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Simultaneous determination of monodesethylchloroquine, chloroquine, cycloguanil and proguanil on dried blood spots by reverse-phase liquid chromatography

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Abstract

A method for simultaneous analysis of chloroquine, proguanil and their metabolites from a whole blood sample (80 μL) dried on a filter paper was developed. Sample preparation included a liquid extraction from the filter paper, followed by a solid-phase extraction (C18 Bond Elut® cartridge). Separation was obtained by reverse-phase liquid chromatography (HPLC) using a gradient elution on an X-Terra® column; UV detection was made at 254 nm. This assay was linear between 150 and 2500 ng mL^{-1} for chloroquine (and metabolite) and 300 and 2500 ng mL^{-1} for proguanil and cycloguanil. The lower limit of quantification was close to 50 ng mL^{-1} for chloroquine (and its metabolite) and 100 ng mL^{-1} for proguanil (and its metabolite). No chromatographic interference from endogenous compounds or other tested anti-malarial drugs was evidenced. Chromatographic separation takes about 40 min with a coefficient of variation below 10.3% for within- and between-batch precision. The paper sampling method was validated in 10 healthy subjects treated by Savarine®. The stability of compounds and metabolites on the filter paper was evaluated at four temperatures (−20, +4, 20 and 50 °C) and for 1, 5 and 20 days. Cycloguanil concentrations were not influenced by storage conditions, whereas, high temperatures and prolonged storage decreased chloroquine and proguanil levels. The proposed HPLC assay is accurate, precise and cost-effective; it can be used for pharmacokinetic and epidemiological studies on anti-malarial treatments.

Keywords: Chloroquine; Proguanil; Dried blood spot; Reverse-phase chromatography

1. Introduction

Chloroquine (CQ) and proguanil (PG) are two main drugs used for the treatment of malaria. PG is a pro-drug activated by the hepatic cytochrome iso-enzyme to cycloguanil (CG), its active form [1]. CQ is metabolized by the same enzymatic system to monodesethyl-chloroquine (MDCQ), which possesses significant anti-malarial activity [2]. Despite the use of Savarine® (an association of CQ and PG) to improve chemoprophylaxis, cases of treatment failure have been reported worldwide [3]. Bad compliance, high cost of treatment and genetic susceptibility in the metabolism of PG have been advocated [4,5]. Pharmacokinetic and epidemiological studies are required to optimize prophylactic treatments and limit the continuous development and spread of resistance of *Plasmodium falciparum* [6].

Filter paper sampling has been proposed to collect blood in hard conditions (such as high temperature), especially in rural areas from poor countries [6,7]. Called "dried blood samples" (DBS), this procedure is less invasive than a veni-puncture. Furthermore, it is a cost-effective choice for both sampling and storage during transport to the laboratory [8]. Several methods have been published on the use of DBS for various anti-malarial drug assays, including quinine [7], mefloquine [9,10], proguanil [6], pyrimethamine [11], and chloroquine [12,13]. No method has been so far reported for other anti-malarial drugs, such as atovaquone, amaodiaquine, doxycycline and piperaquine.

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Since the volume of blood adsorbed on the filter is small (below 200 μL), the extraction step must be particularly efficient. A solid-phase extraction has been reported as the most appropriate and time-saving approach [13]. This method has been successfully applied to the analysis of various anti-malarial drugs [6,7,14].

Several HPLC methods have been published for the measurement of chloroquine and its metabolites [3,14–17], proguanil and cycloguanil [6,18–20] or all these compounds in a single run of biological samples [21,22] or pharmaceutical preparations [23]. The aim of this study was to develop an HPLC procedure for the combined analysis of chloroquine, proguanil (and their main metabolites) in a small volume (80 μL) of blood dried on a filter paper. This method could be applicable to pharmacokinetic and epidemiological studies on anti-malarial drugs and the prophylactic treatment of malaria.

2. Experimental

2.1. Chemical and reagents

Sodium hydroxide, hydrochloric acid 65% (w/w), aqueous ammonia 30% (w/w), dehydrated potassium phosphate, methanol and acetonitrile (all of analytical quality) were purchased from Merck (Nogent-sur-Marne, France). All reagents were prepared in water for intravenous injections (Fresenius France Pharma, Louviers, France). Human lyophilized whole blood was purchased from Bio-Rad (Marnes-la-Coquette, France). Bond Elut® C18 cartridges (200 mg, 3 mL) were obtained from Varian (Les Ulis, France). Borosilicate tubes and heparinized capillaries (reference 9720501) were purchased from VWR International (Fontenay-sous-Bois, France). Blood spot cards (reference 10538414), identical to those used for neonatal screening, were provided by Scleicher and Schuell (Ecquevilly, France).

Chloroquine (CQ) and quinine (as a sulfate salt) were obtained from Sigma–Aldrich (Saint Quentin Fallavier, France). Proguanil (PG), cycloguanil (CG) and bisdemethyled derivative of cycloguanil (internal standard, I.S.) were provided by Laboratoires Astra Zeneca (Paris, France). Monodesethylchloroquine (MDCQ) was a generous gift from the Pharmacology laboratory of Pité-Salpetrière Hospital (Paris, France); all other compounds were kindly provided by the Parasitology laboratory of Bichat hospital (Paris, France) (amodiaquine, 7-monodesethyl amodiaquine, atovaquone, mefloquine, carboxymefloquine). The chemical structures of chloroquine, proguanil, their main metabolites and internal standard are presented in Fig. 1.

