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# Practical HPLC methods for the quantitative determination of common antimalarials in Africa

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**Abstract**

This article describes high-performance liquid chromatographic assays for the quantification of sulfadoxine (SDX), pyrimethamine (PYM), chloroquine (CQ), amodiaquine (AQ) and desethylamodiaquine (AQM) from whole blood. All four assays were set up and validated in Malawi using a common high-performance liquid chromatography platform and column and involved the use of simple mobile phase and extraction reagents. Calibration curves were linear ($r^2 > 0.95$) in the ranges 5–100 μg/ml, 50–1000, 150–1500, 100–1000 and 100–1000 ng/ml for SDX, PYM, CQ, AQ and AQM, respectively. Intra-assay and inter-assay coefficients of variation were <15% at 3 points spanning the concentration range and <20% at the lower limit of quantification. The assays were specific with no interference from the other antimalarials described in this report. All four assays use liquid–liquid extraction, reversed-phase chromatography and UV detection and require between 50 and 200 μl of blood. Because the assays share common instruments and reagents, they are cost-efficient and could be used to optimise antimalarial drug therapies in other resource poor settings.


*Keywords:* Sulfadoxine; Pyrimethamine; Chloroquine; Amodiaquine; Desethylamodiaquine; High-performance liquid chromatography; Malaria

## 1. Introduction

The antimalarial drugs sulfadoxine-pyrimethamine (SDX-PYM) and chloroquine (CQ) have, for several decades, been the mainstay of treatment for *Plasmodium falciparum* malaria in Africa. Because of high levels of parasite resistance to these drugs, many African countries are changing to artemisinin-containing combination therapies (ACTs) as first line therapy for uncomplicated malaria [1]. ACTs are expensive compared to SDX-PYM and CQ, and supplies of artemisinin currently fall short of demand [2]. Amodiaquine (AQ), a 4-aminoquinoline like CQ, retains some activity against CQ-resistant parasites [3]. It is rapidly metabolised to desethylamodiaquine (AQM) which is responsible for most of the antimalarial effect [4]. When used to prevent malaria, the drug caused an unacceptably high prevalence of serious adverse events (agranulocytosis and hepatitis) [5]. However, in the face of widespread CQ and SDX-PYM resistance and few other alternative treatments, AQ has been returned to the armamentarium against malaria. In Africa by the end of 2005, 14 countries had chosen AQ (usually in combination with artesunate) as their first line therapy for uncomplicated malaria [6]. Despite problems with resistance, SDX-PYM is still first line therapy in some countries, usually in combination with AQ or CQ, and is used for intermittent presumptive therapy (IPT) in pregnancy and in the treatment of toxoplasmosis in AIDS patients. CQ remains the treatment of choice for *Plasmodium vivax*, *ovale* and *malariae* infections and it is also used for IPT in pregnancy in a few countries in West Africa. Recent reports of a return of parasite sensitivity to CQ in Malawi following the cessation of its use in 1993 have raised the possibility of the

*Abbreviations:* ACT, Artemisinin-containing combination therapy; AQ, Amodiaquine; AQM, Desethylamodiaquine; CV, Coefficient of variation; CQ, Chloroquine; HPLC, High-performance liquid chromatography; HQC, High QC, IPTIntermittent presumptive therapy; LLOQ, Lower limit of quantification; LQC, Low QC; MQC, Middle QC; PG, Proguanil; PTFE, Polytetrafluroethylene; PYM, Pyrimethamine; QC, Quality control; QND, Quinidine; tR, Retention time; SDX, Sulfadoxine; UV, Ultra-violet

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return of CQ based combinations in future treatment policies for uncomplicated malaria in some settings [7].

Practical assays which could be set up in Africa and used to study the disposition of antimalarials in sick patients would be useful and may help optimise prescribing practices. High-performance liquid chromatography (HPLC) assays for SDX, PYM, CQ, AQ and AQM have all been described before [8–12]. Here, we report extensive modification of these methods to allow (a) a shared common platform, using the same instrumentation and columns, and (b) the analysis of small volumes of whole blood (between 50 and 200 μl, capillary or venous), and stored at −80 °C (range −80 to −86 °C) after collection. We believe this method to be suitable for use in most national reference laboratories in sub-Saharan Africa (and have used the method to analyse samples from a randomised controlled trial which will be reported elsewhere). The use of simple and robust assays similar to those described here, will permit the disposition of AQ, AQM, SDX-PYM and CQ to be studied in patients as part of their assessment under operational conditions in resource poor settings.

