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Short communication

A field-adapted HPLC method for determination of amodiaquine and its metabolite in whole blood dried on filter paper

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Abstract

A reversed-phase high performance liquid chromatographic method was developed and validated for the quantitative determination of amodiaquine (AQ) and its metabolite desethylamodiaquine (DAQ) in whole blood collected on filter paper. The structure analogue 4-(4-dimethylamino-1-methylbutylamino)-7-chloroquinoline was used as internal standard. Upon collection, blood was added to 10% phosphoric acid in a 1:1 ratio and then spotted onto filter paper. The samples were alkalinized (pH ≈ 9.2) with potassium hydroxide at the time of assay and the compounds were extracted together with internal standard into di-isopropyl ether and then re-extracted into an aqueous phase with 0.1 M phosphate buffer at pH 4. The chromatographic analysis was performed using an Agilent Technologies ChemStation LC System. The absorbance of the compounds was monitored at 333 nm. Mean extraction recoveries of AQ and DAQ were 49 and 48%, respectively. Intra-day and inter-day coefficients of variation were <10.5%. The limit of quantification was 50 nM for both compounds (sample size 100 μl). Both AQ and DAQ that were previously reported to be unstable have been stored on filter paper for at least 19 weeks. The method was applied on samples from healthy volunteers.

Keywords: Amodiaquine; Desethylamodiaquine; Filter paper

1. Introduction

Malaria, caused by *Plasmodium falciparum* parasite, transmitted by anopheles mosquito, is one of the most devastating tropical diseases. Every year, between 1.5 and 2.5 million deaths occur due to the disease, most of them being children in sub-Saharan Africa [1]. Malaria accounts for about 25% of all the child mortality in Africa [2]. Despite considerable efforts to eradicate or control it, malaria continues to be a major threat to human health. In the absence of effective and practical preventive measures, the only available options for reducing the morbidity and mortality of malaria are chemoprophylaxis and chemotherapy [3].

In Africa, resistance to chloroquine (CQ), once the mainstay of antimalarial therapy, has spread across the continent and has been associated with malaria-related morbidity and mortality [4]. Many countries are now confronted with the dilemma of shifting drug policies for uncomplicated falciparum malaria from CQ, though the best replacement for first-line therapy is still unclear. Combinations of amodiaquine (AQ) and sulfadoxine-pyrimethamine (SP) or artesunate have been reported to be significantly efficacious. Each regimen could be an appropriate alternative for treatment of uncomplicated malaria for regions of Africa where resistance to the individual drugs remains low [5].

Amodiaquine is chemically related to CQ and its therapeutic use was abandoned due to problematic dosage and severe adverse effects that were associated with its use [6]. Originally it was administered as monotherapy, but its use in combination therapy with artemisinin derivatives or with SP has generated a renewed interest. After oral administration, AQ undergoes rapid and extensive hepatic metabolism by a Cytochrome P450 2C8 *iso*-enzyme [7] to desethylamodiaquine (DAQ), and AQ usually

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becomes undetectable within a few hours. Antimalarial activity is therefore mainly due DAQ making it the most important entity to quantify [8,9].

To be able to perform clinical studies involving AQ under field conditions, methods for quantification of AQ and DAQ in whole blood are necessary. Most of the available methods require plasma or serum for drug analysis [10–15], making them inappropriate for field studies. To the best of our knowledge, only two methods [16,17] have been reported for determination of AQ in whole blood spotted onto filter paper. The authors however, indicate that the drug is very unstable on filter paper at room temperature and stability is only achieved when the filter paper strips are stored in a microscope slide box at 4 °C [16] or when the filter paper spots are stored at –86 °C [17]. This makes the two methods unsuitable for field studies, especially in resource limited settings. In an earlier method published in our laboratory by Minzi et al. [15] for determination of amodiaquine and its metabolite in whole blood, plasma and urine, it was not possible to store both analytes on filter paper.

The aim of the present study therefore, was to develop and validate an analytical procedure suitable for field studies for the determination of AQ and DAQ with HPLC using sampling of whole blood on filter paper. We have however, used similar chromatographic conditions to those published earlier in our laboratory [15].