2.2. Chromatographic system and chromatographic conditions

The chromatographic system consisted of a Waters 600 multisolvent delivery pump (Waters, Saint Quentin-en-Yvelines, France) connected to an automatic injector (Autosampler 712 Waters, Waters, Saint Quentin-en-Yvelines, France). The detector was an UV–vis detector (Waters 2487, Waters, Saint Quentin-en-Yvelines, France) set at 254 nm. Chromatograms

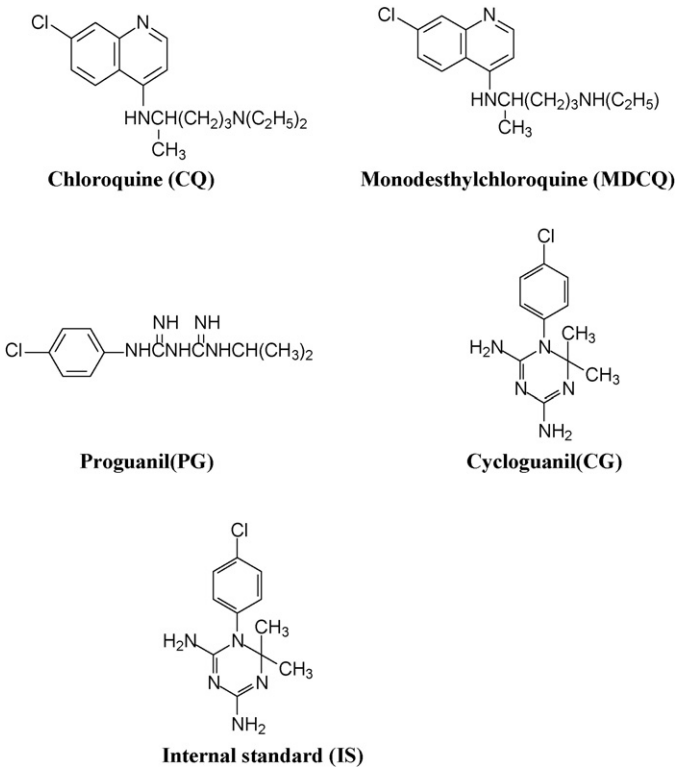

Fig. 1. Chemical structures of chloroquine, proguanil, main metabolites, and internal standard.

were obtained on a 746 data module integrator (Waters, Saint Quentin-en-Yvelines, France) with attenuation set at 4.

Chromatographic separation was performed at ambient temperature on a 5 μm X-Terra® RP 18 100 mm × 4.6 mm i.d. analytical column (Waters, Saint Quentin-en-Yvelines, France). A 5 μm X-Terra® RP18 guard column (10 mm × 4.6 mm i.d.) was inserted between the injector and the analytical column. The mobile phase distributed by the gradient pump consisted of a mixture of (A) acetonitrile–potassium phosphate (pH 5.5, 40 mM) (12:88, v/v) and (B) acetonitrile–potassium phosphate (pH 5.5, 40 mM) (40:60, v/v). The initial ratio of mobile phase components (*A*/*B*) was 98:2; the flow rate was set at 0.6 mL min^{-1}. The gradient elution conditions are presented in Table 1; five successive automated runs of extracted calibration standards assessed its reproducibility.

Before analysis, all liquids were filtered though a 0.45 μm PVDF filter (Waters, Saint Quentin-en-Yvelines, France) with a

Table 1
Gradient elution conditions

Time (min)	Flow-rate (mL min^{-1})	*A* (%)	*B* (%)	Gradient kinetics
0	0.6	98	2	No
5	0.6	80	20	6
12	1	40	60	6
30	1	40	60	6
35	1	98	2	6
40	0.6	98	2	6

Gradient kinetic 6 represents a linear kinetic gradient.

Pyrex filter holder, before degassed for 15 min in an ultrasonic bath (Branson 32000, Prolabo, Paris, France).

2.3. Calibration and quality control samples

Stock standard solutions contained 1000 μg mL^{-1} of each compound (expressed as molecule bases). They were prepared in methanol and stored at −20 °C with a stability of 12 months. Two working solutions (WS_1 = 5 μg mL^{-1} for MDCQ and CQ; WS_2 = 5 μg mL^{-1} for CG and PG) were prepared immediately before use by appropriate dilutions of the different stock standard solutions in methanol.

A stock solution (1000 μg mL^{-1}) of the internal standard (I.S.) was prepared in methanol and stored to −20 °C with a stability of 12 months. The working solution of I.S. was extemporaneously prepared by a 1:100 dilution of the stock solution in methanol.

Standards and DBS quality controls were prepared in lyophilized human whole blood reconstituted with 2 mL of water for intravenous injection.

