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HPLC with ultraviolet detection for the determination of chloroquine and desethylchloroquine in whole blood and finger-prick capillary blood dried on filter paper

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ABSTRACT

A simple, sensitive, selective and reproducible method based on liquid chromatography was developed for the determination of chloroquine (CQ) and its active plasma metabolite desethylchloroquine (DECQ) in finger-pricked capillary blood spot onto filter paper (DBS) and whole blood samples. Both were separated from the internal standard quinine on a reversed phase C18 column, with the mobile phase consisting of a mixture of 1% diethylamine, acetonitrile and methanol (20:55:25, v:v:v) running at a flow rate of 1.0 ml/min. Retention times of QN, DECQ and CQ were 4.5, 5.7 and 6.4 min, respectively. Ultraviolet detection was at the wavelength 256 nm. Sample preparation was done by extraction with hexane and *tert*-butyl methyl ether (1:1, v:v). Good precision and accuracy were obtained for both within-day repeatability and day-to-day reproducibility. Limit of quantification (LOQ) for both CQ and DECQ was accepted as 50 ng/ml using 80 μl DBS sample and 25 ng/ml using 150 μl whole blood sample. The mean recoveries for CQ, DECQ and internal standard for both whole blood and DBS were between 74 and 87%. The method was successfully applied for a pharmacokinetic study of CQ and DECQ in patients with *Plasmodium vivax*. Excellence correlation ($r = 0.997$) was observed between the analysis of both CQ and DECQ in paired whole blood and DBS samples.

1. Introduction

Malaria is one of the leading causes of morbidity and mortality in the tropics with an annual estimate of 500 million clinical cases and 2 million deaths [1]. The treatment of malaria is becoming increasingly difficult due to *Plasmodium falciparum* resistance to commonly used antimalarials, particularly the 4-amoniquinoline chloroquine (CQ). Clinical use of CQ is now mainly as treatment of choice for *P. vivax*, *P. ovale* and *P. malariae* infections in most parts of the world [1].

A number of analytical methods have been reported for measuring CQ and its active main metabolite desethylchloroquine (DECQ) in biological fluids (plasma, serum, whole blood, red cells). Major methods involve high-performance liquid chromatographic techniques (HPLC) coupled with ultraviolet (UV) [2–5] or fluorescence [6–11] detection. The earlier methods with ultraviolet detection lack sensitivity and specificity as they were unable to differentiate the parent CQ from DECQ [2–5]. HPLC methods with fluorescence detection achieve the highest sensitivity and selectivity [6–11]. Several methods (HPLC with UV or fluorescence detection, UV spectrometer, TLC densitometer) have also been developed for simultaneous determination of CQ and its metabolites with other antimalarials. These include CQ with quinine [12]; CQ with primaquine and bulaquine [13]; CQ with DECQ, chlorproguanil and proguanil [14]; CQ with DECQ, bisdesethyl-chloroquine and 4-amino-7-chloroquine [4]; CQ with de-ethylated metabolites [6]; CQ with mefloquine [15] CQ with mono-DECQ and amodiaquine [16]; CQ with quinine, monoDECQ and DECQ [9]; CQ with proguanil and metabolites [17]; and CQ with amodiaquine and metabolites [18]. Sample preparations include both liquid–liquid extraction and solid phase extraction.

To be able to perform clinical pharmacology studies involving CQ and its active plasma metabolite under field conditions, methods for quantification of CQ and DECQ in whole blood are necessary. Most of the above mentioned methods measure CQ and DECQ in whole blood, plasma, serum or red cell samples collected by venipuncture. Whole blood and serum concentrations of CQ and DECQ are, respectively, about 5–10 and 2 times higher than that observed in plasma due to uptake to platelets and granulocytes. Determination of CQ and DECQ in whole blood samples requires no sample preparation with low blood volume collection, and is thus considered the most appropriate biological fluid for pharmacokinetics and application to field monitoring of CQ resistance *P. vivax* malaria. Further advancement was the development of methods

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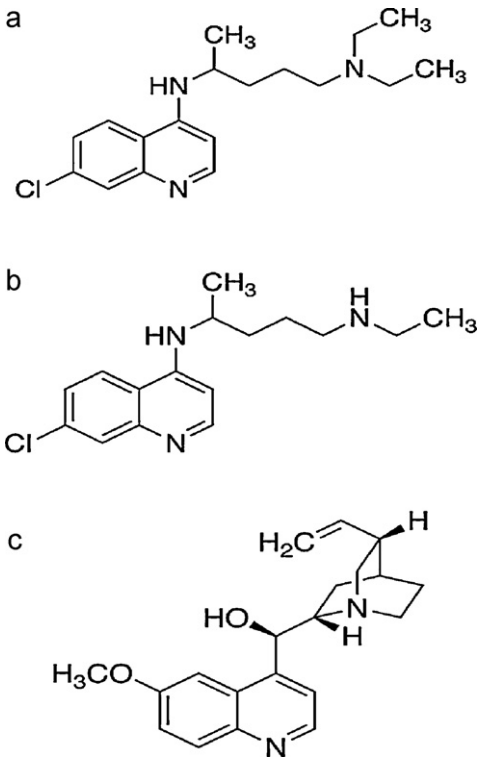

Fig. 1. Chemical structures of (a) chloroquine (CQ), (b) desethylchloroquine (DECQ) and (c) internal standard quinine (QN).

for the determination of CQ and DECQ from capillary blood (finger-prick) spot onto filter paper (dried blood spot: DBS) [14,19–22] as a further step to facilitate the filed studies. A good correlation between analysis of CQ and DECQ in venous and DBS samples has been found [21]. Capillary sampling from a finger prick is much less invasive and generally well accepted for patients with young age below 5 years. The sampling for drug assay can also be collected in parallel with blood smears for malaria microscopy as well as blood samples for isolating DNA for PCR-based genotyping of drug resistance in malaria parasites. In addition, its advantages include the simplicity (no sample preparation), no requirement on facilities and storage conditions (*i.e.* centrifuge, freezer), and small volume of blood (50–200 μl). All these make DBS a cost-effective choice, especially for studies in malaria endemic countries particularly in remote locations.