2. Materials and methods

2.1. Chemicals

AQ, 4-[(7-chloro-4-quinolinyl)amino]-2-[(di-ethylamino) methyl]phenol), dichloride; dihydrate was obtained from Sigma Chemical Company, USA and DAQ was a gift from Dr. Yngve Bergqvist, Dalarna University College, Borlänge, Sweden. The internal standard (I.S.), 4-(4-dimethylamino-1-methylbutylamino)-7-chloroquinoline was synthesized as previously described [14]. Other chemicals (HPLC or analytical grade) were obtained from Merck GmbH (Darmstadt, Germany). Stock solutions of AQ and DAQ were prepared in 0.01 M hydrochloric acid and stored at −70 °C at all times. The I.S. (0.6 μM) was dissolved in 0.5% diethyl amine and kept refrigerated at 4 °C.

2.2. Instrumentation

The chromatographic analysis was carried out using an Agilent Technologies ChemStation LC System (1100 series, Brookside Pkwy Alpharetta, USA) equipped with a photometric detector, a standard flow cell, a binary pump with a degasser and an auto injector. The mobile phase consisted of a methanol-phosphate buffer (0.1 M, pH 2.7) perchloric acid (250:747.5:2.5, v/v). The absorbance was monitored at 333 nm and elution was carried out at room temperature using a flow rate of 1.5 ml per min. A reverse phase column, Zorbax® SB C_{18}, 75 mm × 4.6 mm, particle size 3.5 μm (ChromTech, Hägersten, Sweden) was used.

2.3. Samples

For the development of the method, drug-free venous whole blood from healthy volunteers and phosphoric acid were used. Phosphoric acid (10%, v/v) was spiked with AQ and DAQ using a fresh stock solution to achieve 200 and 1000 nM, respectively. Aliquots of collected blood (100 μl) were mixed with equal amounts of phosphoric acid and 100 μl was spotted onto a filter paper and allowed to dry for 3–4 days at room temperature. For validation, blood from three healthy volunteers was collected before and after oral administration of 600 mg of AQ at 0, 1, 2, 3, 4, 6 and 24 h. The blood also treated as described above. Calibrators and control samples were prepared by adding drug-free whole blood to phosphoric acid spiked to the required concentration of AQ and DAQ and treated it as above. All the filter paper samples were stored at room temperature until assay.

2.4. Analytical procedure

Filter paper containing dried blood spots was cut into 4–6 small pieces and put into 12 ml polypropylene test tubes. Subsequently, 150 μl of I.S. solution and 1 ml of water were added and the contents were sonicated for 15 min. Sodium carbonate buffer (2 ml, 0.2 M, pH 9.7), KOH (120 μl, 1 M) and 8 ml of di-isopropylether were added. Final pH of the water phase was between 9.17 and 9.24. The samples were shaken for 20 min and centrifuged for 10 min at 3500 × *g*. The organic (upper) phase was transferred to a new polypropylene test tube and back-extracted for 10 min with 150 μl of sodium dihydrogen phosphate buffer (0.1 M, pH 4). The phases were separated by centrifugation for 10 min and the organic phase was removed by aspiration. The water phase (130 μl) was injected into the chromatograph.

2.5. Method validation

2.5.1. Recovery, linearity, accuracy and precision

The recovery of AQ and DAQ after application and storage of blood onto filter paper and during extraction was documented using 6 filter paper spots prepared on the same day. The papers were processed as described and the peak areas were compared with those from a directly injected reference solution prepared in 0.01 M hydrochloric acid.

The linearity of the method was evaluated by use of calibrators in the range 50–2000 nM of both AQ and DAQ. The curve was a plot of peak area ratio of each analyte and I.S. against concentration. The regression equation with the slope, intercept and correlation coefficient (r^2) was generated automatically with the ChemStation software. The accuracy and precision were documented using prepared controls at three different concentrations of AQ and DAQ in blood dried on filter paper. The controls were assayed as described before both in one series (intra-day) and on different occasions (inter-day). The variability in the measurement of the analyte was expressed as a coefficient of variation (C.V.).