Standards were prepared at concentrations ranging from 150 to 2500 ng mL^{-1} (MDCQ, CQ) and 300 to 2500 ng mL^{-1} (CG, PG) by evaporation of working solution (15–250 μL WS_1 for MDCQ and CQ; 30–250 μL WS_2 for CG and PG) under a nitrogen steam at +30 °C. Dry residues were dissolved in 500 μL of human whole blood using a vortex (three times 20 s) at room temperature. After a 15-min equilibrium, a volume of 80 μL of each standard (and non-spiked blood for zero) was laid down on a paper filter. Spots were air-dried for 3 h in a dark room at ambient temperature, then cut using a 15-mm diameter steel punch. Dried blood spot standards were stored in closed borosilicate tubes at −20 °C. A calibration curve was constructed from a blank sample (a dried blood spot proceeded with the I.S.) and five non-zero dried blood spots to cover the entire range of concentrations (150–25,000 ng mL^{-1} for MDCQ and CQ; 300–25,000 ng mL^{-1} for PG and CG), including the lower limits of quantification (LOQs). Calibrations curves were generated using drugs or metabolites to the I.S. peak height ratios by weighted ($1/x^2$) least-squares regression on 5 consecutive days.

Three DBS quality control samples, low (C1 = 150 ng mL^{-1} of MDCQ and CQ; 300 ng mL^{-1} of CG and PG), medium (C2 = 800 ng mL^{-1} of each compound) and high (C3 = 2200 ng mL^{-1} of each compound) were prepared and stored in the conditions described for standards.

2.4. Dried blood spot extraction procedure

The extraction procedure was similar to the one described by Bergqvist et al. [6]. In a first step, compounds should be extracted from DBS. Briefly, 5 mL of aqueous ammonia 0.9 M were added to all tubes containing DBS (standards, controls and capillary blood samples) or 80 μL aliquots of venous blood. Tubes were mixed and mechanically shaken for 30 min before placed in an ultrasonic bath for 30 min at room temperature. Tubes were then left standing for 60 min at +4 °C before solid-phase extraction (SPE).

In a second step, drugs were extracted from the aqueous ammonia phase using Bond Elut® C18 cartridges. Before extraction, 50 μL of aqueous internal standard solution (10 μg mL^{-1}) were added to all tubes and mixed. The SPE pretreatment of samples was carried out with a Bond Elut® C18 cartridge connected to a Vac Elut® SPS 21 manifold (Prolabo, Paris, France). SPE columns were preconditioned with 2× 1.0 mL of methanol followed by 2× 1.0 mL of water for intravenous injection; vacuum was applied after the addition of each solvent. The samples were loaded onto the preconditioned cartridges and slowly eluted using gravity instead of vacuum. Columns were washed with 3× 1.0 mL of water for intravenous injection, and 2× 1.0 mL of methanol, successively. Elution of compounds was carried out with 2× 1.0 mL of methanol containing 0.1% (v/v) hydrochloric acid, applying vacuum to dryness. Eluates were evaporated to dryness in borosilicate tubes under a stream of nitrogen at +30 °C. Residues were dissolved in 130 μL of mobile phase A using a vortex; a volume of 70 μL was injected in the HPLC system.

2.5. Validation procedure

Recoveries (absolute, paper elution and column extraction) were evaluated at three different levels for each compound (C1: 150 or 300 ng mL^{-1}, C2: 800 ng mL^{-1} and C3: 2200 ng mL^{-1}). Absolute recovery was calculated by comparing peak height ratios obtained from DBS quality controls (C1–C3) with those obtained by direct injection of methanol standard solutions at the same concentrations. Paper elution recoveries were determined by comparing peak height ratios from DBS with those from 80 μL of whole blood at the same concentrations. Column extraction recovery was calculated by comparing peak heights obtained from 80 μL of spiked whole blood with those obtained by injection of the same methanol standard concentrations, evaporated and dissolved in the mobile phase. All determinations were realized in triplicate.

Method precision was evaluated using three DBS quality controls (C1–C3). For within-batch precision, controls were extracted 10 times in the same series. Between-batch precision was determined in 10 different series over a period of 3 weeks. Accuracy of the method was assessed using bias values.

Detection and quantification limits are defined as the concentration producing a signal-to-background ratio of 3 and 10, respectively. Drug-free paper spots were analyzed (n = 10) and blank mean/standard deviations were calculated.

To study potential interference by other major antimalarial drugs, 80 μL dried blood spots were co-spiked with 1000 ng mL^{-1} of quinine, amodiaquine, monodesethylamodiaquine, atovaquone, méfloquine, and carboxymefloquine. Spots were eluted and analyzed as described above.

The effect of temperature on CQ, MDCQ, CG and PG stability on DBS was studied using three DBS quality controls. Spots were stored at four temperatures (−20, +4, 20 and 50 °C) for 1, 5 and 20 days (the later corresponds to the maximal delay between sampling and analysis). All concentrations (mean of three determinations) were compared to fresh concentration values of C1, C2 or C3 and designed as t_0 concentration.

Table 2
Gradient reproducibility study ($n = 5$)

	Retention time		
	Mean (min)	R.S.D. (min)	CV (%)
MDCQ	8.41	0.6	7.1
CQ	10.40	0.85	8.2
I.S.	20.2	1.6	7.9
CG	22.12	1.92	8.7
PG	27.21	1.45	5.3

Extracted dried blood spot standards (1000 ng mL^{-1}) were measured during five successive automated runs. R.S.D.: relative standard deviation; CV: coefficient of variation.