In the present report, we describe an alternative HPLC with UV detection method, which is relatively simple, rapid, sensitive, accurate and reproducible method for the determination of CQ and DECQ in capillary blood spot onto filter paper. This was performed in comparison with whole blood samples. The method has been applied successfully for pharmacokinetic study of CQ and DECQ in Thai patients with *P. vivax* infection following standard regimen of CQ.

2. Materials and methods

2.1. Chemicals

All solvents were HPLC grade. Organic solvents were purchased from Fison Scientific Equipment (Bishop Meadow Road, Loughborough, U.K.). Hydrochloric acid and diethylamine were obtained from Sigma Chemical Co. (St. Louis, M.O., U.S.A.). Ultrapure analytical grade Type I water ($r > 18\,M\Omega/cm$) was produced by a Milli-Q Plus^{TM} water system (Millipore Corporation, Bedford, MA, USA). Chloroquine (CQ) and desethyl-chloroquine (DECQ) and the internal standard quinine (QN) (Fig. 1a–c) were obtained from Sigma–Aldrich Inc. (St. Louis. MO, USA).

2.2. Standard stock solutions

Stock solutions were prepared for CQ, DECQ and QN by dissolving appropriate amounts of chemicals in distilled water in volumetric flasks. Stock solutions for CQ, DECQ and QN were prepared at the concentration of 1000 ng/μl. The stock solutions were further diluted to make working solutions at concentrations of 10 for CQ, DECQ, and QN. Standard solutions were stored at −80 °C until use.

2.3. Chromatography

The method was developed on a chromatographic system consisting of the elution solvent delivery (SpectraSystem P4000 Quaternary Solvent Delivery/Controller: Thermo Fisher Scientific, CA, USA), equipped with solvent degasser (SpectraSystem SCM1000 Solvent Degasser: Thermo Fisher Scientific, CA, USA), an auto-sampler (SpectraSystem AS3500: Thermo Fisher Scientific, CA, USA) and a UV detector (SpectraSystem UV/Vis 3000: Thermo Fisher Scientific, CA, USA). The wavelength was set at 256 nm. The separation was carried out on a reversed-phase column (Thermo Hypersil Gold C18, 250 mm × 2.1 mm i.d., 5 μm: Fisher Scientific, CA, USA). The elution solvent consisted of 1% diethylamine, acetonitrile and methanol at the ratio of 20:55:25 (v:v:v). The chromatographic analysis was operated at 25 °C. Aliquots of 200 μl samples or standard solutions were injected onto the column with an elution buffer at flow rate of 1.0 ml/min.

2.4. Sample preparation

2.4.1. Whole blood

The procedure was validated on specimens using 150 μl of spiked human whole blood. Outdated human whole blood was obtained from the Blood Bank of Thammasat Chalermprakiet Hospital, and stored frozen in aliquots at −20 °C. To 150 μl whole blood, 35 μl internal standard (QN) working solutions (350 ng) was added. After thoroughly mixing, 500 μl of 0.2 M hydrochloric acid was added. The sample was mixed thoroughly and allowed to stand for 2 min at room temperature (25 °C). One milliliter of 20% sodium hydroxide and 5 ml of the mixture of hexane and *tert*-butyl methyl ether (1:1, v:v) were added, and gently mixed for 30 min. The organic phase (upper layer) was separated through centrifugation at 3000 × *g* (4 °C) for 5 min. The organic phase was transferred to a new polypropylene tube and evaporated to dryness under nitrogen stream at 40 °C. The residue was reconstituted with 200 μl of the mobile phase and 80 μl was injected onto the column.

2.4.2. Dried blood spot

Standards were prepared by spotting CQ-free, finger prick capillary blood (80 μl) onto filter paper (Whatman No. 3, GE Healthcare, CA, USA). After cutting out, each dried spot (DBS) was placed in a 15 ml screw-cap glass tube and 500 μl of 0.5% diethylamine and 350 ng (10 μl working solution) QN (internal standard) were added. The tube was vortexed and 1 ml of 20% sodium hydroxide was added. The tube was mixed by sonicating for 15 min. Thereafter, 5 ml of the mixture of hexane and *tert*-butyl methyl ether (1:1, v:v) was added and gently mixed for 30 min followed by centrifugation at 3000 × *g* (4 °C) for 10 min. The upper organic layer was then transferred to a clean silanized glass tube and dried under a stream of nitrogen at 40 °C. The residue was reconstituted with 200 μl of mobile phase and 80 μl was injected onto the column.

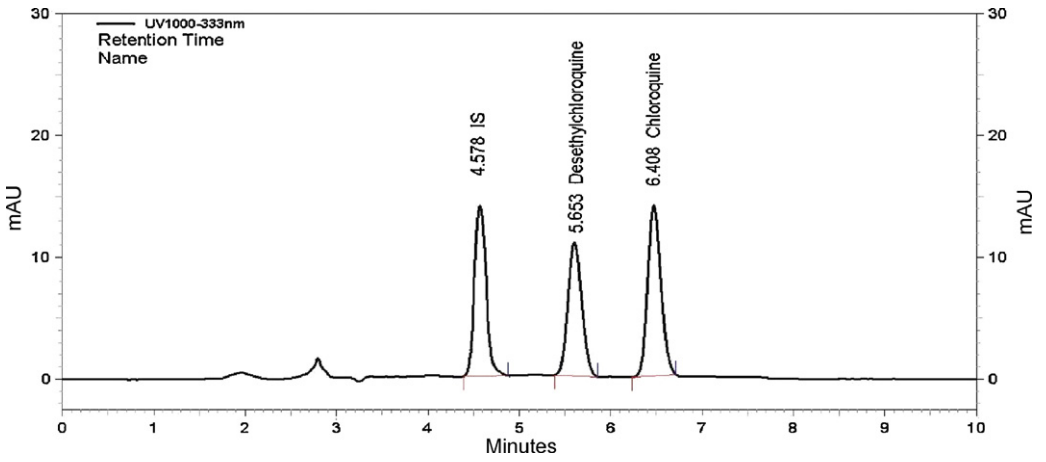

Fig. 2. Chromatogram of standard solution of internal standard QN (350 ng), DECQ (250 ng) and CQ (250 ng).