## 2. Experimental methods common to the analyses of SDX, PYM, CQ, AQ and AQM

### 2.1. Instrumentation and conditions

Chromatography was performed at room temperature, between 20 and 23 °C, using a Thermo Finnigan (San Jose, USA) SpectraSYSTEMS® P1000 isocratic pump and a Thermo Finnigan SpectraSERIES autosampler AS3000 fitted with a 100 μl injection loop. The same column and guard column were used for all analyses. The column was a reversed-phase BDS HYPERSIL C18 column, 150 mm × 4.6 mm, 5 μm particle size (Thermo Hypersil-Keystone, Runcorn, UK) preceded by a LiChrospher 100 RP-18, 5 μm particle, 10 mm × 4.6 mm guard column (Merck, Damstadt, Germany). The column effluent was monitored by a variable wavelength Thermo Finnigan SpectraSYSTEMS® UV1000 detector and peak areas and retention times were calculated using a Thermo Finnigan Chromjet integrator.

### 2.2. Common chemicals and reagents

SDX, PYM, AQ, CQ, quinidine, sulfisoxazole, sodium hydroxide, sodium acetate and 1-octane sulfonic acid were all purchased from Sigma–Aldrich (St. Louis, USA). Proguanil and AQM were gifts from Professor Steve Ward, University of Liverpool, UK. The mobile phases for all 4 assays were constituted from common reagents; varying combinations of deionised water (at varying pHs), methanol, acetonitrile and triethylamine. The mobile phase solvents and the extraction solvents, hexane, ethyl acetate, tert-butyl methyl ether and diethyl ether were purchased from BDH (Poole, UK) and were HPLC grade. We were able to obtain high-purity methanol locally in Malawi manufactured by Associated Chemical Enterprises Ltd. (South Africa). Hydrochloric acid and orthophosphoric acid were purchased from BDH.

For each drug, 10 ml Pyrex glass tubes with PTFE-lined screw-caps were used in the extraction process. To optimise the extraction process, the cleaned glassware was rinsed in a 5% dimethylchlorosilane (FLUKA, Switzerland) solution in toluene (BDH), rinsed in methanol, and then dried.

### 2.3. Calibration

Drug free whole blood was spiked with known amounts of drug and tumble-mixed for 20 min. Calibration curves were constructed by analysing (in duplicate) whole blood samples at known drug concentrations (a minimum of 5 per curve not including zero) within the range of interest and containing internal standard. Peak area ratios (drug/internal standard) were plotted against drug concentrations and linearity was assessed by linear regression. The calibration curve was accepted if $r^2 \geq 95\%$.

### 2.4. Reproducibility and stability

Quality control samples were prepared for each drug by spiking whole blood at 4 concentrations within the calibration curve representing the lower limit of quantification (LLOQ), the low QC (LQC) at 2–3 × the LLOQ, the middle QC (MQC) and a high QC (HQC) at the maximal concentration on the calibration curve. Accuracy and reproducibility were assessed by determining the intra-day and inter-day coefficient of variation (% CV) of the three QCs (6 at each concentration) and LLOQ, over 3 runs, once weekly for 3 weeks. QCs were frozen at −80 °C between analyses and run alongside calibration curve samples freshly made for each of the 3 validation runs.

### 2.5. Recovery

Percentage recovery was determined by comparing the peak area obtained after extraction of a known amount of drug from whole blood with that obtained by injecting an equal mass of drug dissolved in mobile phase directly onto the column.

## 3. Sulfadoxine

### 3.1. Calibration curve and QCs

Stock solutions were prepared by dissolving SDX and sulfisoxazole (internal standard) in methanol. Drug free whole blood was spiked with SDX to produce a calibration curve ranging from 5 to 100 μg/ml. The QCs at concentrations of 15, 50, 100 μg/ml representing the LQC, MQC and HQC, respectively, were made in an identical way.