The results of these tests were evaluated against internationally used acceptance criteria described by Shah et al. [24].

2.6. Application of the method

The paper sampling method was validated in a group of 10 healthy subjects on a 6-week prophylatic treatment with Savarine®. A 2.5 mL venous blood sample was drawn in vacuum tubes containing sodium heparinate as an anti-coagulant. An aliquot of 80 μL was introduced in a borosilicate tube and stored at −20 °C. Capillary blood (80 μL) was sampled by finger puncture using a heparinazed precision capillary and laid down on a sample collection card. Dried in the same conditions as standards and controls, collection cards were placed in zip-closure plastic bags and stored at −20 °C. Before elution, spots were cut in sample collection cards using a 15-mm diameter steel punch and transferred to borosilicate tubes. Elution and extraction procedures are similar to those described above.

The present study was approved by the Ethic Committee of Saint Louis Hospital and was conducted in accordance with the declaration of Helsinki.

3. Results

3.1. Assay performance and validation

The assay performances were assessed by accuracy, precision, specificity, sensitivity, linearity and stability studies.

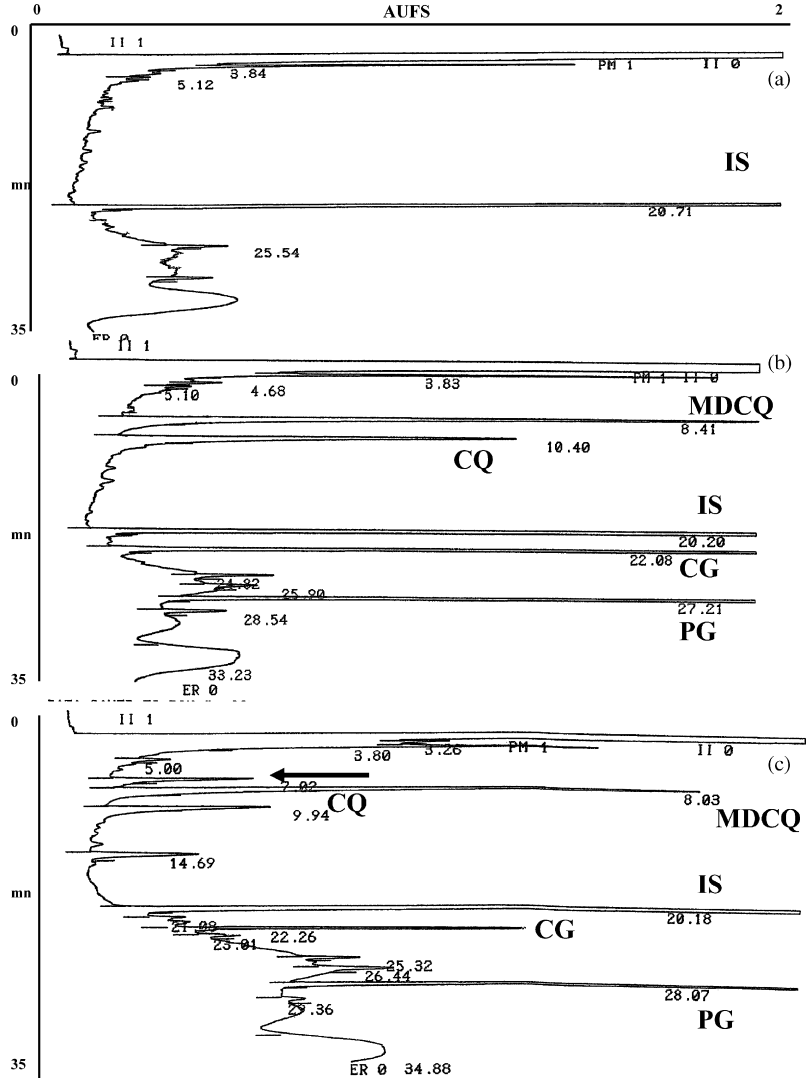

Fig. 2. Typical chromatograms of an extracted blank spot (a), a whole blood spot (500 ng mL^{-1}) (b), and a whole blood spot from a patient under Savarine® prophylaxis (c) spiked with internal standard (500 ng). An arrow indicates bisdesethylchloroquine.

Table 3
Precision and accuracy for calibration samples

Compounds	Concentration added (ng mL^{-1})	Concentration found (mean ± S.D., $n = 5$) (ng mL^{-1})	Precision (% R.S.D.)	Accuracy (% bias)
MDCQ	150	138 ± 13	9.4	−7.0
	500	487 ± 27	5.5	−1.6
	1000	996 ± 41	4.1	−1.4
	1500	1486 ± 112	7.5	−1.9
	2000	2012 ± 105	5.2	−0.4
	2500	2489 ± 183	7.3	−1.4
CQ	150	158 ± 19	12.0	4.3
	500	521 ± 48	9.2	3.2
	1000	1012 ± 55	5.4	0.2
	1500	1486 ± 132	8.9	−1.9
	2000	2011 ± 152	7.6	−0.4
	2500	2542 ± 87	3.4	0.7
CG	300	332 ± 29	8.7	9.7
	500	548 ± 42	7.7	8.6
	1000	998 ± 75	7.5	−0.8
	1500	1525 ± 105	6.8	0.7
	2000	2013 ± 68	3.4	−0.3
	2500	2542 ± 132	5.2	0.7
PG	300	287 ± 26	9.0	−5.3
	500	479 ± 36	7.5	−5.2
	1000	988 ± 85	8.6	−2.2
	1500	1478 ± 125	8.4	−2.5
	2000	1988 ± 73	3.7	−1.6
	2500	2478 ± 142	5.7	−1.9

R.S.D.: relative standard deviation.