Table 1
Accuracy, intra- and inter-assay imprecision for AQ and DAQ

Analyte	Nominal conc. (nM)	Conc. measured (nM) (mean ± S.D.)	C.V. (%)		
			Intra-day	Inter-day	*n*
AQ	50	38 ± 2	3.8	4.4	6
	100	93 ± 4	4.4	6.8	10
	250	231 ± 17	7.2	4.0	8
DAQ	250	248 ± 19	7.7	10.5	8
	500	503 ± 30	6.0	2.8	10
	1000	984 ± 67	6.8	6.3	8

2.5.2. Method application in healthy volunteers

In order to test the applicability of the method for the analysis of AQ and DAQ, three adult healthy volunteers were used. One volunteer was female and two were males. Each was given three tablets equivalent to 600 mg of amodiaquine hydrochloride base after over-night fasting. Whole blood samples were collected at 0 h and at 1, 2, 3, 4, 6 and 24 h after single oral administration of AQ. Aliquots of blood were treated with phosphoric acid as described.

3. Results and discussion

AQ has regained renewed interest as an effective antimalarial and methods for monitoring drug concentrations are needed. The available analytical methods for its determination comprise of plasma, urine and whole blood samples, where the drug has been reported to be unstable at room temperature [15]. Drying of blood onto filter paper is known to reduce the chances of infection by viruses and is also good for storage and transportation purposes [18,19].

Apparently, only two methods have been reported for determination of AQ on filter paper [16,17]. Unfortunately, they are not suitable for resource-limited settings because of immediate need of refrigeration of samples as reported for the two methods. In this work, effort has been made to stabilize the drug and its metabolite with phosphoric acid and store them onto filter paper at room temperature (≈25 °C) for at least 4 months. With our method earlier reported problems that the drug was possibly being oxidized in air [15] and could not be stored on filter paper have been solved.

3.1. Chromatography

Chromatographic conditions used were similar to those published earlier from the same laboratory [15]. The three substances chromatographed as symmetrical peaks as before, well separated from the front. Retention times were about 3.7, 4.5 and 6.0 min for I.S., DAQ and AQ, respectively.

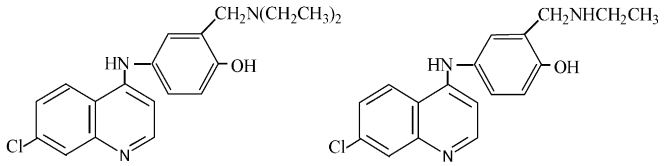
3.2. Method validation

With our method, the recovery of AQ and DAQ was 49% and 48%, respectively. However, the low recovery was acceptable since it does not substantially affect the performance of the method as observed from both the accuracy and precision (Table 1). The recovery value includes both extractions of analytes from filter paper into organic solvent and back to phosphate buffer. A lower recovery for AQ (47%) and DAQ (40%) from filter paper has been previously reported [17]. With our method, a higher recovery was sacrificed in order to achieve simultaneous and comparable extraction efficiency of both analytes. The recovery of either AQ or DAQ could be improved well above the recommended minimum of 50% [20] by choosing a pH above 9.24 or one below 9.17 for AQ and DAQ, respectively. This observation is explainable by the p*K*a values of the two compounds (Fig. 1). A volume of 120 μl of potassium hydroxide added to the filter paper samples during the extraction process seemed to give the optimum pH for simultaneous extraction of AQ and DAQ.

The linearity of the method as measured by the correlation coefficient of inter-assay linear regression curves (r^2) was better than 0.99 in all cases in the measured range of 50 and 2000 nM for both AQ and DAQ. The (mean ± S.D.) regression equation for the calibration curves prepared from calibrators was $y = (0.00186 \pm 0.00143)x + (0.03348 \pm 0.00829)$, $r^2 = (0.9948 \pm 0.0037)$ ($n = 4$) for AQ and $y = (0.00103 \pm 0.00133)\ x + (0.0154 \pm 0.00380)$, $r^2 = (0.9981 \pm 0.00102)$ ($n = 4$) for DAQ (y = area ratio of AQ or DAQ to I.S., x = concentration of AQ or DAQ in nmol l^{-1}). The limit of quantification was 50 nM for both AQ and DAQ.