### 3.2. Extraction procedure

Whole blood (50 μl) was pipetted into a silanized Pyrex glass tube and 5 μg sulfisoxazole (SS) added as an internal standard. One millilitre of 0.1 M sodium acetate buffer (pH 3.5) was added and vortex mixed for 5 s followed by 5 min of incubation.

SDX and SS were extracted with 5 mls of hexane–ethyl acetate (1:1 v/v) by gently mixing for 30 min. After centrifugation at 3000 × *g* for 10 min, the organic (upper) phase was transferred into a clean silanized glass tube and dried under a stream of nitrogen in a water bath at 37 °C.The residue was reconstituted in 100 μl of mobile phase and 50 μl was injected onto the column.

### 3.3. Chromatography

The mobile phase consisted of deionised water containing 4.6 mM 1-octane sulfonic acid, methanol and acetonitrile (70:19:11% v/v), sonicated to de-gas and flowing at 1 ml/min. The UV detector was set at a wavelength of 274 nm.

## 4. Pyrimethamine

### 4.1. Calibration curves and QCs

Stock solutions of PYM and proguanil (internal standard) were made by dissolving in methanol. Drug free whole blood was spiked with PYM to produce a calibration curve ranging from 50 to 1000 ng/ml. The QCs at concentrations of 150, 500 and 1000 ng/ml, representing the LQC, MQC and HQC, respectively, were made in an identical way.

### 4.2. Extraction procedure

Whole blood (150 μl) was mixed with 50 ng of proguanil (PG) in silanized Pyrex tubes. One millilitre of 1 M sodium hydroxide was added followed by 10 s of vortex mixing followed by 5 min of static incubation. PYM and PG were extracted with 4 ml of ethyl acetate by gently mixing for 30 min. The organic solvent (upper phase) was transferred to a clean silanized glass tube after centrifugation at 3000 g for 10 min and dried under a stream of nitrogen in a 37 °C water bath. The residue was reconstituted with 100 μl of mobile phase and 50 μl was injected onto the column

### 4.3. Chromatography

The mobile phase consisted of deionised water containing 5 mM of 1-octane sulfonic acid (adjusted to pH 2.5 with concentrated orthophosphoric acid), methanol and acetonitrile (60:10:30% v/v), sonicated to de-gas and flowing at 3 ml/min. The UV detector was adjusted to 254 nm.

### 4.4. Chloroquine

#### 4.4.1. Calibration curve and QCs

Stock solutions were prepared by dissolving CQ diphosphate salt and anhydrous quinidine (internal standard) in distilled water and methanol, respectively. Drug free whole blood was spiked with CQ to make a calibration curve ranging from 150 to 1500 ng/ml. QCs were made in an identical way at concentration of 300, 750 and 1500 μg/ml corresponding to the LQC, MQC and HQC, respectively.

#### 4.4.2. Extraction procedure

Whole blood (150 μl) was mixed with 750 ng of quinidine in silanized Pyrex tubes. Five hundred microlitres of 0.2 M hydrochloric acid was added and incubated for 2 min followed by 1 ml of 20% sodium hydroxide. CQ and QND were extracted with 5 mls hexane – tert-butyl methyl ether (1:1 v/v) by gently mixing for 30 min and centrifugation at 3000 g for 10 min. The upper organic phase was transferred to a clean silanized glass tube and dried under a stream of nitrogen at 37 °C. The residue was reconstituted with 100 μl mobile phase and 50 μl was injected onto the column.

#### 4.4.3. Chromatography

The mobile phase consisted of deionised water (adjusted to pH 2.8 with concentrated orthophosphoric acid) and acetonitrile (85:15% v/v) and 1% triethylamine, de-gassed and flowing at 3 ml/min. The UV detector was set at 340 nm.

## 5. Amodiaquine and desethylamodiaquine

### 5.1. Calibration curve and QCs

Stock Solutions of AQ, AQM and quinidine (internal standard) were made by dissolving the drugs in distilled water. A solution containing both AQ and AQM in equal concentrations was used to make calibration curves in the range of 100–1000 ng/ml for both drugs. Quality controls, containing both AQ and AQM, were made in an identical way at concentrations of 300, 500 and 1000 ng/ml for the LQC, MQC and HQC, respectively.

