Validation and Pharmacokinetic Application of a High-Performance Liquid Chromatographic Technique for Determining the Concentrations of Amodiaquine and Its Metabolite in Plasma of Patients Treated with Oral Fixed-Dose Amodiaquine-Artesunate Combination in Areas of Malaria Endemicity

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Artemisinin-based combination therapies (ACTs) have been adopted by most African countries, including Nigeria, as first-line treatments for uncomplicated falciparum malaria. Fixed-dose combinations of these ACTs, amodiaquine-artesunate (FDC AQAS) and artemether-lumefantrine (AL), were introduced in Nigeria to improve compliance and achieve positive outcomes of malaria treatment. In order to achieve clinical success with AQAS, we developed and validated a simple and sensitive high-performance liquid chromatography (HPLC) method with UV detection for determination of amodiaquine (AQ) and desethylamodiaquine (DAQ) in plasma using liquid-liquid extraction of the drugs with diethyl ether following protein precipitation with acetonitrile. Chromatographic separation was achieved using an Agilent Zorbax C₁₈ column and a mobile phase consisting of distilled water-methanol (80:20 [vol/vol]) with 2% (vol/vol) triethylamine, pH 2.2, at a flow rate of 1 ml/min. Calibration curves in spiked plasma were linear from 100 to 1,000 ng/ml ($r > 0.99$) for both AQ and DAQ. The limit of detection was 1 ng (sample size, 20 μ l). The intra- and interday coefficients of variation at 150, 300, and 900 ng/ml ranged from 1.3 to 4.8%, and the biases were between 6.4 and 9.5%. The mean extraction recoveries of AQ and DAQ were 80.0% and 68.9%, respectively. The results for the pharmacokinetic parameters of DAQ following oral administration of FDC AQAS (612/200 mg) for 3 days in female and male patients with uncomplicated falciparum malaria showed that the maximum plasma concentrations ($C_{\max}$) (740 ± 197 versus 767 ± 185 ng/ml), areas under the plasma concentration-time curve (AUC) ($185,080 \pm 20,813$ versus $184,940 \pm 16,370$ h · ng/ml), and elimination half-life values ($T_{1/2}$) (212 ± 1.14 versus 214 ± 0.84 h) were similar ($P > 0.05$).

Extensive deployment of antimalarial drugs in the past 50 years has led to high selection pressure on human malaria parasites to evolve mechanisms of resistance. *Plasmodium falciparum* is now highly resistant to chloroquine (a one-time first-line antimalarial) and other antimalarials in most malaria-affected areas. Due to the relentless increase in resistance of malaria parasites to conventional drugs, many African countries have adopted the WHO recommendation to use artemisinin-based combination therapies (ACTs) as the first-line treatment for uncomplicated falciparum malaria (1). Artemisinin derivatives rapidly reduce the biomass of multidrug-resistant parasites, leaving the partner drugs, which usually have longer half-lives and therefore are eliminated more slowly, to kill the remaining residual parasites. The complete clearance of all parasites is therefore dependent on the partner drugs, such as amodiaquine (AQ), lumefantrine, piperaquine, and mefloquine, being effective and persisting at parasitocidal concentrations until all infecting parasites are killed. A fixed-dose combination (FDC) of AQ and artesunate (AS) tablets (Diasunate; Emzor, Lagos, Nigeria) was introduced to the Nigerian market to improve patient compliance and achieve a positive outcome of malaria treatment. Following oral administration of AQ, there is rapid and extensive metabolism of the AQ to its pharmacologically active derivative desethylamodiaquine (DAQ), which is assumed to be responsible for most of the therapeutic effect (2); *in vitro* studies (3–5), however, suggest synergism between AQ and DAQ (Fig. 1). This underscores the importance of routine thera-

peutic drug monitoring when artesunate is coadministered with AQ, to ensure not only appropriate drug dosing, but also that the dosing regimen results in concentrations in the blood sufficiently high to kill the residual parasites. A diseased state significantly alters the disposition of antimalarial drugs, yet several analytical techniques that reported the quantification of AQ and DAQ (2, 6–13) in various biological fluids demonstrated its applicability only either in healthy volunteers or in patients administered AQ as monotherapy. To our knowledge, there is only one published analytical method (14) for AQ that has demonstrated its applicability for use in malaria patients taking an amodiaquine-artesunate (AQAS) combination. Dried blood spot (DBS) was the technique

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FIG 1 Structures of amodiaquine and desethylamodiaquine.