Regarding chromatographic performance, the gradient reproducibility displayed a coefficient of variation (CV) below 10% for each compound during five consecutive automated runs (Table 2). The specificity of the method was established by representative chromatograms (Fig. 2) of an extracted blank spot (a), a calibration whole blood spot (b), and a whole blood spot obtained from a patient treated by Savarine® (c), spiked with internal standard. For MDCQ, CQ, internal standard, CG, and PG, retention times were 8.4, 10.4, 20.2, 22.1 and 27.2 min, respectively. The patient chromatogram showed a peak at 7.02 min (arrow, Fig. 2c), corresponding to bidesethylchloroquine (the second metabolite of chloroquine). Among other anti-malarial drugs, some were not extracted or not detected at 254 nm, as atovaquone, mefloquine and its first metabolite, carboxymefloquine. Quinine, amodiaquine and 7-monodesthylamodiaquine were detected and eluted at 18, 15 and

Table 4
Recoveries of different extraction steps ($n = 3$)

	Recovery (C1 = 150 ng mL^{-1})			Recovery (C1 = 300 ng mL^{-1})			Recovery (C2 = 800 ng mL^{-1})			Recovery (C3 = 2200 ng mL^{-1})		
	Absolute	Paper	Column	Absolute	Paper	Column	Absolute	Paper	Column	Absolute	Paper	Column
MDCQ												
Mean (%)	90	94	96	ND	ND	ND	91	93	98	91	93	98
R.S.D. (%)	5.0	4.2	1.2	ND	ND	ND	3.4	2.6	1.3	1.9	2.1	0.9
CQ												
Mean (%)	85	90	95	ND	ND	ND	89	92	97	88	92	96
R.S.D. (%)	4.1	3.7	1.1	ND	ND	ND	3.4	2.4	1.4	2.2	2.4	0.9
CG												
Mean (%)	ND	ND	ND	80	92	87	81	93	87	78	90	87
R.S.D. (%)	ND	ND	ND	7.1	3.1	2.3	3.8	2.4	1.6	3.6	2.4	1.5
PG												
Mean (%)	ND	ND	ND	78	89	88	76	88	87	77	91	85
R.S.D. (%)	ND	ND	ND	3.4	2.3	1.5	6.7	3.2	2.1	5.7	2.7	2.1

ND: not determined, R.S.D.: relative standard deviation.

Table 5
Precision and accuracy for DBS samples

Compounds	Concentration added (ng mL^{-1})	Within-batch precision ($n = 10$)			Between-batch precision ($n = 10$)		
		Concentration found (mean ± S.D.) (ng mL^{-1})	Precision (% R.S.D.)	Accuracy (% bias)	Concentration found (mean ± S.D.) (ng mL^{-1})	Precision (% R.S.D.)	Accuracy (% bias)
MDCQ	150 (C1)	159 ± 16	10.1	5.0	157 ± 12	7.6	3.7
	800 (C2)	785 ± 66	8.4	−2.9	792 ± 68	8.6	−2.0
	2200 (C3)	2245 ± 186	8.3	1.0	2198 ± 125	5.7	−1.1
CQ	150 (C1)	148 ± 11	7.4	−2.3	155 ± 12	7.7	2.3
	800 (C2)	794 ± 53	6.7	−1.7	812 ± 29	3.6	0.5
	2200 (C3)	2148 ± 105	4.9	−3.4	2254 ± 104	4.6	1.5
CG	300 (C1)	315 ± 26	8.2	4.0	288 ± 25	8.7	−5.0
	800 (C2)	842 ± 48	5.7	4.2	814 ± 41	5.0	0.7
	2200 (C3)	2275 ± 158	6.9	2.4	2245 ± 89	4.0	1.0
PG	300 (C1)	297 ± 19	6.4	0.1	292 ± 18	6.2	−3.7
	800 (C2)	786 ± 35	4.4	−2.7	802 ± 37	4.6	−0.7
	2200 (C3)	2169 ± 132	6.1	−2.4	2258 ± 123	5.4	1.6

R.S.D.: relative standard deviation.

12 min, respectively. No other physiological or pharmacological compounds seemed to interfere with the extraction method or the chromatographic separation.