### 5.2. Extraction procedure

Whole blood (200 μl) containing AQ and AQM were spiked with 500 ng of QND. Distilled water was added and vortex mixed followed by 10 min static incubation in the dark. Acetonitrile (400 μl) was added to precipitate the plasma proteins and improve the drug recovery. The drugs were then extracted with 4 ml of diethyl ether by gently mixing for 30 min in the dark. After centrifuging the mixture at 3000 × *g* for 10 min, the upper organic phase was transferred into clean silanized tubes and dried under a stream of nitrogen in a water bath at 37 °C. The residue was reconstituted in 100 μl of mobile phase and 50 μl injected onto the column.

### 5.3. Chromatography

The mobile phase consisted of water, methanol and triethylamine (83:16:1% v/v), adjusted to pH 2.2 with concentrated orthophosphoric acid, de-gassed and flowing at 1.5 ml/min. The UV detector was set to 340 nm.

## 6. Results

The chromatography, recovery and reproducibility data is described below for each of the assays separately. In addition, a single concentration–time profile from a child in the randomised

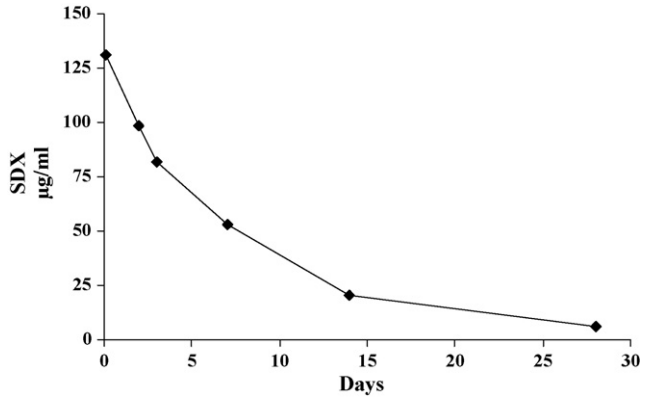


Fig. 1. Whole blood sulfadoxine concentration in a 12.1 kg child treated with oral sulfadoxine-pyrimethamine (containing 500 mg SDX) taken as a single dose.

controlled trial is shown for each of the drugs for illustrative purposes (Figs. 1–4)

### 6.1. Sulfadoxine

Sulfadoxine and sulfisoxazole were baseline resolved with typical retention times of around 5.4 and 3.5 min, respectively (Fig. 5A). Using a 50 μl blood sample, the LLOQ for SDX was 5 μg/ml (250 ng on column), and the calibration curve was linear in the range 5–100 μg/ml ($r^2 > 0.97$) in all 3 runs. The inter-assay and intra-assay CV values at 5, 15, 50 and 100 μg/ml were <15% and the mean recovery was 62.7% (Table 1). Whole blood spiked with sulfamethoxizole (the sulpha component of cotrimoxazole), AQ, AQM, PYM or CQ and extracted by the same method did not produce any peaks which interfered with the SDX or SS peaks.

### 6.2. Pyrimethamine

PYM and PG were baseline resolved with typical retention times of around 3.4 and 5.9 min, respectively (Fig. 5B). Using a 150 μl blood sample, the LLOQ for PYM was 50 ng/ml (7.5 ng on column), and the calibration curves were linear in the range 50–1000 ng/ml ($r^2 > 0.95$) in all 3 runs. The inter-assay and intra-assay CV values at 50, 150, 500 and 1000 ng/ml were <15% and the mean recovery was 73.8% (Table 1). Whole blood spiked with AQ, AQM, SDX or CQ and extracted by the same method did not produce any peaks which interfered with the PYM or PG peaks.