used, but the blood spot size and hematocrit are challenges for consideration when validating the performance of this technique. Some of the reported methods (9, 10, 15, 16) are also fraught with challenges, being expensive, laborious, or time-consuming, making them unsatisfactory in areas of malaria endemicity in sub-Saharan Africa, where time and financial resources are essential major constraints. Minzi et al. (10) reported a high-performance liquid chromatography (HPLC) method for AQ and DAQ in biological fluids using synthetic 4-(4-dimethylamino-1-methylbutylamino)-7-chloroquinoline, which is not easily accessible, as an internal standard (IS). The extraction and retention times reported for AQ and DAQ by Gitau et al. (7) were too long to make the method desirable when time is of the essence. The analytical technique developed by Mihaly et al. (9) for quantifying AQ and DAQ used a large sample volume (1 ml of plasma) and may not be suitable for pharmacokinetic (PK) studies in patients from areas of malaria endemicity who may be anemic. While the method described by Bell et al. (15) may be cost-effective, the process of extraction is cumbersome and the yield low. In the HPLC method reported by Navaratnam et al. (17), the electrochemical detection used is not available in the part of the continent where this study took place due to its high cost of procurement. Sourcing of reagents for these methods (2, 6, 7, 10–13) is also capital intensive, and this may further deplete the already meager resources of poor African countries where malaria is endemic.

Nonetheless, the challenges of these analytical techniques were considered in coming up with a suitable method. The aim of this study, therefore, was to develop and validate a simple, sensitive analytical method that is realistic for use in pharmacokinetic studies and determination of AQ and DAQ in the plasma of patients treated with amodiaquine-artesunate (FDC AQAS) in resource-poor areas of malaria endemicity.

MATERIALS AND METHODS

Chemicals and reagents. AQ, chloroquine diphosphate, paracetamol, artesunate, and the IS, quinidine, were obtained from Sigma (Stockholm, Sweden). Sulfadoxine was obtained from Swipha (Lagos, Nigeria), while DAQ was obtained from G. O. Kokwaro of the Kenya Medical Research Institute/Wellcome Laboratory, Nairobi, Kenya. Diasunate (a fixed-dose combination of 100 mg artesunate and 400 mg amodiaquine hydrochloride [batch number, 945 K; National Agency for Food and Drug Administration and Control registration no. 04-6911]) and amodiaquine tablets (Camoquine; 200 mg) were purchased locally from Emzor (Lagos, Nigeria) and Parke-Davis, Senegal, respectively. High-performance liquid chromatography (HPLC) grade methanol (Chromasol), HPLC grade diethyl ether, and acetonitrile were obtained from Sigma-Aldrich Chemie, GmbH, Germany. Triethylamine and orthophosphoric acid were from Pharmacos Limited (Essex, England) and BDH (Poole, United Kingdom), respectively.

Instrumentation and chromatography. Chromatography was performed at ambient temperature in the Therapeutic Drug Monitoring (TDM) Unit of the Obafemi Awolowo University Teaching Hospital, Ile-Ife, Nigeria. The chromatographic system was an Agilent 1100 series liquid chromatography system (Agilent Technologies Deutschland GmbH) made up of a quaternary gradient pump fitted with a gradient mixer, a system purge, and a diode array detector (DAD). Sample injections were performed on a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA) with a 20- μ l sample loop. The mobile phase consisted of water-methanol (80:20 [vol/vol]) containing 2% (vol/vol) triethylamine adjusted to pH 2.2 with concentrated orthophosphoric acid. Prior to use, the mobile phase was filtered, degassed, and pumped through the column at 1 ml/min. Chromatographic separation was achieved with a reversed-phase Agilent Zorbax C₁₈ column (150 mm by 4.6-mm inside diameter [i.d.]; particle size, 5 μ m), and the column effluent was monitored with the DAD at 340 nm. The detector output was linked to a Hewlett Packard personal computer through the ChemStation software program (Agilent Technologies Deutschland GmbH) to analyze and record the chromatograms on an integrator (Chromjet data integrator; Chrom Tech, Inc., USA).

Sample preparation. Stock solutions containing 1 mg base/ml AQ, DAQ, and IS were prepared in methanol in 25-ml volumetric flasks, followed by sonication for 10 min. Working solutions of different concentrations were obtained from the stock by appropriate serial dilution with distilled water and stored at -20°C . For calibrators, 50- μ g/ml working solutions of AQ, DAQ, and IS were used. Drug-free human plasma (blank) was spiked with working solutions of AQ and DAQ to yield final concentrations of 25, 50, 100, 200, 400, 600, 800, and 1,000 ng/ml. In addition, quality control (QC) solutions were prepared by weighing separately standard reference AQ and DAQ. Blank plasma was spiked with the QC solutions to obtain QC sample concentrations of 150, 300, and 900 ng/ml. The samples were used in developing and evaluating the method. Aliquots (200 μ l) of sample were stored at -20°C until analysis, when they were brought to room temperature before use.