2.5. Calibration curves

2.5.1. Detector linearity

Solutions of CQ, DECQ and QN in distilled water at concentrations ranging from 25 to 1500 ng/ml were injected into the HPLC system in order to assess detector linearity. Peak heights were plotted against the quantities of all standards injected. All were found to be linear ($r > 0.999$) over the concentration range observed.

2.5.2. Whole blood

For analysis of CQ and DECQ in whole blood samples, calibration curves were prepared by replicate analysis of seven whole blood samples (150 μl each) spiked with varying concentrations of CQ and DECQ (25, 50, 100, 250, 500, 1000, 1500) and a fixed concentration of the internal standard (350 ng). Samples were analyzed as described in Section 2.4.1.

2.5.3. Dried blood spot

For analysis of CQ and DECQ in DBS samples, calibration curves were prepared by replicate analysis of six dried blood spot samples (80 μl of capillary blood each) spiked with varying concentrations of CQ and DECQ (50, 100, 250, 500, 1000, 1500 ng/ml) and a fixed concentration of the internal standard (350 ng). Samples were analyzed as described in Section 2.4.2.

2.6. Data analysis

Peak height ratios of CQ/internal standard and DECQ/internal standard were calculated. Concentrations of CQ and DECQ in both whole blood and DBS samples were determined by matching peak height responses against a calibration curve of response ratio (height of CQ or DECQ/height of internal standard) *vs* concentration, obtained from standard sample injection. The

Table 1
Summary of assay precision and accuracy (intra-assay and inter-assay) for CQ and DECQ assay in whole blood and dried blood spot samples.

	Concentration added (nmol/100 μl)	Precision (%C.V.)		Accuracy (%DMV)[a]	
		Intra-assay	Inter-assay	Intra-assay	Inter-assay
(a) Whole blood					
CQ	25	2.2	3.3	6.1	7.0
	50	1.4	3.6	5.5	5.2
	100	0.9	2.0	3.3	2.4
	250	0.6	0.6	1.6	1.9
	500	2.9	2.2	0.2	2.9
	1000	1.3	2.5	3.1	5.3
	1500	1.1	2.0	−3.4	−5.3
DECQ	25	2.0	2.6	6.3	9.1
	50	1.3	4.7	4.8	4.9
	100	0.9	2.0	4.6	2.4
	250	0.4	0.9	2.0	0.9
	500	4.9	1.5	−3.0	4.3
	1000	1.8	1.6	−0.3	−0.4
	1500	1.1	1.7	−3.4	−5.5
(b) Dried blood spot					
CQ	50	1.8	1.9	2.3	4.9
	100	0.8	1.5	0.2	5.2
	250	1.1	1.2	−1.4	1.0
	500	3.6	2.3	−2.7	2.1
	1000	1.9	1.2	0.09	−1.8
	1500	3.2	1.7	−2.9	−5.0
DECQ	50	1.3	3.4	2.3	5.1
	100	0.9	1.7	2.2	3.0
	250	0.7	2.8	−0.3	0.7
	500	2.2	0.9	−2.5	2.6
	1000	2.0	1.1	−2.6	−1.4
	1500	0.9	1.7	−3.0	−5.7

[a] %DMV = deviation of mean value from theoretical value (%).