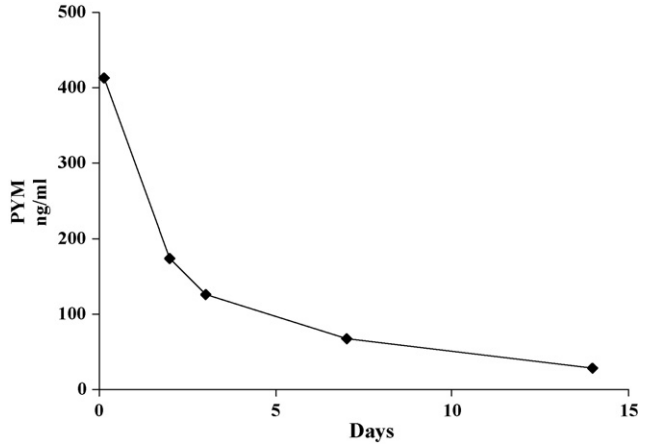


Fig. 2. Whole blood pyrimethamine concentration in a 12.1 kg child treated with oral sulfadoxine-pyrimethamine (containing 25 mg of PYM) taken as a single dose.

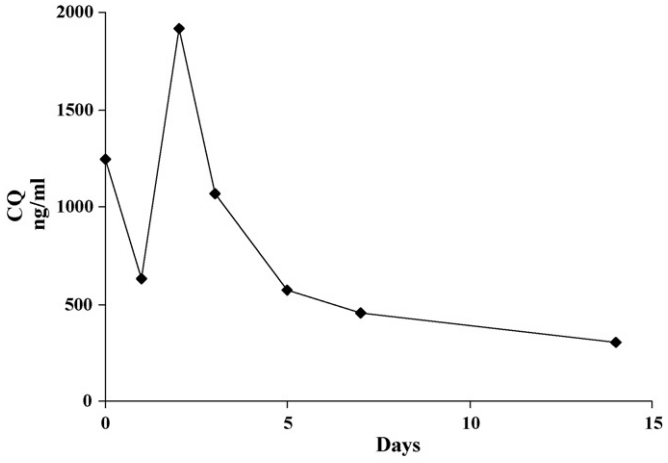


Fig. 3. Whole blood chloroquine concentration in a 10 kg child treated with oral chloroquine 75 mg (base) once daily for 3 days.

### 6.3. Chloroquine

Chloroquine was baseline resolved with a typical retention time of around 3.0 min. Quinidine was also baseline resolved and gave two distinct peaks around 5.5 and 7.3 min (Fig. 5C). The area of the first quinidine peak was used for determination of the CQ concentration. Using a 150 μl blood sample, the LLOQ for CQ was 100 ng/ml (15 ng on column), and the calibration curves were linear in the range 100–1500 ng/ml ($r^2 > 97\%$) in all 3 runs. The inter-assay and intra-assay CV

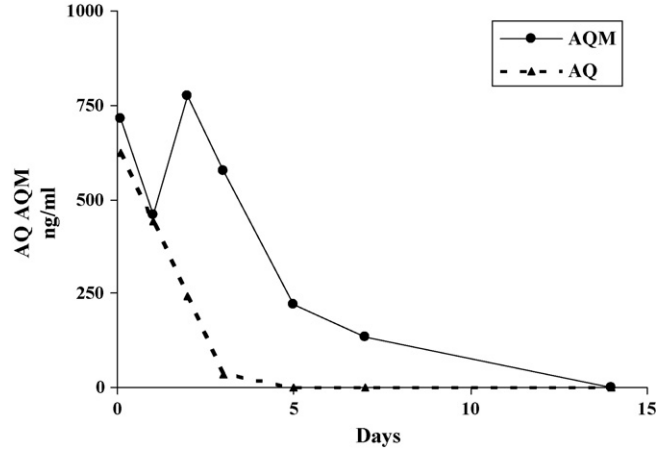


Fig. 4. Whole blood amodiaquine (■) and desethylamodiaquine (●) concentrations in a child treated with oral amodiaquine 100 mg (base) once daily for 3 days.