Analytical procedure. Extraction of the drugs from human plasma was carried out in screw-cap 15-ml polypropylene centrifuge tubes. Frozen aliquots of the calibrators, QC, and other plasma samples were each vortex mixed thoroughly after allowing them to thaw at room temperature. To 0.2 ml plasma in a centrifuge tube, the IS (10 μ l at 50 μ g/ml) was added, and the final volume was made up to 1 ml with distilled water. The tubes and their contents were vortex mixed for 60 s, followed by precipitation of the plasma protein with 400 μ l of acetonitrile. The mixture was shaken again for 2 min, after which 3 ml diethyl ether was added and vortex mixed for 90 s before centrifuging at $3,000 \times g$ for 10 min. The upper organic phase was aspirated with a Pasteur pipette into a clean polypropylene centrifuge tube, while the aqueous phase was reextracted with another 3 ml of diethyl ether and centrifuged as described above. The pooled organic phases were evaporated to dryness with a current of air. The residue was reconstituted in 100 μ l of the mobile phase, and a 20- μ l aliquot was injected into the HPLC system.

Validation of the method. Calibration curves were prepared from the calibrators, drug-free plasma spiked with the drugs to achieve concentrations in the range of 100 to 1,000 ng/ml for AQ and DAQ. Sample prep-

aration and extraction were performed as described above on six replicates at each concentration of AQ and DAQ on six separate days. Blank samples were included with each replicate. The peak area ratio of each analyte (AQ and DAQ) to the IS was plotted against the corresponding concentration to construct calibration curves to evaluate the linearity of the method on each day. Unweighted regression analysis was performed using SPSS software version 16.0 for Windows to determine the slope, intercept, and correlation coefficient (r) of the calibration curves. The concentrations of AQ and DAQ in the test samples were calculated using the regression parameters obtained from the equations of the calibration curves.

The limits of quantification (LOQ) and detection (LOD) were determined by the signal-to-noise (S/N) ratio evaluations of analytes spiked from 10 to 100 ng/ml. The LOQ is defined as the lowest concentration of the analyte with an S/N ratio of at least 10 and acceptable accuracy and precision ($<15\%$) (18); it was set as the first point for the calibration curve. The LOD provides the lowest concentration of analyte that can be discriminated from the background noise but not quantified and with a S/N ratio of at least 3. The criteria for determining this concentration were based on background interferences (S/N) and the reproducibility and variability of the response (signal) (19).

The intra- and interday assay precision, accuracy, and recovery were determined using the replicate samples ($n = 6$) of prepared QCs at three concentration levels spanning the low, medium, and upper ranges of the calibration curves. Each sample was spiked with the IS (10 μ l at 50 μ g/ml) and taken through the extraction process as described above. Six replicates of each concentration of QC samples were assayed in one run for the intraday variation, while two replicates of each QC sample were assayed for 3 days in case of interday variation. The intra- and interday precision and accuracy of the method were expressed as the relative standard deviation (RSD) or percent coefficient of variation (CV) and percent bias values, respectively. The coefficient of variation was determined from the following equation: $CV (\%) = (\text{standard deviation [SD]}/\text{mean}) \times 100$. The percent bias value was calculated as follows: $\text{bias} (\%) = [(\text{calculated concentration} - \text{theoretical concentration})/\text{theoretical concentration}] \times 100$. The acceptance criteria for precision and accuracy were that the CV was lower than 15% (except for the LOQ, where a CV of $<20\%$ was allowed) and the bias was within $\pm 15\%$, in that order.

The absolute recovery of the extraction procedure was determined by comparing the peak area ratios of the drugs in the extracted samples with those of the corresponding pure standards (direct/neat injection) of the same concentrations, which were not subjected to sample pretreatment, as follows: $\text{absolute recovery} = (\text{response of analyte spiked into matrix} [\text{processed}]/\text{response of analyte as pure standard}) \times 100$.

Stability. The stability of AQ and DAQ in the stock solutions and human plasma at concentrations of 150, 300, and 900 ng/ml was investigated over a period of 3 months under the storage conditions used for study samples, as recommended by Blessborn et al. (20). The short-term, long-term, and freeze-thaw stabilities of AQ and DAQ in human plasma were also evaluated at the three QC concentrations. The short-term stability was conducted by analyzing triplicates of the QC samples stored at -20°C and kept at room temperature (25°C) on the benchtop for 24 h before sample preparation. The long-term stability was assessed with triplicates of the QC samples stored at -20°C for 1 month and 3 months. The freeze-thaw stability was examined over three cycles. In each freeze-thaw cycle, the spiked plasma samples were allowed to thaw unassisted at room temperature after storage at -20°C and then analyzed at the third cycle. For the studies, all the samples were analyzed, and the results were compared with those obtained for freshly prepared samples. The bias was calculated from the calculated and theoretical concentrations, and the acceptance criterion for all the stability studies was $\pm 15\%$ from the theoretical value.

