQ in areas where malaria is highly endemic and technical facilities are limited.

2. Materials and methods

2.1. Chemicals

AQ was obtained from Sigma (Stockholm, Sweden). DAQ and bisdesethylamodiaquine (BDAQ) were gifts from Assoc. Professor Yngve Bergqvist, Dalarna University College, Borlänge, Sweden. CQ was from Pharmacia, Stockholm, Sweden and DCQ was obtained from Sterling Winthrop, New York, USA. The internal standard (I.S.) 4-(4-dimethylamino-1-methylbutylamino)-7-chloroquinoline was synthesized as previously [9]. Solvents and chemicals (HPLC or analytical grade, as required) were purchased from Merck (Darmstadt, Germany). Stock solutions were made by dissolving AQ, DAQ, CQ and DCQ in 0.01 *M* hydrochloric acid. The I.S. was dissolved in diethylamine (DEA) 0.5% in water.

2.2. Instrumentation

The method was devised using a Gilson model 231 sample injector with a 200-μl loop (Villiers le Bel, France), a Gilson model 118 UV–Vis absorbance detector and a Kontron model 422 high-pressure pump (Milan, Italy).

A reversed-phase column, Zorbax® SB C_{18}, 75× 4.6 mm I.D, 3.5 μm (ChromTech, Hägersten, Sweden) was used. The mobile phase consisted of methanol–phosphate buffer (0.1 *M*, pH 3)–perchloric acid (250:747.5:2.5, v/v). Elution was carried out at room temperature using a flow-rate of 1.5 ml/min. Detection was achieved at 333 nm.

2.3. Samples

For the development and evaluation of the method, drug-free whole blood, plasma and urine were spiked with AQ and its metabolite DAQ. Samples from a healthy volunteer who took a single oral dose AQ (600 mg AQ base) were also used. Urine samples were diluted 10–100 times before extraction while the blood and plasma samples were extracted directly.

Venous blood samples (2×10 ml) were obtained from a volunteer and collected in heparinised plastic tubes before and at 1, 1.5, 2, 2.5, 3, 6, 8, 24, 48, 72 and 168 h after a single oral dose of 600 mg AQ base. The first (10 ml) collected blood samples were immediately centrifuged at 3500 *g* for 15 min to get plasma. The remaining blood samples (10 ml) and the obtained plasma samples were stored at −70 °C until assay.

Urine samples were collected before and 0–4, 4–8, 8–12 and 12–24 h after drug intake and then at 24-h intervals for 3 weeks. The urine volumes from each collection were measured and aliquots of 10 ml were kept at −70 °C until assay.

CQ samples were prepared by applying 100-μl

aliquots of whole blood spiked with CQ and DCQ onto filter paper strips (Whatman chromatography paper, Kebo, Spånga, Sweden).

2.4. Analytical procedure for AQ and DAQ

To 100-μl samples (whole blood, plasma and diluted urine) 900 μl water and 50 μl of 1.6 μ*M* I.S. were added; thereafter, 2000 μl of carbonate buffer, pH 9.5, were added. The samples were extracted using 7 ml of diisopropyl ether on a reciprocal shaker for 20 min. After centrifugation for 10 min at 3500 *g*, the organic (upper) phase was transferred to a new tube and extracted as above for 10 min with 150 μl of 0.1 *M* phosphate buffer (pH 4). The phases were separated by centrifugation as above and the organic phase was removed by aspiration. Into the chromatograph, 100–120 μl were injected.

To 1000-μl samples, 1000 μl water, 50 μl 1.6 μ*M* I.S. (in 0.5%DEA) were added; thereafter, 2000 μl of carbonate buffer, pH 9.5, were added. The samples were extracted and injected into the chromatograph as described above.