The validated method was used to determine whole blood concentrations of both AQ and DAQ in healthy volunteers after oral administration of three tablets equivalent to 600 mg of amodiaquine hydrochloride base to three adult healthy volunteers. The data were used to obtain both the concentration-time profiles (Fig. 2) and the pharmacokinetic parameters (Table 2) of the analytes in whole blood spotted onto filter paper. Only one volunteer had quantifiable amounts of AQ.

The parameters compare well with those reported in literature [11,12,21], for both AQ and DAQ, an indication that the devel-



Amodiaquine, pKa = 8.72 Desethylamodiaquine, pKa = 8.66

Fig. 1. Structures of amodiaquine and desethylamodiaquine.

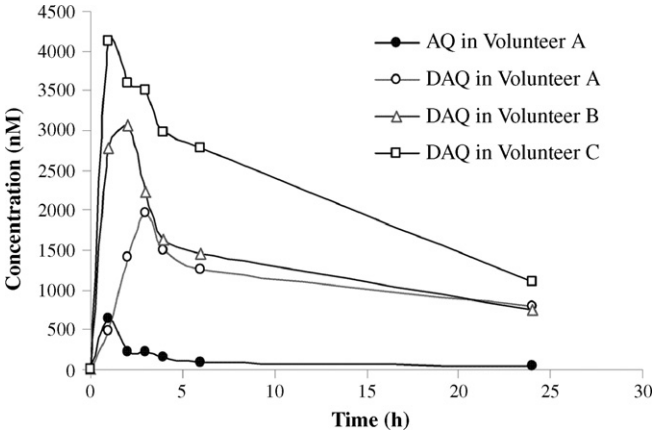


Fig. 2. Whole blood concentration-time profiles of AQ and DAQ in three healthy volunteers (0–24 h) following administration of a single oral dose of 600 mg of AQ.

Table 2
Pharmacokinetic parameters of AQ and DAQ in three adult Ugandan healthy volunteers following a single oral dose of 600 mg of AQ base

Analyte	T_{max} (h)	C_{max} (nmol/l)	$AUC_{0-24\,h}$ (nmol h/l)	$T_{1/2}$ (h)
AQ ($n=1$)	1	632	2494	18.65
DAQ ($n=3$)	2 ± 1	3055 ± 1080	36935 ± 14434	19 ± 5

Data are mean values ± standard deviation (S.D.) for DAQ.

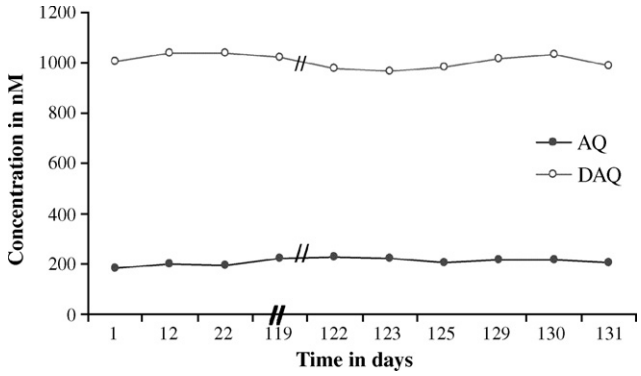


Fig. 3. Stability of AQ and DAQ in whole blood stored on filter paper for 131 days.

oped method is reliable and can be applied in pharmacokinetic studies involving AQ.

3.3. Stability of AQ and DAQ on filter paper

The stability of the analytes at 200 nM, AQ and 1000 nM, DAQ was investigated by analyzing the spots over a period of 4 months. This was done at storage conditions to be used for study samples as recommended [22]. Some of the filter paper spots were stored in Uganda at room temperature (≈25 °C) for 2 months whereas others were stored in Sweden, at room temperature (≈21 °C) before they were analyzed. No significant decrease in concentration was observed during storage for both analytes at all conditions for at least 131 days (Fig. 3).

In conclusion, an HPLC method has been developed for the simultaneous determination of AQ and its major metabolite DAQ in blood applied and stored onto filter paper. The method is simple, cost-effective and involves stabilizing the drugs in blood and spotting it onto filter paper, making it good field studies in resource limited settings. The assay requires a small sample volume (100 μl), which is important in clinical studies especially those involving children. Since AQ combination therapy has been recommended as an alternative for malaria chemotherapy, the method will enable field studies involving AQ.

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