Clinical application of the method to pharmacokinetic study. The Medical Ethics Committee of the Olabisi Onabanjo University Teaching Hospital, Sagamu, Nigeria, approved the study protocol to conduct the

pharmacokinetic study. Written informed consent was administered to all patients who presented with fever to the study site during the study period. Patients who consented to participate in the study underwent both clinical and laboratory examination for malaria prior to inclusion. In order to demonstrate the applicability of the assay in the pharmacokinetic study of AQ and DAQ, 6 patients (3 females and 3 males) with a mean (SD) weight of 62.2 (4.4) kg and a mean age of 23.3 (1.2) years and with acute uncomplicated falciparum malaria were recruited into the study. Although the study was not primarily an efficacy study, 1,000 asexual parasites/ μ l was set as the minimum parasitemia to be eligible for inclusion. The patients had no history of antimalarial drug use in the preceding 28 days. The demographic data of the patients, such as baseline temperature, age, body weight, and gender, were recorded prior to drug administration. Each patient received two tablets of FDC AQAS (each consisting of 306 mg AQ base and 100 mg artesunate) over 3 days with a time interval of 24 h between doses. The doses were calculated based on body weight. Venous blood samples (5 ml) were withdrawn into heparinized tubes predose and at 0.5, 1, 2, 4, 6, and 12 h after the first dose on day 0 and on days 1, 2, 3, 7, and 14 at predefined times. Venous blood samples were obtained through an indwelling cannula in the first 12 h and subsequently by direct venipuncture with needle and syringe. The sampling schedule was designed to allow the capture of the concentration-time profile of AQ for the first 12 h after the first dose on day 0, taking into consideration its short elimination half-life. The subsequent sampling was at 24-h intervals before drug intake to follow up on the characterization of the DAQ elimination phase for at least three terminal half-lives. This reduced the discomfort to the patients of having to reinsert the cannula on subsequent days. Blood samples were centrifuged at 3,000 rpm for 10 min within 30 min of sample collection, and the resulting plasma was stored at -20°C until analysis. The samples were analyzed as described above.

The individual plasma concentration-time data obtained from the analysis were used to generate the PK parameters of AQ and DAQ by noncompartmental analysis using the computer program WinNonlin version 5 (Pharsight Corporation, California, USA). The maximum plasma concentration (C_{max}) and the time to C_{max} (T_{max}) were direct readings from the measured data. The linear trapezoidal with linear interpolation method was used to enumerate the area under the plasma concentration-time curve from time zero to the time of the last measured concentration (AUC_{0-t}). The log-linear regression of 3 to 5 terminal concentration-time data points was used to estimate the elimination rate constant (λ) and the corresponding elimination half-life ($T_{1/2}$). The AUC extrapolated from the last data point to infinity ($\text{AUC}_{t-\infty}$) was calculated by dividing the estimated concentration at the last data point (Clast) with the elimination rate constant. The total drug exposure ($\text{AUC}_{0-\infty}$) was computed as $\text{AUC}_{0-t} + \text{AUC}_{t-\infty}$. Data were described using the statistical program SPSS for Windows v. 16. The between-group comparison of the mean values of the pharmacokinetic parameters following administration of FDC AQAS in female and male patients was examined by Student's t test. All tests of statistical significance, except where specifically indicated, were two tailed. A P value of <0.05 was taken to indicate statistically significant differences.

RESULTS AND DISCUSSION

Method development and chromatography. Optimization was achieved by monitoring varying chromatographic conditions in terms of appropriate chromatographic columns, mobile phases and their constitution, extraction solvents, and solvent for reconstitution before arriving at suitable conditions that gave satisfactory results.

The development of the method started with the evaluation of three chromatographic columns against different compositions of mobile-phase systems to identify a suitable stationary phase. Both Agilent Zorbax C_{18} and Hypersil C_4 columns resulted in sharp peaks, while a PLRP-S column produced broad peaks. The Agilent

FIG 2 HPLC chromatograms of a standard solution containing 500 ng/ml of IS (QND) and 1,000 ng/ml each of DAQ and AQ (A), extracted blank human plasma (B), extracted plasma spiked with 500 ng/ml IS and 1,000 ng/ml of DAQ and AQ (C), and an extracted plasma sample from a patient obtained 1 h following a single oral dose (612/200 mg) of AQAS tablets and spiked with 500 ng/ml IS (D). mAU, milliabsorbance units.