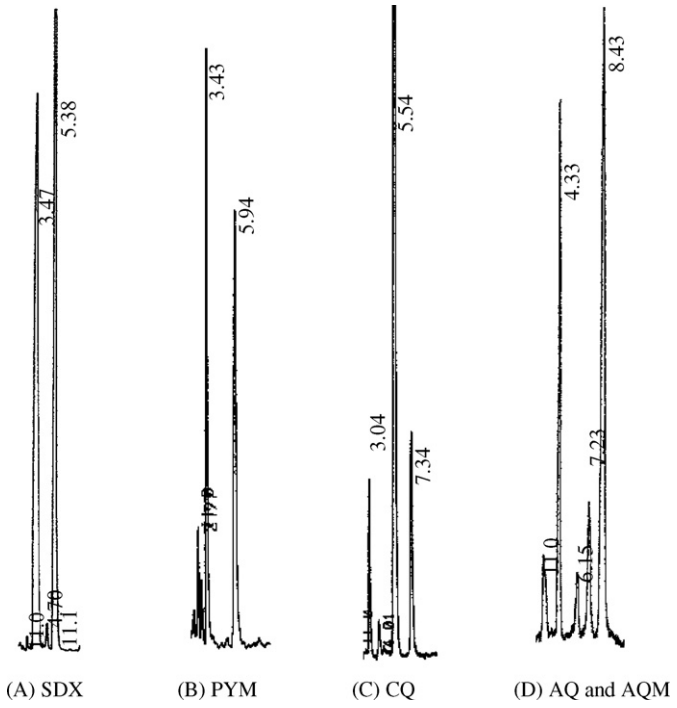


Fig. 5. Chromatograms showing peaks after extraction from of drugs from whole blood using each of the four assays described. Retention times (tRs) in min are shown adjacent to the peaks. (A) Sulfadoxine (tR 5.4) and sulfisoxazole (tR 3.5), (B) pyrimethamine (tR 3.4) and proguanil (tR 5.9), (C) chloroquine (tR 3.0) and quinidine (tRs 5.5 and 7.3) and (D) amodiaquine (tR 8.4), desethylamodiaquine (tR 7.2) and quinidine (tRs 4.3 and 6.2).

values at 100, 300, 750 and 1500 ng/ml were <15% and the mean recovery was 80.6% (Table 1). Whole blood spiked with AQ, AQM, SDX or PYM and extracted by the same method did not produce any peaks which interfered with the CQ or QND peaks.

Table 1

| Drug | QC Sample | Concentration | Inter-assay CV (%) | Intra-assay CV (%) | Recovery % |
|---|---|---|---|---|---|
| SDX | LLOQ | 5 μg/ml | 10.9 | 9.3 | |
| | LQC | 15 μg/ml | 11.1 | 10.8 | 56.9 |
| | MQC | 50 μg/ml | 13.8 | 9.3 | 63.8 |
| | HQC | 100 μg/ml | 10.3 | 13.1 | 67.5 |
| PYM | LLOQ | 50 ng/ml | 14.0 | 12.6 | |
| | LQC | 150 ng/ml | 11.3 | 10.5 | 70.2 |
| | MQC | 500 ng/ml | 9.3 | 9.0 | 73.2 |
| | HQC | 1000 ng/ml | 7.0 | 5.6 | 78.1 |
| CQ | LLOQ | 150 ng/ml | 13.3 | 13.3 | |
| | LQC | 300 ng/ml | 13.3 | 12.9 | 79.8 |
| | MQC | 750 ng/ml | 11.2 | 12.0 | 81.4 |
| | HQC | 1500 ng/ml | 8.3 | 8.1 | 80.5 |
| AQ | LLOQ | 100 ng/ml | 14.8 | 18.7 | |
| | LQC | 300 ng/ml | 11.9 | 8.5 | 38.6 |
| | MQC | 500 ng/ml | 12.2 | 12.6 | 47.1 |
| | HQC | 1000 ng/ml | 14.5 | 12.9 | 37.0 |
| AQM | LLOQ | 100 ng/ml | 14.7 | 14.8 | |
| | LQC | 300 ng/ml | 13.8 | 13.0 | 27.0 |
| | MQC | 500 ng/ml | 13.1 | 12.0 | 32.3 |
| | HQC | 1000 ng/ml | 14.7 | 12.8 | 25.2 |