2.5. Analysis of CQ and DCQ in samples dried on filter paper

Analysis of CQ and DCQ in blood samples dried on filter paper was carried out in a slightly different way. Filter paper containing 100 μl of dried blood spots was cut into pieces and put into centrifuge tubes. A volume of 500 μl 0.5% DEA in water, 50 μl of 1.6 μ*M* I.S. (for samples with concentrations ranging between 50 and 400 n*M*), or 75 μl of 3.2 μ*M* I.S. (for samples having concentrations of between 500 and 4000 n*M*), and 2000 μl of 1 *M* KOH were added. The mixture was sonicated for 15 min. A volume of 7 ml of diisopropyl ether was added and extraction was made as above except that phosphate buffer, with a pH 2.5 instead of pH 4, was used for back extraction.

2.6. Standard curves

Standard curves were prepared by adding known amounts of AQ, DAQ (0–40 μl) standard solution containing AQ and DAQ as well as 50 μl of I.S. to blank whole blood, plasma and urine. The range of the standard curves used for validation of the method was 2.5–40 n*M* for 10 n*M* samples, 50–400 n*M* for 100 n*M* samples and 0.5–4 μ*M* for 1 μ*M* samples. The resulting peak area ratios between the internal standard and the analytes were plotted versus the concentrations.

Standard curves for determination of CQ and DCQ in dried blood spots were prepared by adding known amounts of CQ, DCQ and I.S. to 0.5% DEA in water. The extraction procedure was carried out as described. The resulting peak area ratios were plotted versus the concentrations.

2.7. Extraction recovery

To check the extraction recovery, standard AQ and DAQ were added to whole blood, plasma and urine. CQ and DCQ were added to whole blood and 100-μl aliquots were dried on filter paper. The concentrations are given in Table 1. Samples were extracted according to the above procedure, and the peak areas were compared with those of directly injected standards.

2.8. Imprecision and accuracy

Known amounts of AQ and DAQ were added to blank whole blood, plasma, and urine. CQ and DCQ were added to whole blood and 100-μl aliquots were dried on filter paper. The concentrations are given in

Table 1
Extraction recovery for AQ and DAQ

Sample size and conc.		Recovery [(%)±SD]		
		I.S.	AQ	DAQ
Blood 100 μl (n=6)	100 n*M*	83±2	103±2	78±7
Blood 100 μl (n=10)	1 000 n*M*	92±5	84±6	68±9
Blood 1000 μl (n=10)	10 n*M*	73±9	82±9	71±7
Plasma 100 μl (n=8)	100 n*M*	88±6	97±4	75±6
Plasma 100 μl (n=8)	1 000 n*M*	84±6	87±2	77±6
Plasma 1000 μl (n=9)	10 n*M*	88±5	70±8	81±6
Urine 100 μl (n=9)	100 n*M*	93±6	94±5	73±9
Urine 100 μl (n=9)	1 000 n*M*	97±7	91±7	73±8

Table 2
Accuracy, within- and between-assay imprecision ($n=7$) for AQ and DAQ

Sample size and conc.		Found[a] [(n*M*)±SD]		Within-assay C.V. (%)		Between-assay C.V. (%)	
		AQ	DAQ	AQ	DAQ	AQ	DAQ
Blood 100 μl	100 n*M*	97±7	98±4	7	4	9	5
Blood 100 μl	1000 n*M*	1023±31	1005±20	3	2	4	5
Blood 1000 μl	10 n*M*	11±1	10.5±1	8	8	10	9
Plasma 100 μl	100 n*M*	102±4	98±7	4	7	5	4
Plasma 100 μl	1000 n*M*	984±30	990±30	3	3	2	3
Plasma 1000 μl	10 n*M*	10±1	10±1	4	5	8	5
Urine 100 μl	100 n*M*	102±4	108±8	4	7	9	6
Urine 100 μl	1000 *M*	980±20	972±19	2	2	6	5

[a] Mean.

Table 2. The samples were extracted and analyzed as described above both in one series (within assay imprecision and accuracy) and on different occasions (between assay imprecision and accuracy). The standard deviation and coefficient of variations were calculated.