Zorbax C₁₈ was eventually chosen based on the peak symmetry and retention time obtained with the optimization of the mobile phase. The choice of mobile phase for analysis is important in order to achieve good resolution between the peaks and to pro-

duce analytes with distinct sharp peaks without interference from endogenous substances. The initial mobile phase tested included various concentrations of phosphate buffer (0.02 to 0.08 M) with methanol and triethylamine adjusted to varying pH values be-

TABLE 1 Regression parameters of calibration curves for AQ and DAQ in plasma

Calibration curve and statistics	AQ			DAQ		
	Slope	Intercept	Correlation coefficient	Slope	Intercept	Correlation coefficient
1	0.00480	0.140	0.9984	0.0034	0.0847	0.9987
2	0.00470	0.165	0.9998	0.0034	0.1021	0.9984
3	0.00480	0.168	0.9989	0.0035	0.1024	0.9955
4	0.00470	0.157	0.9997	0.0035	0.1015	0.9973
5	0.00480	0.151	0.9993	0.0034	0.0986	0.9984
6	0.00490	0.132	0.9996	0.0035	0.0969	0.9989
Mean ($n = 6$)	0.00478	0.152	0.9993	0.00345	0.0977	0.9979
SD	0.0001	0.0140	0.0005	0.0001	0.00673	0.00128
CV (%)	1.57	9.19	0.0519	1.59	6.89	0.128

TABLE 2 Accuracy, precision, and recovery for the determination of AQ and DAQ in plasma ($n = 6$)

Analyte	C_{theo}^a (ng/ml)	C_{cal}^b (ng/ml)	Bias (%)	Precision (CV [%])		Recovery (mean $\pm$ SD)
				Intraday	Interday	
AQ	150.0	162	7.93	4.81	3.87	92.7 $\pm$ 6.38
	300.0	279	-6.99	3.88	3.93	73.3 $\pm$ 3.38
	900.0	927	2.95	2.12	4.25	71.0 $\pm$ 1.05
DAQ	150.0	145	-3.15	3.49	1.30	77.9 $\pm$ 3.13
	300.0	281	-6.45	2.69	5.20	62.7 $\pm$ 2.22
	900.0	962	6.84	2.98	4.88	66.2 $\pm$ 1.52

^a C_{theo} , theoretical concentration.^b C_{cal} , calculated concentration.

tween 2 and 4 with orthophosphoric acid. Though the mobile phase, consisting of phosphate buffer (0.025 M KH_2PO_4)-methanol (80:20 [vol/vol]) containing 1% (vol/vol) triethylamine, pH 2.7, produced sharp peaks, the presence of multiple peaks indicated interference with the peaks of the solvent front or endogenous peaks from plasma. Thereafter, a mobile phase consisting of water-methanol-triethylamine in varying proportions and adjusted to different pH values between 2 and 3 was optimized with the chosen column. Eventually, the final mobile phase used for the separation was a mixture of water and methanol (80:20 [vol/vol]) containing 2% (vol/vol) triethylamine adjusted to pH 2.2 with orthophosphoric acid. The mobile phase was able to separate the IS, DAQ, and AQ from interfering peaks within a 6.0-min run time with the UV absorption spectrum set at 340 nm. Quinidine was selected as the IS because it did not interfere with the analysis of amodiaquine, as it was unlikely that individuals would have previously taken the drug to treat malaria, unlike commonly used antimalarials, such as quinine, chloroquine, or sulfadoxine-pyrimethamine, which if used as the IS could have interfered with the analysis of the analytes. The retention times of the drugs on the chromatograms were 3.9 min for the IS, 4.8 min for DAQ, and 5.8 min for AQ. Figure 2 shows representative chromatograms of AQ, DAQ, and the IS in solution and plasma.

TABLE 3 Stability of AQ and DAQ in spiked plasma samples ($n = 3$)

Stability	Bias (%) by concn		
	Low (150 ng/ml)	Medium (300 ng/ml)	High (900 ng/ml)
Storage stability (-20°C)			
DAQ			
30 days	2.83	-4.12	6.95
90 days	3.40	-8.14	6.51
AQ			
30 days	6.44	-6.69	4.22
90 days	7.62	-6.68	3.92
Freeze-thaw stability (-20°C and 25°C)			
DAQ	0.85	-4.32	7.95
AQ	9.17	-5.97	4.10
Bench stability (25°C ; 24 h)			
DAQ	-7.81	-7.76	8.29
AQ	8.51	-6.30	3.99

TABLE 4 Pharmacokinetic parameters of AQ and DAQ in 6 patients with falciparum malaria who received FDC AQAS (612/200 mg for 3 days)

Subject	AQ				DAQ			
	C_{max} (ng/ml)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{0-t} (h $\cdot$ ng/ml)	C_{max} (ng/ml)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{0-t} (h $\cdot$ ng/ml)
1	252	0.50	7.26	1,084	549	48	211	107,766
2	274	1.00	7.39	1,031	729	48	213	116,125
3	256	0.50	10.6	1,001	942	48	212	138,628
4	237	1.00	7.70	1,033	553	48	213	107,182
5	277	0.50	7.83	1,076	858	48	214	123,218
6	267	1.00	11.1	986	889	48	215	128,846
Mean ^a	261	0.75	8.65	1,035	753	48	213	120,294
SD	15.1	0.27	1.74	39.3	172		1.33	12,362

^a Mean pharmacokinetic parameters of AQ and DAQ for 6 patients with SD.