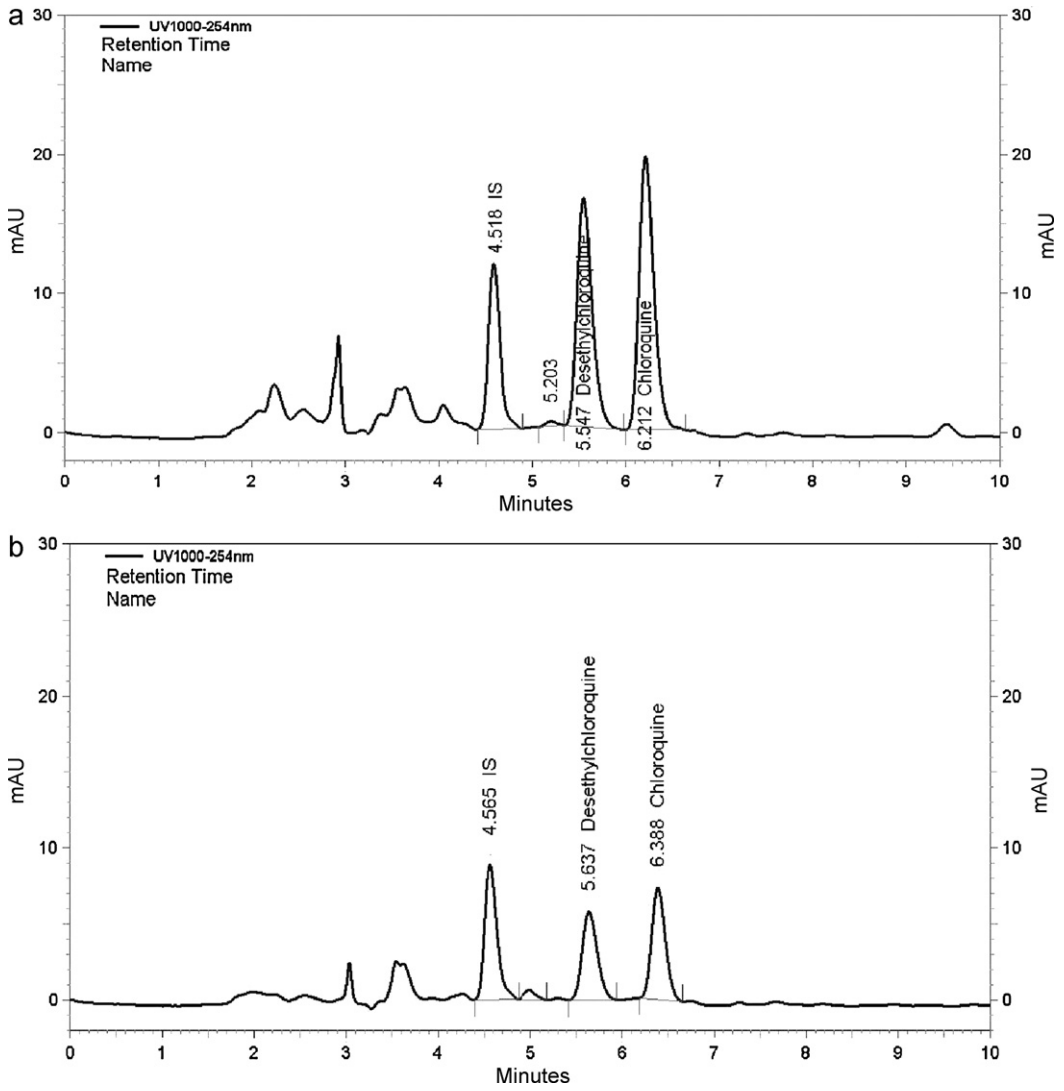

Fig. 3. Chromatograms of (a) whole blood spiked with 350 ng QN (internal standard), 250 ng DECQ, and 250 ng CQ, 1000 ng paracetamol, 1000 ng dimenhydrinate, 1000 ng mefloquine, 1000 mg sulfadoxine, 1000 mg pyrimethamine, 1000 mg artemisinin, 1000 mg primaquine, 1000 mg praziquantel and 1000 mg albendazole; and (b) whole blood sample collected from one patient after 1 h of chloroquine administration on the first day (spiked with 350 ng QN).

internal standard corrected for variation in the sample preparation (protein precipitation/dilution) steps used. Peak detection, peak height integration, peak height ratio calculation, calibration curve fitting (least square regression without weighting) and calculation of sample concentrations were performed by the Millenium 2000 Chromatograph™ software (Waters, Milford, MA, USA).

2.7. Method validation

2.7.1. Precision

The precision of the methods for the determination of CQ and DECQ in whole blood and DBS samples based on *within-day repeatability* was determined by replicate analysis of six sets of samples spiked with seven different concentrations of CQ and DECQ for whole blood (25, 50, 100, 250, 500, 1000 and 1500 ng/ml) and six different concentrations of CQ and DECQ for DBS (50, 100, 250, 500, 1000 and 1500 ng/ml) samples. The *reproducibility (day-to-day variation)* of the method was validated using the same concentration ranges of whole blood and DBS samples as described above but only a single determination of each concentration was made on six different days. Coefficient of variation (CV) were calculated from the ratios of standard deviation (SD) to the mean and expressed as percentage.

2.7.2. Accuracy

The accuracy of the methods for the determination of CQ and DECQ in whole blood and DBS samples was determined by replicate analysis of six sets of samples spiked with seven different concentrations of CQ and DECQ for whole blood (25, 50, 100, 250, 500, 1000 and 1500 ng/ml) and six different concentrations of CQ and DECQ for DBS (50, 100, 250, 500, 1000 and 1500 ng/ml) samples, and comparing the difference between spiked value and that actually found (theoretical value).

2.7.3. Recovery

The analytical recovery of sample preparation procedures for CQ and DECQ in whole blood and DBS samples was estimated by comparing the peak heights obtained from samples (whole blood, DBS) prepared as described in Section 2.4, with those measured with post-extraction sample spiked with equivalent amounts of CQ, DECQ or QN. For both whole blood and DBS samples, triplicate analysis of CQ and DECQ was performed at the concentrations of 50, 500, and 1500 ng/ml. The tripli-