The peak area ratio of each compound to I.S. in DBS was linear over the concentration range of 150 (or 300) to 2500 ng mL^{-1}. The calibration mode was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors ($1/x$, $1/x^2$, $1/\sqrt{x}$). The residuals were improved by weighted ($1/x^2$) least-squares linear regression. The best fits for calibration curves were achieved by linear equation, as follows: MDCQ, $y = 0.0012x + 0.00445$; CQ, $y = 0.0014x - 0.00521$; CG, $y = 0.00072x - 0.0265$: PG, $y = 0.0034x + 0.0083$. Over the entire concentration range, linearity correlation coefficients ranged from 0.998 to 0.999. Table 3 summarizes calibration curves results for each molecule. These curves were suitable for the generation of acceptable data for the concentrations of each compound in DBS samples during between and within-batch validations.

Results of recovery studies ranged from 76 to 91%. For all compounds, the paper elution step showed 89–94% recovery. Lower values were observed for CG and PG during the column extraction procedure at all tested concentrations (Table 4).

The limit of detection (LOD) with a signal-to-noise ratio higher than 3 was 50 ng mL^{-1} for MDCQ or CQ and 100 ng mL^{-1} for CG or PG. The lower limit of quantification (LOQ), the lowest concentration in standard curves which can be measured with an acceptable accuracy and precision for analytes from DBS, was 150 ng mL^{-1} for MDCQ and CQ, and 300 ng mL^{-1} for CG and PG. Mean responses for molecule peaks at the assay sensitivity limit (150 or 300 ng mL^{-1}) were almost 15 (MDCQ and CQ) and 10 (CG and PG) folds greater than those in 10 DBS blank samples at the retention times of the compounds.

Accuracy and precision (within- and between-batch) were determined using 10 determinations for each concentration level (Table 5). Precision was determined as the within- and between-assay relative standard deviation (R.S.D.). Accuracy was expressed as percentage of bias and was within the acceptable ±20% limits of the theoretical value at the LOQs. At all other concentration levels, bias were within ±15% of those limits. The precision around the mean values never exceeded 10% at any of studied concentrations.

3.2. Stability studies

DBS quality controls were subjected to short (1 day), medium (5 days) and long (20 days) storage at different temperatures (−20 °C, +4 °C, +20 °C and +50 °C). Stability studies were carried out at three concentration levels (150 or 300, 800 and 2200 ng mL^{-1}) with three determinations and compared to fresh values (noted t_0). Results are reported in Table 6. For all molecules, no significant variation of concentrations was observed at all temperatures after 1 day conservation. For monodesethylchloroquine, the response was decreased at +20 °C, and completely inhibited at +50 °C, after 5 and 20 days of storage. A significant decrease of chloroquine concentrations (upper 50%) was observed at +20 and +50 °C during medium (5 days) and prolonged (20 days) storage. Temperature and storage had no impact on cycloguanil levels. A temperature of +50 °C induced a 30% decrease of the proguanil response after 5 days of storage; it reached near 80% after 20 days.

3.3. Application of the method

Concentrations of each compound in DBS and whole blood obtained from healthy subjects treated by Savarine® are presented in Table 7. The correlation between DBS (capillary blood) and whole blood concentrations were comprised between 0.94 and 0.98. Using Bland–Altman analysis [25], the difference between the two sampling methods was significant (by repeated measure ANOVA, $p < 0.05$).