During method development, several options were also investigated in order to obtain a suitable liquid-liquid extraction procedure for AQ and DAQ. Following the spiking of plasma with the drugs, a number of options were explored. They included precipitation of plasma proteins with different reagents, like methanol, acetonitrile, and perchloric acid; extraction with di-isopropyl-ether or diethyl ether; and varying the pH of the sample by basification with different phosphate buffers or sodium hydroxide (1 M or 2 M). Back extraction of the drugs from organic solvent with 0.1 M phosphate buffer, 0.1 N HCl, and the mobile phase was also explored. Most of these options, however, gave poor recovery, and the best recovery was obtained by adding 400 μ l of acetonitrile to 200 μ l of plasma and extracting with two 3-ml aliquots of diethyl ether. The efficiency of the recovery was improved by extraction with two aliquots (3 ml each) of diethyl ether. The residue upon evaporation of the organic solvent was reconstituted in 100 μ l of the mobile phase, and 20 μ l was injected into the chromatograph. With this method, the total time spent on the extraction steps and the elution of the last analyte, AQ, was about 32 min, which is shorter than those reported by previous authors (6, 7, 10), with corresponding analysis times of 60, 71, and 61 min.

The specificity of the method was assessed by injecting commonly used antimalaria and analgesic drugs. Some of these drugs, such as artesunate, sulfadoxine, pyrimethamine, and paracetamol, did not produce any peak, while chloroquine produced a peak that did not interfere with the AQ, DAQ, and IS peaks. This indicates that there were no interferences from the commonly used antimalaria and analgesic drugs.

Method validation. The calibration curves of AQ and DAQ showed linearity in the range of 100 to 1,000 ng/ml with correlation coefficients (r) of >0.99 . Representative (mean $\pm$ SD) regression equations for the calibration curves prepared from the calibrators were as follows: $y = (0.0048 \pm 0.0001)x + (0.1521 \pm 0.0140)$, $r = (0.9993 \pm 0.0005)$, for AQ and $y = (0.0035 \pm 0.0001)x + (0.0977 \pm 0.0067)$, $r = (0.9979 \pm 0.0013)$, for DAQ, where y is the peak area ratio of AQ or DAQ to IS, x is the concentration of AQ or DAQ in nanograms per milliliter, and r is the correlation coefficient. The CV (%) obtained for regression parameters following repeated runs for AQ were 1.57% (slope), 9.12% (intercept), and 0.05% (correlation coefficient), while the corresponding values for DAQ were 1.59%, 6.89%, and 0.13% for the slope, intercept, and correlation coefficient, respectively. The results showed good linearity after unweighted simple linear re-

FIG 3 Plasma concentration-time curves of AQ (▲) and DAQ (●) in 6 subjects with falciparum malaria who received FDC AQAS (612/200 mg for 3 days). The error bars indicate SD.

gression analysis (Table 1). The concentrations of six spiked calibration standards from 10 to 100 ng/ml were determined to establish the LOQ of the method. The mean $\pm$ SD of the peak area ratios obtained when plasma samples ($n = 6$) were spiked with concentrations of 100 ng/ml of both AQ and DAQ were 0.6168 ± 0.0291 and 0.3950 ± 0.0010 , respectively, with corresponding CV of 4.73% and 2.53% and biases of -2.85% and -13.8% . The spiked plasma samples were found to have S/N ratios of >10 , with precision and accuracy of $<15\%$. Thus, using an injection volume of 20 μ l, the LOQ of the present method was 2 ng on column for both AQ and DAQ in plasma. The LOD of the drugs based on the S/N of >3 was 1 ng on column using an injection volume of 20 μ l. The accuracy and precision assayed at this concentration were

-34.0 and 21.1% for AQ and -30.9 and 22.2% for DAQ, respectively. The values were above acceptable precision for the LOQ. The on-column sensitivity confirmed the method to be as sensitive as those reported by Ntale et al. (14) and Minzi et al. (10), whose on-column minimum detectable limits were 1.78 ng for AQ and 1.64 ng for DAQ, and 3.6 ng for AQ and 3.3 ng for DAQ, respectively. It is noteworthy that Gitau et al. (7) reported on-column minimum detectable limits of 5 ng for AQ and 10 ng for DAQ.

The intra- and interassay precision and accuracy of the method were evaluated with QC samples of the drugs at concentrations of 150, 300, and 900 ng/ml. At all levels, the intra- and interassay precision levels in plasma ranged from 0.418 to 4.809% and from 1.302 to 5.203% (CV), in that order, while the intra- and interassay accuracy levels ranged from -6.348 to 9.494% and from -6.098 to 9.22% , respectively. These results showed that the proposed method was precise and accurate. The extraction procedure for AQ and DAQ yielded mean recoveries of 80.0 and 68.9%, respectively. The recovery was above the recommended minimum of 50% (21) and therefore acceptable. In other studies, Lindgarth et al. (22) reported recoveries of 47% and 40% for AQ and DAQ, respectively, while Ntale and coworkers (16) reported corresponding recoveries of 49% and 48%. Further details of the intra- and interassay precision, accuracy, and extraction recovery are summarized in Table 2.