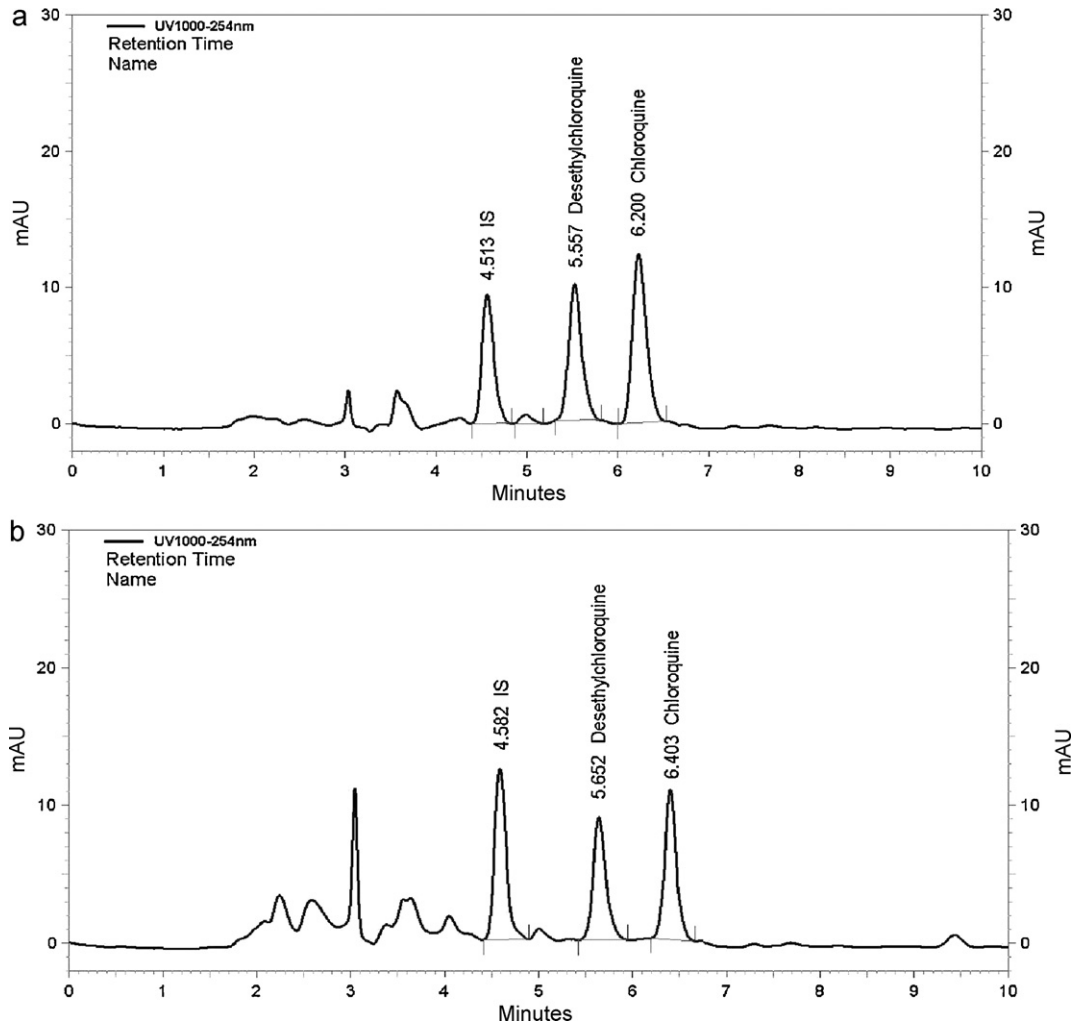

Fig. 4. Chromatogram of dried blood spot samples spiked with (a) 350 ng QN (internal standard), 250 ng DECQ, and 250 ng CQ, 1000 ng paracetamol, 1000 ng dimenhydrinate, 1000 ng mefloquine, 1000 mg sulfadoxine, 1000 mg pyrimethamine, 1000 mg primaquine, 1000 mg artemisinin, 1000 mg praziquantel and 1000 mg albendazole; and (b) dried blood spot collected from one patient after 1 h of the administration of chloroquine (spiked with 350 ng QN).

cate analysis of QN was performed at a single concentration of 350 ng/ml.

2.7.4. Selectivity

The selectivity of the methods (whole blood and DBS) was verified by checking for interference by commonly used drugs, *i.e.*, antimalarials (mefloquine, sulfadoxine, pyrimethamine, primaquine, artemisinin), anthelminthics (albendazole, praziquantel), paracetamol and dimenhydrinate after subjecting them to sample preparation procedures.

2.7.5. Limit of quantification

The limit of quantification (LOQ) of the assay procedure was determined from the lowest concentration of CQ or DECQ (in spiked whole blood or DBS samples) that produced a peak height ten times the baseline noise at a sensitivity of −0.2 μA in a 150 (whole blood) or 80 (DBS) μl sample.

2.7.6. Stability

The stability of CQ and DECQ in whole blood samples was determined by storing spiked whole blood samples (concentrations 50, 500, and 1500 ng/ml; triplicate analysis for each concentration) in a −80 °C freezer (Sanyo, Japan) for three months. Concentrations were measured periodically (1, 15 days and 1 and 3 months). For freeze and thaw stability, whole blood samples were frozen at −80 °C for at least 24 h and thawed unassisted at room temperature (25 °C). When completely thawed, the samples were transferred back to the original freezer and refrozen for at least 24 h. The process was repeated for three cycles.

The stability of CQ and DECQ in DBS samples was determined by storing spiked DBS samples (concentrations 50, 500, and 1500 ng/ml; triplicate analysis for each concentration) and stored in plastic zipper bags in desiccators at room temperature (25 °C) for three months. Concentrations were measured periodically (1, 15 days and 1 and 3 months).

2.8. Quality control

Quality control (QC) samples for CQ and DECQ were made up in whole blood and DBS samples using a stock solution separate from that used to prepare the calibration curve, at the concentrations 75, 500 and 1500 ng/ml and 150, 500 and 1500 ng/ml for whole blood and DBS sample, respectively (triplicate each). For whole blood, samples were aliquotted into cryovials, and stored frozen at −80 °C for use with each analytical run. DBS samples were stored in plastic zipper bags in a desiccators at room temperature (25 °C).

The results of the QC samples provided the basis of accepting or rejecting the run. At least four of every six QC samples had to be within ±20% of their respective nominal value. Two of the six

Table 2
Storage stability data of CQ and DECQ in (a) whole blood and (b) dried blood spot at concentrations 50, 500, and 1000 ng/ml.

		CQ			DECQ		
		Mean (S.D.)	SD	%DMV[a]	Mean (S.D.)	SD	%DMV[a]
(a) Whole blood							
1 day	50	53.4	1.7	6.8	50.3	1.5	0.7
	500	478.2	23.0	−4.3	501.9	7.4	0.3
	1000	999.0	58.8	−0.1	967.7	15.2	−3.2
15 days	50	54.2	1.9	8.4	52.7	1.0	5.4
	500	492.6	12.8	−1.46	460.4	11.9	−7.9
	1000	1054.6	10.2	5.4	1023.9	9.9	2.4
30 days	50	55.4	52.8	2.8	51.2	2.7	2.5
	500	496.9	512.4	19.5	488.7	35.1	−2.2
	1000	1103.7	1061.7	36.9	984.4	34.3	−1.5
90 days	50	56.9	53.8	5.6	52.8	2.8	5.6
	500	510.3	520.9	20.8	508.7	19.1	1.7
	1000	1131.3	1079.1	83.8	1045.8	12.3	4.5
(b) Dried blood spot							
1 day	50	49.9	2.21	−0.1	48.3	0.7	−3.2
	500	494.5	13.5	−1.0	499.8	15.1	−0.02
	1000	1063.8	11.9	6.3	1052.6	20.8	5.2
15 days	50	52.3	1.3	4.6	49.2	1.0	−1.4
	500	516.5	8.5	3.3	507.6	13.1	1.5
	1000	975.2	7.6	−2.4	982.1	11.3	−1.7
30 days	50	50.3	3.9	0.7	48.2	1.5	−2.1
	500	508.4	13.7	1.6	495.2	24.1	−0.9
	1000	1025.7	7.0	2.5	1028.0	18.4	2.8
90 days	50	50.6	1.8	1.3	52.3	2.4	4.7
	500	516.1	10.7	3.2	508.2	17.1	1.6
	1000	968.3	16.2	−3.1	941.8	11.1	−5.8

[a] %DMV = deviation of mean value from theoretical value (%).