Table 6
Stability study

Time storage	Nominal concentration ($ng\,mL^{-1}$)		MDCQ ($ng\,mL^{-1}$)				CQ ($ng\,mL^{-1}$)				CG ($ng\,mL^{-1}$)				PG ($ng\,mL^{-1}$)			
			−20 °C	+4 °C	+20 °C	+50 °C	−20 °C	+4 °C	+20 °C	+50 °C	−20 °C	+4 °C	+20 °C	+50 °C	−20 °C	+4 °C	+20 °C	+50 °C
1 Day	C1	Concentration (t_0)	152	158	149	155	155	156	154	149	305	311	302	299	306	287	295	302
	150 or 300	Concentration recovered																
		Mean ± S.D.	148 ± 12	149 ± 14	152 ± 13	144 ± 12	152 ± 13	159 ± 15	148 ± 11	156 ± 10	321 ± 19	302 ± 25	312 ± 18	302 ± 26	312 ± 15	305 ± 18	298 ± 14	303 ± 19
		R.S.D. (%)	8.1	9.3	8.6	8.3	8.5	9.4	7.4	6.4	5.9	8.3	5.8	8.6	4.8	5.9	4.7	6.3
	C2	Concentration (t_0)	789	803	805	793	805	812	802	786	802	8015	799	806	817	802	795	797
	800	Concentration recovered																
		Mean ± S.D.	802 ± 26	812 ± 34	795 ± 23	803 ± 32	812 ± 19	805 ± 22	795 ± 35	801 ± 35	798 ± 35	816 ± 34	792 ± 46	816 ± 32	802 ± 25	787 ± 31	806 ± 42	805 ± 36
		R.S.D. (%)	3.2	4.2	2.9	4.0	2.3	2.7	4.4	4.4	4.4	4.2	5.8	3.9	3.1	3.9	5.2	4.4
	C3	Concentration (t_0)	2178	2206	2235	2224	2189	2206	2214	2185	2205	2.174	2198	2.209	2196	2.205	2.2012	2.002
	2200	Concentration recovered																
		Mean ± S.D.	2208 ± 79	2215 ± 102	2224 ± 91	2216 ± 95	2189 ± 56	2205 ± 69	2212 ± 102	2226 ± 79	2156 ± 86	2199 ± 104	2185 ± 78	2206 ± 83	2188 ± 131	2204 ± 106	2187 ± 95	221 ± 87
		R.S.D. (%)	3.6	4.6	4.1	4.3	2.5	3.1	4.8	3.5	4.0	4.7	3.6	3.8	6.0	4.8	4.3	3.9
5 Days	C1	Concentration (t_0)	155	152	149	156	159	161	148	156	302	295	305	304	298	295	302	294
	150 or 300	Concentration recovered																
		Mean ± S.D.	153 ± 12	156 ± 13	103 ± 12	ND	155 ± 12	86 ± 11	92 ± 10	88 ± 9	298 ± 26	299 ± 23	305 ± 28	297 ± 19	302 ± 13	315 ± 12	297 ± 16	152 ± 14
		R.S.D. (%)	7.8	8.3	11.6	ND	7.7	12.8	10.9	10.2	8.7	7.7	9.2	6.4	4.3	3.8	5.4	9.2
	C2	Concentration (t_0)	802	786	795	789	805	806	802	791	805	799	802	802	806	798	785	806
	800	Concentration recovered																
		Mean ± S.D.	798 ± 35	810 ± 26	546 ± 47	152 ± 28	798 ± 41	412 ± 56	398 ± 42	451 ± 36	806 ± 35	811 ± 42	789 ± 52	812 ± 42	795 ± 35	804 ± 26	796 ± 31	452 ± 31
		R.S.D. (%)	4.3	3.2	8.6	18.4	5.1	13.6	10.5	8.0	4.3	5.2	6.6	5.2	4.4	3.2	3.9	6.8
	C3	Concentration (t_0)	2225	2189	2231	2242	2220	2206	2198	2225	2206	2.189	2.206	2.219	2205	2.214	2.208	2.185
	2200	Concentration recovered																
		Mean ± S.D.	2205 ± 75	2026 ± 52	1450 ± 154	402 ± 55	2235 ± 68	1215 ± 56	1198 ± 65	1095 ± 89	2189 ± 102	2206 ± 78	2201 ± 89	2223 ± 99	2189 ± 103	2205 ± 95	2206 ± 102	1478 ± 137
		R.S.D. (%)	3.4	2.6	10.6	13.7	3.0	4.6	5.4	8.1	4.6	3.5	4.0	4.4	4.7	4.3	4.6	9.3
20 Days	C1	Concentration (t_0)	156	149	152	159	155	158	149	152	300	302	300	309	297	305	302	318
	150 or 300	Concentration recovered																
		Mean ± S.D.	155 ± 13	152 ± 14	ND	ND	156 ± 14	ND	ND	ND	301 ± 15	309 ± 14	297 ± 26	299 ± 32	302 ± 11	294 ± 9	298 ± 12	ND
		R.S.D. (%)	8.4	9.2	ND	ND	8.9	ND	ND	ND	5.0	4.5	8.7	10.7	3.6	3.1	4.0	ND
	C2	Concentration (t_0)	812	799	805	803	795	805	802	800	802	789	790	805	806	795	806	803
	800	Concentration recovered																
		Mean ± S.D.	799 ± 24	803 ± 30	ND	ND	802 ± 35	127 ± 25	135 ± 12	112 ± 19	798 ± 35	789 ± 41	805 ± 39	811 ± 34	798 ± 31	803 ± 48	800 ± 32	165 ± 19
		R.S.D. (%)	3.0	3.7	ND	ND	4.4	19.6	8.9	16.9	4.4	5.2	4.8	4.2	3.9	6.0	4.0	11.5
	C3	Concentration (t_0)	2205	2178	2194	2215	2206	2194	2188	2212	2156	2.204	2.199	2.188	2205	2.198	2.221	2.204
	2200	Concentration recovered																
		Mean ± S.D.	2178 ± 89	2195 ± 66	ND	ND	2185 ± 102	224 ± 31	246 ± 29	198 ± 35	2203 ± 102	2210 ± 79	2155 ± 125	2012 ± 89	2289 ± 78	2201 ± 103	2214 ± 85	425 ± 21
		R.S.D. (%)	4.0	3.0	ND	ND	4.7	13.8	11.8	17.7	4.6	3.6	5.8	4.4	3.4	4.7	3.8	4.9

ND: not detectable.