2.9. Interference

Drug-free whole blood samples were separately spiked with aspirin, paracetamol, sulfamethoxazole, sulfadoxine, sulfadimidine, pyrimethamine, chlorpheniramine, promethazine, quinine and trimethoprim to achieve therapeutic concentrations. Samples from patients treated with quinine were also tested. The drugs were extracted according to the method described above. The presence of peaks was monitored after injection of 100–120 μl of aqueous solution.

2.10. Stability

The stability of AQ and DAQ in whole blood, plasma and urine samples was studied at 37, 20, 4 and −20 °C for up to 30 days. The samples were also studied in blood mixed with ascorbic acid or sodium thiosulfate at different concentrations and dried. Concentration changes in plasma and urine samples stored at −20 °C were further followed for 16 months. The stability of CQ blood samples dried on filter paper has been reported previously [10].

3. Results

3.1. Evaluation of the analytical procedure

Typical chromatograms of AQ, DAQ, BDAQ, CQ, DCQ and I.S. from spiked plasma, are shown in Fig. 1A. The chromatograms of extracts of plasma samples of the healthy volunteer at 6 h after an oral dose of 600 mg of AQ base are shown in Fig. 1D. The retention times corresponded to those obtained in spiked samples.

Excellent linearity in plasma, blood and urine samples was seen in response for AQ, DAQ, CQ and DCQ over the concentration range studied. The correlation coefficients were between 0.991 and 0.999 in all runs (see Fig. 2A–C).

Extraction recoveries are given in Table 1. The within-day and day-to-day coefficients of variation (C.V.) using spiked AQ, CQ and their metabolites are shown in Tables 2 and 3. The precision of the method was satisfactory over the concentration range studied (Tables 2 and 3). Accuracy studies demonstrated good accuracy for AQ, DAQ, CQ and DCQ (cf. Tables 2 and 3). Limits of quantification were 100 and 10 n*M* for all analytes using 100 and 1000 μl biological samples, respectively, with an intra- and inter-assay variation of <10%.

3.2. Interference

The drugs tested were selected due to their importance in treating different diseases in tropical regions. There was interference of AQ with quinine

