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ANALYSIS OF FILTER-PAPER-ABSORBED, FINGER-STICK BLOOD SAMPLES FOR CHLOROQUINE AND ITS MAJOR METABOLITE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

Methodology has been developed to facilitate the collection, transport, and analysis of blood samples in studies of chloroquine absorption and metabolism. The method utilizes high-performance liquid chromatography (HPLC) with fluorescence detection to quantify chloroquine and its major metabolite, desethylchloroquine, in 100-μl quantities of blood collected on filter paper. Detection limits are 5 ng/ml for both analytes. No loss of either analyte occurred from filter-paper-collected blood spots stored over a twelve-weeks' period at room temperature. Filter-paper-collected, finger-stick blood spots give values for each analyte comparable to corresponding determinations on venous, whole-blood samples. The HPLC mobile phase selected has general applicability to the separation of antimalarial drugs. The methodology permits effective assessment of chloroquine prophylaxis compliance and parasite drug resistance in remote, malaria-endemic regions.

INTRODUCTION

Recent advances in the analysis of chloroquine in body fluids have made possible important investigations into its use as an antimalarial and antirheumatic drug [1]. The most sensitive of the methods have utilized high-performance liquid chromatography (HPLC) with fluorescence detection [2,3]. These methods are also sufficiently selective to quantify both chloroquine and its major metabolite, desethylchloroquine. A major deterrent to the field applicability of chloroquine analysis methods has been the requirement for a volume of fresh whole blood, serum, or plasma. Therefore, an analytic method

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was developed to accurately and reproducibly determine the concentration of chloroquine and desethylchloroquine in a small sample of blood collected on filter paper from a finger puncture, such as could be collected under field conditions and transported to the analytical laboratory. HPLC analysis with fluorescence detection was employed, because of its sensitivity. Investigations were performed to demonstrate the storage stability of chloroquine and desethylchloroquine in blood-spot samples and the comparability of analytical results between finger-stick and venipuncture samples.

EXPERIMENTAL*

Standards

Chloroquine (Cq) diphosphate was purchased from Sigma (St. Louis, MO, U.S.A.). Before its use as a standard, this material was dried under vacuum over phosphorus pentoxide and the melting point determined using a Fisher-Johns apparatus, calibrated with Fisher TherMetric standards. The melting-point range was 192.1—193.3°C. Desethylchloroquine (DECq) base was supplied by Sterling-Winthrop Research Institute (Rennselaer, NY, U.S.A.). The internal-standard compound, 7-chloro-4-(1′-methyl-4′-isopropylaminobutylamino)-quinoline was provided by Walter Reed Army Institute of Research (Washington, DC, U.S.A.). This compound is the isopropyl analogue of desethylchloroquine and is designated IPA for brevity. Quinine base was formed from commercially available quinine hydrochloride from Merck, Sharp and Dohme (Rahway, NJ, U.S.A.) by extraction from aqueous base using diethyl ether—hexane (1 : 1). The quinine was crystallized from this solvent pair after partial evaporation followed by cooling to room temperature. Amodiaquine was provided by Parke, Davis and Company (Detroit, MI, U.S.A.) and pyrimethamine was from Burroughs Wellcome (Research Triangle Park, NC, U.S.A.).

Reagents and materials

Spectroscopic quality hexane, methyl-*tert.*-butyl ether (MTBE), and methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) were glass-distilled. The diethylamine (Aldrich, Milwaukee, WI, U.S.A.) was distilled before use. Human blood used for standards was freshly collected using acid—citrate—dextrose solution (ACD) as an anticoagulant. All other chemicals used were of reagent grade or better. The filter paper used was rectangular (25 mm × 75 mm) cut from sheets of paper (catalogue number 1023-.038, James River Rochester, Rochester, MI, U.S.A.). The 100-μl pipets were Corning catalog number 70995-100 from Corning Glass Works (Corning, NY, U.S.A.).