Table 7
Comparison between capillary venous blood and dried blood spots samples in healthy subjects treated by Savarine®

Patient number	MDCQ			CQ			CG			PG		
	Whole blood concentration ($ng\,mL^{-1}$)	DBS concentration ($ng\,mL^{-1}$)	Recovery (%)	Whole blood concentration ($ng\,mL^{-1}$)	DBS concentration ($ng\,mL^{-1}$)	Recovery (%)	Whole blood concentration ($ng\,mL^{-1}$)	DBS concentration ($ng\,mL^{-1}$)	Recovery (%)	Whole blood concentration ($ng\,mL^{-1}$)	DBS concentration ($ng\,mL^{-1}$)	Recovery (%)
1	265	247	93.2	145	122	84.1	101	95	94.1	182	155	85.2
2	390	367	94.1	154	132	85.7	137	118	86.1	271	241	88.9
3	238	222	93.3	208	195	93.8	124	116	93.5	245	201	82.0
4	315	302	95.9	174	141	81.0	146	131	89.7	205	181	88.3
5	265	221	83.4	168	154	91.7	108	94	87.0	178	152	85.4
6	236	204	86.4	111	94	84.7	120	110	91.7	221	195	88.2
7	354	321	90.7	107	97	90.7	112	92	82.1	156	131	84.0
8	285	258	90.5	192	161	83.9	108	98	90.7	212	198	93.4
9	229	198	86.5	121	110	90.9	126	107	84.9	147	137	93.2
10	245	236	96.3	138	113	81.9	109	95	87.2	195	167	85.6
Regression curve												
Slope		0.9957			0.9035			0.8565			0.8632	
Intercept		23.39			5.25			3.59			2.12	
Correlation coefficient		0.98			0.97			0.94			0.97	
Bland–Altman analysis												
Mean difference		−24.60			−19.90			−13.50			−25.40	
95% Confidence interval		−45.7; −3.5			−35.9; −3.8			−23.1; −3.9			−43.2; −7.5	
Significance (ANOVA)		$p<0.01$			$p<0.01$			$p<0.01$			$p<0.01$	

This table reports recovery values obtained in capillary DBS *vs.* whole venous blood samples in 10 subjects. Correlation results and statistical analysis (ANOVA test, significant value, $p<0.05$) are also presented.

4. Discussion and conclusion

Numerous papers have reported the use of dried blood spot (DBS) as a convenient way to measure biological parameters [26–28] and drugs [28,29] in blood. Ease of collection, transport and storage make DBS a cost-effective choice, especially for studies in poor countries [30]. Frequently used for anti-malarial drugs [7–12], no publication has so far described a DBS procedure for the combined analysis of chloroquine and proguanil (and their main metabolites).

A reverse HPLC method to the simultaneous measure chloroquine, proguanil and their first metabolites in DBS was successfully developed. To extract compounds from DBS, a liquid extraction was followed by a SPE extraction. For chloroquine and its first metabolite (MDCQ), extraction recoveries were excellent (above 85% for CQ and MDCQ at all DBS quality control levels) and similar to those described in previous paper [11,12]. Lower values were observed for proguanil (76–81%), but similar to those reported to Berquist et al. [6]. This could be related to the column extraction itself, rather than elution from the filter paper. Some studies have reported the use of silanised glass tubes instead borosilicate tubes to avoid non-specific adsorption [31–33]. Silanised tubes have been specially recommended for artemisinin derivatives [31,32] and in one publication for chloroquine and metabolites [33]. Since recoveries obtained with borosilicate tubes were excellent and similar to those reported in the literature [11,12], silanised tubes were not tested.

To improve chromatographic separation, a gradient elution was selected [23,34] for this assay. This approach appears compatible with routine use, as reflected by the coefficient of variation of retention times for all molecules (CVs: 5.3–8.7%). Resolution was highly satisfactory and better than reported by Chaulet et al. [22] with a similar total analysis time. Combining a gradient elution with new chromatographic materials (X-terra® column, Waters) increased the resolution without counter ion in the mobile phase [23,24]. The present HPLC procedure displayed an excellent precision and accuracy, even at the lowest concentration levels. Furthermore, this method requires only a small blood volume (80 μL), lower than most other techniques published [6,7], with the exception of CQ and MDCQ (75 μL) [12]. Concerning sample preparation, our procedure is still time-consuming and does not represent an improvement over others methods [6,12].

As in other studies [6,7], concentrations measured in capillary blood dried on a filter paper (DBS) highly correlated with those from venous blood. They were, however, significantly lower in DBS. Only, Lindström et al. [12] have reported CQ and MDCQ venous concentrations lower than dried blood spots.

Interestingly, storage conditions had a different impact on the measured levels of each compounds. Except for cycloguanil, a significant decrease of their concentrations for was noted at +50 °C (including a total inhibition of response for MDCQ). At +20 °C, there was no impact on proguanil concentration, but a significant one for chloroquine and its metabolite. This could be related to a heat destruction of the molecules (especially at +50 °C) or alternatively their adsorption on the paper filter. These data contrast with those published by Lindström et al. [12] who reported an excellent stability for CQ and MDCQ during 7 weeks at +20 °C (no other temperature was studied). At +37 °C, no variations of proguanil concentration was reported in plasma DBS [7] or in whole blood DBS [6]. Our data suggest that these compounds are very sensitive to high temperature (+50 °C) after 4 days of storage. Under equatorial latitudes, this extreme temperature is frequently observed. A special attention must be paid to avoid a prolonged exposition of samples to high temperature to limit the destruction of these compounds.

In conclusion, this HPLC assay has the required sensitivity to measure chloroquine, proguanil and their main metabolites in 80 μL of whole blood dried on a paper filter (dried blood spots). Despite the heat sensitivity of dried blood spots, this approach seems suitable for use in pharmacokinetic and epidemiological studies on anti-malarial drugs on the field.

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