QC samples could be outside the ±20% of their respective nominal value, but not at the same concentration.

2.9. Application of the method to biological samples

The method was applied to the investigation of the pharmacokinetics of CQ and DECQ in whole blood and DBS samples in a total of 10 Thai patients with *P. vivax* malaria (aged 20–28 years, weighing 50–62 kg) who received treatment with chloroquine (Government Pharmaceutical Organization of Thailand) at the dose of 2000 mg chloroquine phosphate given over 3 days (500 mg at 0, 6–12 h, and on days 2 and 3). This was part of a large clinical trial to monitor clinical efficacy of chloroquine for treatment of *P. vivax* infection in Thailand. The study was approved by the Ethics Committee of Ministry of Public Health of Thailand. Written informed consents for study participation were obtained from all patients. Venous blood samples (2 ml) were collected into heparinized plastic tubes at the following time points: day-1 (0, 1, 6, 12 h), day-2 (0, 1, 6, 12 h), day-3 (0, 1 h), day-4, -7, -14, -21, -28, -35 and -42. Finger-prick capillary blood samples were also collected from each patient at the same schedule. Whole blood samples were stored immediately after collection at −80 °C until analysis. DBS samples were stored in plastic zipper bags in a desiccators until analysis.

2.10. Correlation between CQ/DECQ concentrations in whole blood and dried blood spot samples

Correlation between the analysis of CQ and DECQ concentrations in whole blood and DBS samples were determined from paired samples collected from three patients as described in Section 2.9.

3. Results and discussion

We describe a HPLC assay procedure based on reversed phase chromatography with UV detection for the specific, sensitive, accurate and reproducible quantitative analysis of CQ and DECQ in

Table 3
Freeze and thaw stability for CQ and DECQ in spiked whole blood samples at concentrations of 50, 500 and 1500 ng/ml at room temperature (freshly prepared) and following three freeze (−80 °C)–thaw cycles.

Storage condition	Target concentration (ng/ml)	Measured concentration (ng/ml) (mean ± SD; $n = 3$)	
		CQ	DECQ
Freshly prepared	50	50.1 ± 0.8	50.3 ± 1.6
	500	501.3 ± 1.2	501.4 ± 1.9
	1500	1503.4 ± 2.9	1489.6 ± 5.3
6 h at room temperature (25 °C)	50	50.1 ± 0.3	50.5 ± 0.2
	500	502.2 ± 1.2	500.3 ± 1.8
	1500	1495.0 ± 1.6	1490.0 ± 2.7
1 freeze–thaw cycle	50	50.4 ± 0.7	50.0 ± 0.6
	500	492.1 ± 1.0	489.1 ± 1.4
	1500	1497.9 ± 3.1	1490.7 ± 4.0
2 freeze–thaw cycle	50	50.4 ± 0.9	50.0 ± 0.6
	500	501.6 ± 1.5	489.1 ± 1.4
	1500	1508.9 ± 4.7	1510.7 ± 5.0
3 freeze–thaw cycle	50	50.0 ± 1.1	50.0 ± 1.6
	500	509.3 ± 1.2	508.1 ± 1.4
	1500	1493.8 ± 4.0	1489.7 ± 5.0

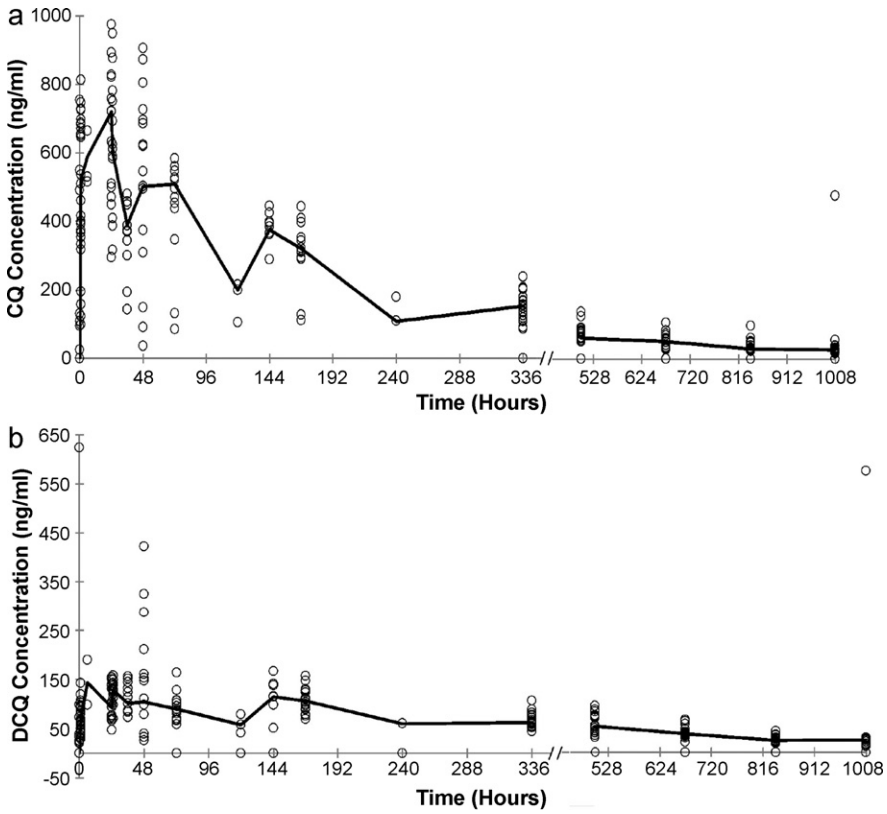

Fig. 5. Whole blood concentrations of (a) CQ and (b) DECQ in 10 Thai patients with P. vivax malaria. Data are presented as median (line) and individual (circle) values.