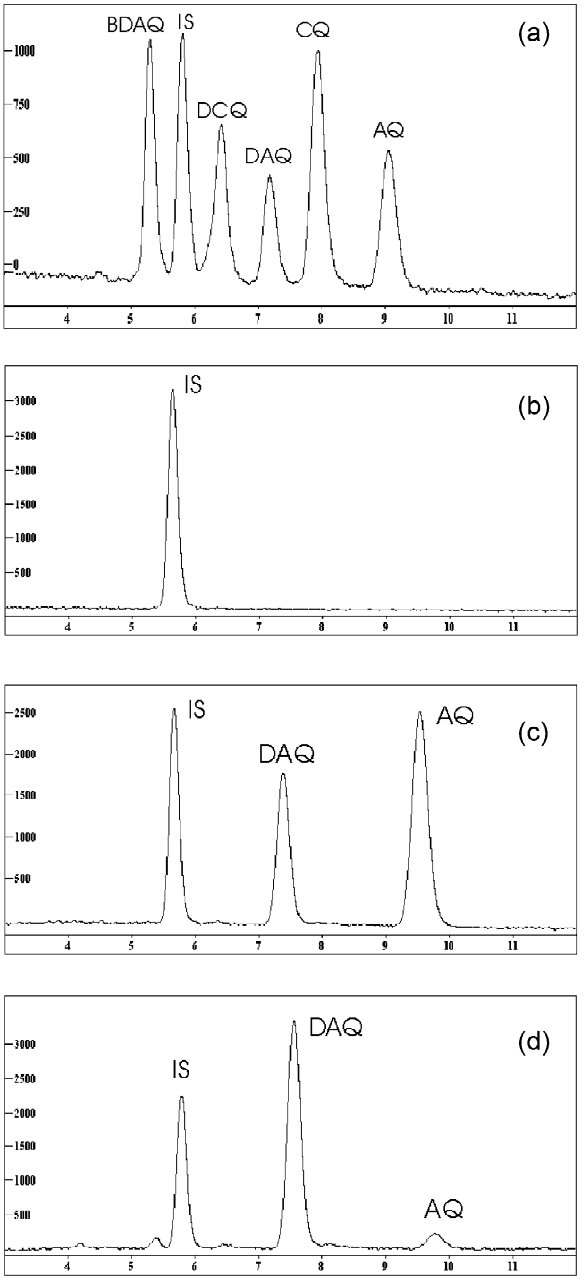

Fig. 1. (A) Separation of BDAQ, I.S., DCQ, DAQ, CQ and AQ in a blood sample. (B) Blank plasma (spiked with internal standard) from a volunteer. (C) Separation of I.S., DAQ and AQ in spiked plasma. (D) Plasma sample from a volunteer 6 h after an oral intake of 600 mg of AQ.

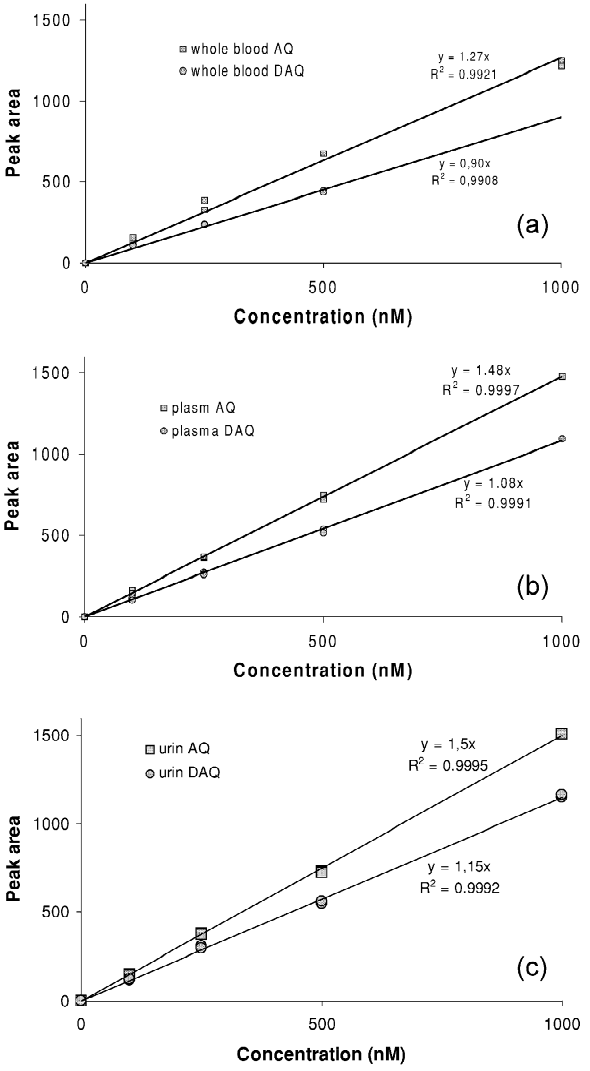

Fig. 2. Calibrations curves of AQ and DAQ in whole blood, plasma and urine. (A) Calibration curves of AQ and DAQ in whole blood (B) Calibration curves of AQ and DAQ in plasma. (C) Calibration curves of AQ and DAQ in urine.

but the other drugs did not show any interference. Endogenous substances did not show any interference.

3.3. Stability

AQ and DAQ were not stable in spiked blood samples and blood samples from the volunteer at any of the temperatures studied (Fig. 3A,B). Whole blood

Table 3
Accuracy, within-assay imprecision and extraction recovery for CQ and DCQ in blood samples dried on filter paper ($n=10$)

Samples 100 μl	Added (n*M*)	Found[a] [(n*M*)±SD]	Within-assay C.V. (%)	Recovery (%)±SD)
CQ	100	96±2	2	84±6
	200	198±8	4	72±8
	2000	2002±100	5	92±4
DCQ	50	49±2	5	104±2
	100	99±2	2	101±5
	1000	990±40	4	105±6

[a] Mean.

samples may be kept for a maximum of 1 week in the refrigerator. AQ and DAQ concentrations showed a slow decline in plasma and urine samples stored at −20 °C and we recommend that samples should not be stored at this temperature for more than 3 months (see Fig. 3B).