### *6.4. Amodiaquine and desethylamodiaquine*

Desethylamodiaquine and amodiaquine were baseline resolved with typical retention times of around 7.2 and 8.4 min, respectively. Quinidine was also baseline resolved and produced 2 peaks on the chromatogram around 4.3 and 6.2 min (Fig. 5D). The area of the first quinidine peak was used for determination of the AQ and AQM concentrations. Using a 200 μl blood sample, the LLOQ for AQ was 100 ng/ml, (20 ng on column), and the calibration curve was linear in the range 100–1000 ng/ml ($r^2 > 0.97$) in all 3 runs. The inter-assay and intra-assay CV values at 300, 500 and 1000 ng/ml were <15% and the mean recovery was 40.9% (Table 1). At the LLOQ (100 ng/ml), the inter-assay and intra-assay CV values were <20%. For AQM, using 200 μl of blood, the LLOQ was 100 ng/ml (20 ng on column), and the calibration curves were linear in the range 100–1000 ng/ml ($r^2 > 0.95$) in all 3 runs. The inter-assay and intra-assay CV values at 300, 500 and 1000 ng/ml were all <15% and the mean recovery was 28.2% (Table 1). At the LLOQ concentration for AQM, 100 ng/ml, the inter-assay and intra-assay CV values were <15%. Whole blood spiked with SDX or PYM and extracted by the same method did not produce any peaks which interfered with the AQ, AQM or QND peaks. CQ extracted by this method produced a peak at 5.5 min but this did not interfere with the QND, AQ or AQM peaks.

## 7. Discussion

We have described four different assays to measure some of the most common antimalarial drugs currently in use in Africa. The assays were set up and validated in Malawi to internationally recognised standards, with inter-day and intra-day % CV of less than 15% at all levels of the QCs and <20% at the LLOQ concentration [13]. The assays are sensitive; the LLOQ's on column being 250, 7.5, 15, 20 and 20 ng for SDX, PYM, CQ, AQ and AQM and are specific with no interference detected in the chromatograms with the other drugs described here. We did not run the assays with quinine, artesunate or mefloquine so we cannot say if there would have been any interference with these drugs. Mefloquine is rarely used in Africa and artesunate has no UV absorbance at concentrations found in humans so it is unlikely either of these drugs would interfere with the operational use of these assays.

The LLOQs and calibration curve ranges for the SDX, PYM and CQ assays are in keeping with those reported previously [9,11]. For AQ and AQM, the mean percent recoveries we achieved from whole blood were 40.8 and 28.2%, respectively. These are far below those reported by Gitau et al from blood spots on filter paper; 83.9% for AQ and 74.3% for AQM [8]. We tried to replicate this extraction method using the same filter paper, but the recoveries were always lower than those we had obtained from whole blood.

We have seen no deterioration in the QC samples stored at −80 °C over a 2 year period suggesting that these drugs are stable in whole blood for prolonged periods at this temperature. The assays require only small volumes of blood and so are applicable to studies involving young children. In some countries in Africa,

collecting and storing venous blood samples at −80 °C may not be possible and we did not evaluate the stability of samples stored at −20 °C. Clearly it is important that this is done in the future. In addition, the assays should be tested using dried blood samples stored on filter paper rather than venous blood as this would further increase their practical utility in Africa. Our attempts to extract AQ and AQM from filter paper suggested difficulties with this process and we opted to use venous blood.

We have used these methods to measure drug concentrations in children in an antimalarial drug efficacy study in Malawi (to be reported elsewhere). The four concentration–time profiles (Figs. 1–4), one profile for each of the drugs described in this report, are from children in this study. The graphs show the typical disposition characteristics of these drugs, rapid absorption followed by long elimination phases (one or more compartmental), and are in keeping with other published data [10,11,14]. We have over 400 kinetic profiles for these drugs from children in our study and these will be described in detail elsewhere.

Pharmacokinetic studies of antimalarials in operational use have led to changes in prescribing practices in the past. For example, the oral bioavailability of mefloquine was found to be increased if the 25 mg/kg treatment dose was split by a period of up to 24 h [15], and the oral bioavailability of lumefantrine is improved if taken with food [16]. The use of a loading dose of quinine for the management of severe malaria was recommended after it was demonstrated that this led to the more rapid achievement of optimal blood quinine concentrations [17]. Indeed some reports of parasite 'resistance' to drugs may in fact be due to subtherapeutic drug concentrations [18] rather then true resistance. The four methods described here are cost-efficient and could be set up in other developing world countries and may lead to further improvements in prescribing practices.

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