Stability of AQ and DAQ in plasma. Stability studies carried out with the plasma QC samples of both AQ and DAQ to determine whether any sample deterioration occurred showed no significant decrease in the concentration at -20°C for 3 months. Stock solutions of the drugs stored under the same condition were stable. The evaluated stability period was more than our storage

TABLE 5 Pharmacokinetics of AQ and DAQ in the present and previous studies

				PK result (±SD)						
				AQ			DAQ			
Subject(s)	No. of subjects	Sampling period (h)	Dosage regimen	C _{max} (ng/ml)	T _{max} (h)	T _{1/2} (h)	C _{max} (ng/ml)	T _{max} (h)	T _{1/2} (h)	Reference
Adult patients	6	336	Single oral dose for 3 days ^a	261 ± 15.1	0.75 ± 0.27	8.65 ± 1.74	753 ± 172	48.0	213 ± 1.33	Present study
Healthy volunteer	1	24	Single oral dose for 1 day ^b	258	2.00	NA ^d	1,138	2.00	NA	7
Healthy volunteers	3	1,440	Single oral dose for 1 day ^a	47.6 ± 14.8	0.58 ± 0.14	2.07 ± 1.29	802 ± 501	0.83 ± 0.29	114 ± 77.7	8
Healthy volunteer	1	168	Single oral dose for 1 day ^b	11.2	1.00	14.5	822	2.00	149	10
Healthy volunteers	13	480	Single oral dose for 1 day ^b	29.2 ± 10.9	2.32 ± 1.16	5.50 ± 4.10	269 ± 70.8	3.68 ± 1.85	241 ± 147	11
Healthy volunteers	7	96	Single oral dose for 1 day ^b	32.0 ± 3.00	0.50 ± 0.03	5.20 ± 1.70	181 ± 26.0	3.40 ± 0.80	NA	13
Pediatric patients	12	672	Single oral dose for 3 days ^c	618–1,454	72	NA	575–3,940	72	NA	14
Healthy volunteers	3	24	Single oral dose for 1 day ^b	225	1	18.61	1,001 ± 354	2 ± 1	19 ± 5	16
Healthy volunteers	23	1,440	Single oral dose for 1 day ^c	61.7 ± 24.8	1.44 ± 1.11	2.2 ± 1.1	973 ± 511	1.7 ± 0.77	224 ± 102	17

^a Amodiaquine administered as a fixed-dose combination (AQAS).

^b Amodiaquine administered as monotherapy (AQ).

^c Amodiaquine administered as a non-fixed-dose combination (AQ-AS).

^d NA, not available.

FIG 4 (A) Scatter matrix plot showing correlation of age, body weight (BW), DAQ AUC_{7-∞}, DAQ AUC_{0-∞}, and DAQ AUC_{0-t} with the DAQ concentration on day 7. (B to E) Scatter dot plots comparing DAQ AUC_{0-t} (B), DAQ AUC_{0-∞} (C), DAQ AUC_{7-∞} (D), and DAQ concentration on day 7 (E) in female and male patients treated with FDC AQAS.

period, i.e., the time from the first sample collection to the last day of our analysis. The analytes were also stable on the bench at the ambient temperature at which the study was conducted. The results of the stability test, which included long-term, short-term, freeze-thaw, and bench stabilities, are summarized in Table 3. The result suggests that plasma samples containing the drugs did not deteriorate under the laboratory conditions used in the study.