human whole blood and DBS samples. The developed method was relatively simple compared with the previous methods [19–22]. With UV detection, sensitivity was also comparable to that with fluorescence detection [19]. Sampling finger-prick capillary blood on filter paper is considered easier to perform, requires less training (finger puncture with a lancet), less invasive than conventional venous collection, and involves little discomfort or risk to the participant. In addition, storage and transportation of samples do not require cold chain or a concern of degradation as observed with other plasma, serum or whole blood samples. There is also very limited biohazard risk for laboratory workers.

3.1. Chromatographic separation

A number of HPLC chromatographic systems were investigated to optimize the separation of CQ, DECQ and the internal standard QN. Retention maps were generated for all compounds as a function of stationary phase and elution solvent (mobile phase). For, the elution solvent, composition of 1% diethylamine: acetonitrile: methanol (20:55:25, v:v:v) with a reversed phase (5 μm, 250 mm × 2.1 mm; Thermo Hypersil Gold) column resulted in good separation with optimal separation. The retention times of QN, DECQ and CQ were approximately 4.6, 5.7, and 6.4 min, respectively. The chromatograms showed a good baseline separation. Chromatogram of standard solution of QN (350 ng), DECQ (250 ng/ml) and CQ (250 ng/ml) is shown in Fig. 2.

3.2. Sample preparation

A number of sample preparation procedures were investigated to optimize the extraction of CQ, DECQ and the internal standard QN from whole blood and DBS samples. The optimal sample preparation procedure used in this study was simple involving only a single step liquid–liquid extraction by organic solvents (hexane:*tert*-butyl methylether = 1:1, v:v), which resulted in clean chromatograms.

3.3. Calibration curves

Whole blood analysis of CQ and DECQ was calibrated using the concentration range of 25–1500 ng/ml, whereas DBS sample analysis was calibrated using the concentration range of 50–1500 ng/ml. All calibration ranges yielded linear relationships with correlation coefficients (r) of 0.999 or better.

3.4. Method validation

3.4.1. Precision

Little variation of CQ and DECQ assays in whole blood or DBS samples was observed; coefficients of variation (CV) for six or seven analysis at the concentration range of 25–1500 or 50–1500 ng/ml were all below 5%.

The intra-assay (within-day) and inter-assay (day-to-day) variation for CQ and DECQ assay in whole blood or DBS samples at the concentration ranges 25–1500 or 50–1500 ng/ml are summarized in Table 1a and b.

For intra-day assay validation in whole blood samples, the coefficients of variation (CV) for both CQ and DECQ varied between 0.6 and 4.9%. The inter-day assay CV varied between 0.6 and 4.7%.

For intra-day assay validation in DBS samples, the CV for both CQ and DECQ varied between 0.7 and 4.7%. The inter-day assay CV varied between 0.9 and 3.4%.

3.4.2. Accuracy

Good accuracy was observed from both the intra-day and the inter-day assays, as indicated by the minimal deviation of mean values found with measured samples from that of the theoretical values (actual amount added).

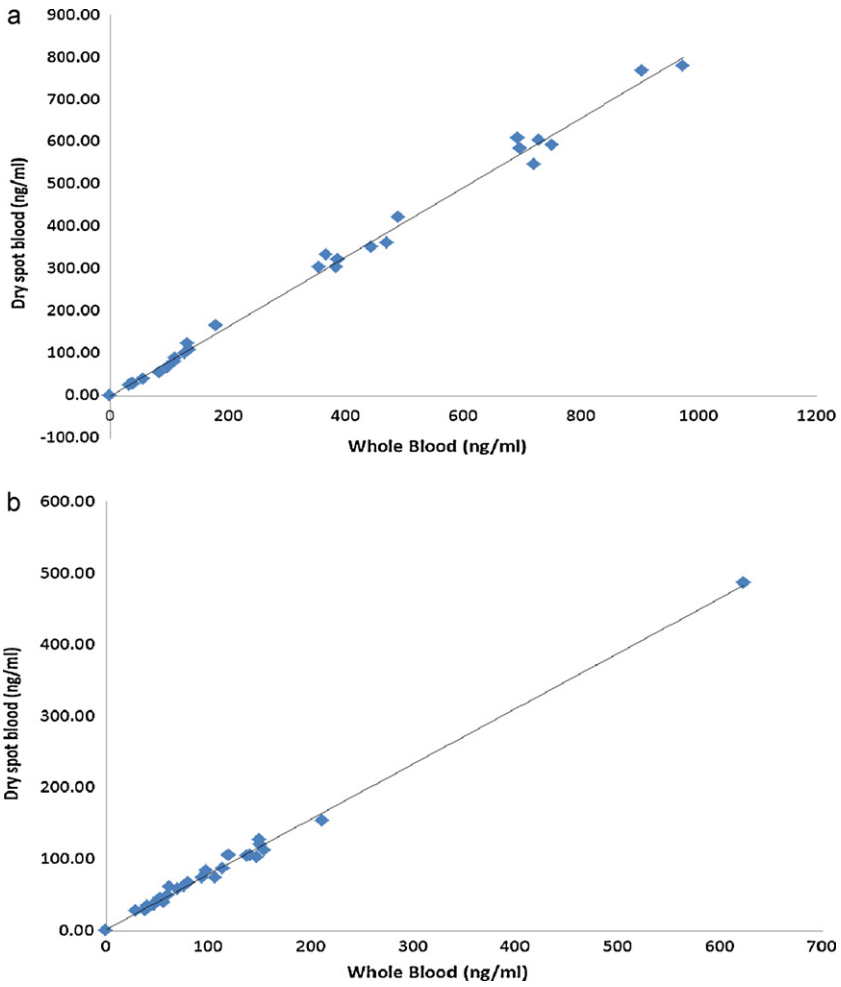

Fig. 6. Correlation between concentrations of (a) CQ ($r = 0.997$) and (b) DECQ ($r = 0.997$) in whole blood and dried blood spot samples

The intra-assay (within-day) and inter-assay (day-to-day) accuracy for CQ and DECQ assay in whole blood or DBS samples at the concentration ranges 25–1500 or 50–1500 ng/ml are summarized in Table 1a and b.