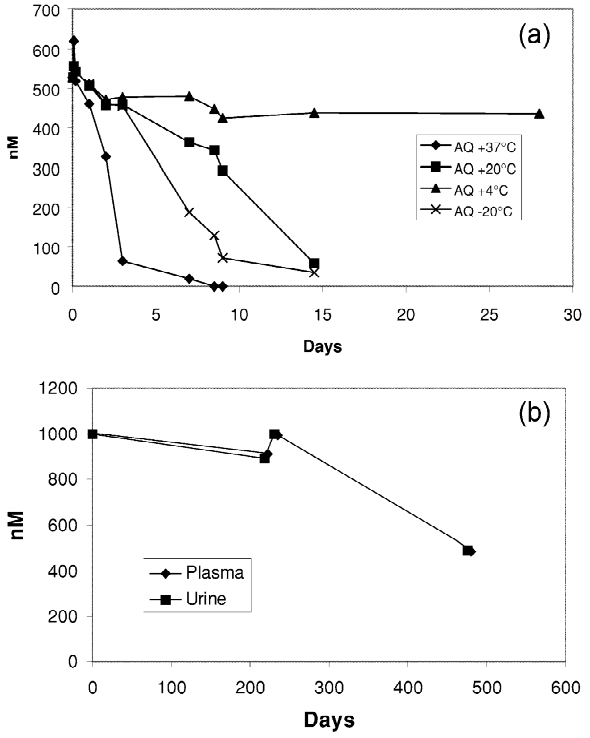

Fig. 3. Stability of AQ and DAQ in whole blood, plasma and urine. (A) Stability of AQ in whole blood*. (B) Stability of AQ in plasma and urine at −20 °C*. *The stability of DAQ in whole blood, plasma and urine does not differ significantly from that of AQ.

Standards and stock solutions were always stored at −70 °C and were found to remain stable for more than a year.

3.4. Pharmacokinetics

The pharmacokinetic parameters of AQ and DAQ after an oral intake of AQ 600 mg base by the healthy volunteer are shown in Table 4. The parameters were calculated using whole blood data. The average whole blood to plasma ratio in this individual was 1.7±0.4 (mean±SD) for AQ and 2.3±0.4 for DAQ.

4. Discussion

The chromatographic system used affords complete separation of AQ, CQ and their active metabolites. Despite the good chromatographic selectivity, simultaneous determination of AQ and CQ is not possible since different pH has to be used for the liquid–liquid extraction.

Within-day and day-to-day variations were always less than 10%, demonstrating that the precision of the method is good under these conditions. Assays of spiked samples always gave good results (see Tables 2 and 3) confirming good accuracy of the method. The peaks present in the chromatograms of samples from the healthy subject correspond to those observed in the spiked samples.

Quinine interfered with AQ peaks. In studies involving determinations of AQ, only volunteers without quinine intake prior to the study should be recruited. Even though there is no separation be-

Table 4
Pharmacokinetic parameters of AQ and DAQ in whole blood after an oral intake of a single 600-mg dose of AQ base by a healthy volunteer

	T_{max} (h)	C_{max} (n*M*)	$T_{1/2}$ (h)	AUC (nmol·h l^{-1})		
				$0-t$[a]	$t\rightarrow\infty$	Total
AQ	1.0	31.4	14.5	168	417	585
DAQ	2.0	2507	149	72 954	43 805	116 759

[a] $(0-t)$=AUC 0–∞ and 0–168 h for AQ and DAQ, respectively.

tween AQ and quinine an interference by quinine can in most cases be revealed by inspection of the chromatograms since a peak due to dihydroquinine is present.