Pharmacokinetic application. The validated analytical method was applied to quantify the plasma concentrations of both AQ and DAQ when amodiaquine was coadministered with artesunate as a fixed-dose combination to treat patients with uncomplicated falciparum malaria. A total of 72 plasma samples were obtained from six patients over a period of 14 days after oral administration of FDC AQAS. The data obtained were used to generate pharmacokinetic parameters (Table 4) in individual patients and pharmacokinetic profiles by plotting the plasma drug concentrations of AQ and DAQ in plasma versus time (Fig. 3). Although the pharmacokinetic parameters of AQ and DAQ observed in our study compared well with those from previous studies, there were some variations in the values reported among the studies (7, 8, 10, 11, 13, 14, 16, 17) from malaria patient or healthy adult volunteers (Table 5). The disparities could be due to differences in race, study designs, subjects, sampling duration, and dosage regimen. DAQ was detected in the plasma 0.5 h after administration of FDC AQAS. This has lent credence to the observation (2) that AQ is rapidly and extensively metabolized to its pharmacologically active derivative DAQ, which is probably responsible for most of the therapeutic effect, and hence, the suggestion to monitor DAQ and not AQ *in vivo* (23). For a slowly eliminated drug like DAQ, the AUC is an important pharmacokinetic parameter for parasite clearance, and a correlation between the plasma concentration on day 7 and the AUC has been observed (24). It is, however, easier to characterize the AUC after day 7 ($AUC_{7-\infty}$) than the total AUC ($AUC_{0-\infty}$), as the latter is affected by more variable factors, like the rate and extent of absorption, the initial volume of distribution, and the elimination phase of the drug (25). In the present study (Fig. 4A), we observed that the DAQ concentration on day 7 had highly significant positive relationships with the $AUC_{7-\infty}$ (Pearson $r = 0.999$; $P = 0.0001$), the AUC on day 7 (Pearson $r = 0.861$; $P = 0.028$), AUC_{0-t} (Pearson $r = 0.930$; $P = 0.007$), $AUC_{0-\infty}$ (Pearson $r = 0.903$; $P = 0.014$), and body weight (Pearson $r = 0.882$; $P = 0.02$). There was, however, a nonsignificant negative relationship with age (Pearson $r = -0.616$; $P = 0.193$). The strong association between the DAQ concentration on day 7 and the $AUC_{7-\infty}$ could also suggest the latter is an important pharmacokinetic parameter in the determination of treatment outcome. The $AUC_{7-\infty}$ captures both the time above which the plasma or blood concentration of an antimalarial drug exceeds the minimum parasitocidal concentration ($T > MPC$) and the amount of drug to which the residual parasites are exposed during the elimination phase of slowly eliminated antimalarial drugs. The study also showed that body weight contributed about 78% of the variability observed in the DAQ concentration on day 7 and therefore, providing further support for the use of a weight-based regimen in dosing. The role of gender in the disposition of drugs remains controversial. Some sources suggest that the activity of drug-metabolizing enzymes is higher in women than in men (26), while others found no effect (27). In the present study, though no statistically significant difference (Table 6) was found in the disposition of DAQ between female and male subjects with falcipa-

TABLE 6 Comparative pharmacokinetic parameters of DAQ in female and male patients with uncomplicated malaria

Parameter	Value (mean $\pm$ SD) ($n = 3/\text{group}$)		<i>P</i> value
	Female	Male	
$C_{\max}$ (ng/ml)	740 $\pm$ 197	767 $\pm$ 185	0.872
$T_{\max}$ (h)	48.0	48.0	
$T_{1/2}$ (h)	212 $\pm$ 1.14	214 $\pm$ 0.84	0.093
AUC_{0-t} (h $\cdot$ ng/ml)	120,840 $\pm$ 15,962	119,750 $\pm$ 11,241	0.928
$AUC_{0-\infty}$ (h $\cdot$ ng/ml)	185,080 $\pm$ 20,813	184,940 $\pm$ 16,370	0.993
AUC on day 7 (h $\cdot$ ng/ml)	73,790 $\pm$ 9,669	74,153 $\pm$ 7,631	0.962
Concn on day 7 (ng/ml)	350 $\pm$ 63.5	332 $\pm$ 27.9	0.667

rum malaria following treatment with FDC AQAS, the intraindividual variability in the DAQ AUC_{0-p} , $AUC_{0-\infty}$, $AUC_{7-\infty}$, and AUC on day 7 was higher in females (Fig. 4B to E). This observation needs further confirmation in larger studies, since a small number of patients ($n = 3/\text{group}$) were recruited.

In conclusion, the liquid chromatographic method we have described not only retains the precision and accuracy of other methods for the simultaneous determination of AQ and its major metabolite, but also adds to the body of knowledge available for determination of the drugs when AQ is coadministered with artesunate as a fixed-dose combination to treat patients with uncomplicated malaria. The method was successfully applied to study the disposition of AQ and DAQ following single oral administration of FDC AQAS (612/200 mg) for 3 days in patients with uncomplicated falciparum malaria, and the pharmacokinetic parameters of the drugs were consistent with previously published values. Easy access to HPLC with UV detection compared to electrochemical detection in most research centers in sub-Saharan Africa and the composition of the mobile phase (mainly [80%] distilled water and a small volume of commonly available methanol and triethylamine) have made the method affordable and practical for use for TDM in resource-poor areas of sub-Saharan Africa where malaria is endemic.

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We declare that we have no competing interests.

O.N.A. participated in the conceptualization of the study and its design, drug assay, data analysis, and manuscript preparation. O.O.B. supervised drug assay and manuscript preparation. C.O.F. was involved in the clinical evaluation of the method and in preparation of the manuscript. O.A.O. recruited medical personnel and monitored the study. O.G.A. participated in the conceptualization of the study and its design and in manuscript preparation.

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