For intra-day assay validation in whole blood samples, the mean deviation from the theoretical values (MDV) varied between −3.4 and +6.1%. The inter-day assay MDV varied between −5.5 and +7.0%.

For intra-day assay validation in DBS samples, the MDV varied between −3.0 and +2.3%. The inter-day assay MDV varied between −5.7 and +5.2%.

3.4.3. Recovery

For whole blood samples, the mean recoveries for CQ at 50, 500 and 1500 ng/ml were 79.99, 80.79 and 85.33%, respectively. The corresponding values for DECQ were 74.88, 85.08 and 83.12%, respectively. The recovery of QN at the concentration of 500 ng/ml was 87.49%. Since the volume of blood adsorbed on the filter paper is smaller than 100 μl, the extraction step must be particularly efficient. Furthermore, finger-prick specimens are subject to dilution by interstitial fluid, giving a lower drug concentration. The recovery of extraction (liquid–liquid) of CQ and DECQ from DBS samples was found to be satisfactory (74–87%). The mean recoveries for CQ at 50, 500 and 1500 ng/ml were 78.19, 77.52 and 75.03%, respectively. The corresponding values for DECQ were 73.44, 76.31 and 74.89%, respectively. The recovery of QN at the concentration of 350 ng/ml was 81.32%. The results reflect essentially high recovery for all compounds from the spiked whole blood and DBS and indicate lack of interference from sample preparation procedure. The choice of filter paper has been reported to influence recovery of amodiaquine, proguanil and sulfadoxine [17] and Whatman ET 31 CHR paper appears to give an ideal combination of blood wet ability, absorption and elution properties. In our study, the method was developed using Whatman number 3 which is much cheaper and provided comparable recovery.

3.4.4. Selectivity

Selectivity of the chromatographic separation was demonstrated by the absence of interferences from endogenous peaks and commonly used drugs in whole blood and DBS samples both in spiked samples (Figs. 3a and 4a) and samples collected from one patient after 1 h after the first dose of chloroquine on the first day (Figs. 3b and 4b).

3.4.5. Limit of quantification

The limit of quantification (LOQ) in human whole blood and DBS samples for CQ was accepted as 25 ng/ml using 150 μl blood. The LOQ of DECQ in human whole blood and DBS were accepted as 50 ng/ml using 80 μl capillary blood.

3.4.6. Stability

Results from the present study show that whole blood samples containing CQ and DECQ at concentrations of 50, 500 and 1500 ng/ml were found to be stable when stored in a −80°C freezer for a minimum of three months without significant decomposition of the drug. DBS samples containing CQ and DECQ at the same

concentrations were also found to be stable when stored in zipper plastic bags in plastic in a desiccator at room temperature (25 °C) for a minimum of three months without significant decomposition of the drug. DBS samples result in an enhancement of drug stability, because of the dehydration of the sample on the filter paper and consequent minimization of enzymatic and chemical hydrolyses of the drugs. Nevertheless, the specimens should be dried at ambient temperature and humidity for at least 3–4 h before storage. The stability of CQ in whole spots on paper at room temperature was seen to be stable and only decease by approximately 20% after 20 years (Bergqvist personal communication). It is noted however for the possibility of high sampling variation, which often depends on the difference in concentration between finger-prick and venous results (Table 2).

Freeze and thaw stability of CQ and DECQ in whole blood expressed as the deviation of the mean from the values before three freeze and thaw cycles procedure were determined by repeated analysis of three aliquots of samples at three different concentrations (50, 500, 1500 ng/ml). Aliquots of each sample were stored at the intended storage temperature for 24 h and thawed at room temperature. When completely thawed, they were again refrozen for 24 h under the same conditions. The freeze–thaw cycle was being repeated three times, and then analyzed after third cycle (Table 3).

3.5. Application of assay and analysis of specimens

The methods for simultaneous analysis of CQ and DECQ developed in the current study were applied to samples collected for pharmacokinetic study with good accuracy and precision. Excellent correlation between analysis in whole blood and DBS samples was also found. Whole blood concentration-time profiles of CQ and DECQ following 2000 mg CQ phosphate over 3 days in Thai patients with *P. vivax* infection were similar to that reported previously [23]. Median whole blood concentration-time profiles of CQ and DECQ in whole blood are shown in Fig. 5a and b, respectively. All patients responded well to treatment with no reappearance of parasitemia within the 42 day follow-up period.

3.6. Correlation between concentrations of CQ and DECQ in whole blood and dried blood spot samples

Excellent correlation ($r = 0.997$) was observed between the analysis of both CQ and DECQ in whole blood and DBS samples collected from three patients with *P. vivax* following the standard regimen of CQ (Fig. 6). The mean ratios of CQ and DECQ concentrations in whole blood and DBS were 1.13 and 1.21, respectively.

4. Conclusions

The analytical method for the determination of CQ and DECQ in biological fluids established in this study meets the criteria for application to routine clinical drug level monitoring or pharmacokinetic study [24,25]. Although liquid–liquid extraction is time-consuming, solid phase extraction (SPE) is relatively expensive, which is not applicable for most of malaria endemic countries. The advantage of the method over previously reported ones is basically, its rapidity, simplicity and high sensitivity with simple UV detection. In addition, sample preparation procedure is simple, faster and less expensive.

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