The interference cannot be avoided even by using fluorimetric since only quinine but not AQ possesses strong fluorescence. Rarely used antimalarial drugs like artemisinin and mefloquine were not tested for interference. Furthermore artemisinin has no UV absorption characteristics and therefore cannot be detected in our system.

The stability of AQ and DAQ in whole blood is poor even at −20 °C. The concentration decline was actually found to be more rapid at this temperature than at +4 °C (Fig. 3A). Repeated freezing and thawing did not markedly affect stability. We have no explanation for this unusual observation. Whole blood samples can be kept in a refrigerator for 1 week, but we recommend that plasma is separated and frozen as soon as possible. Plasma should thus be used for analysis of AQ and DAQ in studies in areas where −70 °C facilities are not accessible. We recommend that plasma samples should not be stored for more than 3 months at −20 °C.

We attempted to dry 100-μl EDTA and heparin whole blood samples spiked with AQ and DAQ on filter paper as reported in the CQ, quinine and sulfadoxine methods [10–12]. No peaks were obtained after extraction of the dried blood samples. However, we could recover these substances when we extracted them from plasma samples and standard solution dried on filter paper and in plastic tubes. We suspect that AQ and DAQ undergo oxidation during the drying process in the presence of blood or there could be a complex formation of these compounds with blood constituents. Attempts to use antioxidants like ascorbic acid and thiols to protect AQ and DAQ from the suspected oxidation in the process of drying the blood did not give positive results.

During the preparation of this manuscript, Bergqvist et al. published an automated solid-phase extraction method for determination of AQ and CQ from blood samples dried on filter paper [14]. However the method remains too expensive to be adapted in analytical laboratories in countries where malaria is endemic. A simple and cost-effective method which can be set up in analytical laboratories in developing world is needed. More studies should be carried out to achieve a simple extraction of AQ from dried whole blood samples.

We aimed to use small (100 μl) blood samples since the method is intended to be applied in areas where malaria is predominant especially in children who are likely to be anemic due to malnutrition. It is also more convenient to collect small blood samples obtained by finger prick. By using 100-μl samples we successfully studied the pharmacokinetic parameters of DAQ after an oral intake of 600 mg of AQ base. However, it was impossible to assay AQ, which is rapidly metabolized to DAQ [4,5], for more than 4 h after AQ intake by using this sample size. In an on-going interaction study we want to follow the concentrations of AQ and DAQ for up to 8 and 216 h, respectively. We therefore validated the method also for 1000 μl whole blood and plasma samples. Using this sample volume we were able to quantify AQ for up to 8 h after an oral intake of 600 AQ.

BDAQ could be detected using blood samples of 100 μl (Fig. 2C), but no attempts to quantify this compound were made. DAQ was observed shortly after intake of tablets. In clinical studies in which only DAQ is targeted, 100 μl blood and urine samples can be successfully used to assay this compound. Assessment of the pharmacokinetic parameters of AQ after a single oral intake of 600 mg of AQ base requires use of 1000-μl samples.

The pilot analysis of samples from the volunteer after an oral intake of AQ has indicated that the

concentrations chosen for the evaluation of the method are relevant for the planned studies.

Our method was further modified to permit the assay of CQ blood samples dried on filter paper, since in areas of high malaria endemicity, medication with CQ is highly expected [13]. A filter paper method has previously been successfully used in assaying CQ [10]. The new method was found to exhibit good accuracy and reproducibility with respect to CQ and DCQ as shown in Table 3.

The ability of our method to detect AQ and BDAQ and to be able to quantify AQ even 8 h after a single oral dose suggests that our method is more sensitive than the methods previously published [4–7]. The method can be used in different pharmacokinetic and epidemiological studies involving CQ and AQ as well as therapeutic monitoring of these drugs in malaria patients in areas where malaria is highly endemic with high rate of self-medication.

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