Equipment

The HPLC apparatus consisted of two Waters Model M-6000 A solvent delivery systems and a Waters Model 660 solvent programmer, coupled to a Waters

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Model U6K loop injector, a Dupont Zorbax Sil column (5—6 μm particle diameter; 25 cm × 4.6 mm), a Varian Vari-chrom variable-wavelength absorbance detector, and a Perkin-Elmer Model 650S fluorescence detector, connected in series. A Varian Model 9176 dual-pen recorder was used to display both an ultraviolet (UV) absorbance trace and a fluorescence trace. Integration of fluorescence peaks was performed using a Varian CDS-111 integrator.

Analysis of filter-paper-collected blood spots

Standards were prepared by spotting chloroquine-free, ACD-anticoagulated blood onto filter paper, cutting out the dried spots, placing each spot in a 57 × 25 mm I.D. glass vial (screwcaps contained PTFE inserts), and adding 50-μl volumes of chloroquine and desethylchloroquine standard solutions to the filter paper in each vial. Sample blood spots on filter paper were similarly cut out and placed in vials. To each vial were added 50 μl of IPA internal-standard solution (1.02 ng/μl in 0.002 *M* hydrochloric acid) and 3 ml of 0.2 *M* hydrochloric acid. The vials were vortexed and allowed to stand for at least 1 h. (For convenience papers could stand in the vials overnight.) The paper in each vial was then macerated using a clean glass rod. Each vial was vortexed and the contents filtered through a plug of silanized glass wool in a Pasteur pipet. The filtrates were collected in 15-ml conical glass centrifuge tubes.

To each of the filtrates were added 3.0 ml of MTBE—hexane (1:1) and 0.5 ml of 5.0 *M* sodium hydroxide. The centrifuge tubes were placed on a Glas-Col Model RD-250 rotator equipped with a Model RD-60 rotator head (Terre Haute, IN, U.S.A.) that inverted the capped tubes at the rate of 30 inversions per min for a period of 15 min. Centrifugation for 5 min was employed as necessary to break any mild emulsions that occurred. Most of each organic layer was transferred to a second centrifuge tube using a Pasteur pipet. The solvent was evaporated by heating on a water bath combined with a gentle flow of dry nitrogen. The samples and standards were reconstituted in 100-μl quantities of freshly prepared mobile phase, and 30-μl quantities of the resulting solutions were injected into the HPLC.

The mobile phase used was hexane—MTBE—methanol—diethylamine (37.25:37.25:25.0:0.5). The flow-rate was 1.0 ml/min (68 bar). The UV detector was set at 340 nm. The UV signal was monitored on the second pen of the dual-pen recorder to provide a check on the quantification of samples that contained high concentrations of chloroquine. For the fluorescence detector, the excitation wavelength was 320 nm and the emission wavelength 380 nm with each slit width set to 5 nm. Fluorescence peak-area-ratio standard curves were calculated for both chloroquine and desethylchloroquine, and the samples were quantified with reference to these curves.

Collection of volunteer blood samples

A researcher traveling to Africa in connection with ongoing malaria studies underwent the recommended chemoprophylactic regimen with chloroquine diphosphate (300 mg/week as base). The researcher, who weighed 190 pounds, volunteered to provide blood samples by venipuncture (collected in heparinized tubes) at intervals during the first two weeks of his regimen and for a number of weeks after his return, including several weeks subsequent to the last

administration of chloroquine. Additionally, filter-paper-collected, finger-stick blood samples were taken at the same time as certain venipuncture samples. Studies to evaluate the storage-stability of chloroquine in filter-paper-collected blood spots and the comparability of venipuncture and finger-stick samples utilized certain of the samples from this volunteer.

Storage study and venipuncture/finger-stick comparability study

Two 5-ml quantities of blood from the volunteer who was undergoing chloroquine chemoprophylaxis were used in this study — one taken 24 h, the other 10 days after the 25th and final weekly chloroquine dose. The samples were analyzed initially by a procedure in which a 500-μl quantity of each whole-blood sample was placed in a 15-ml centrifuge tube and diluted with 2 ml of demineralized water. The 3.0 ml of MTBE—hexane (1:1) and 0.5 ml of 5 *M* sodium hydroxide were added, and the analysis was completed as outlined above for the filter-paper, blood-spot method. Standards were processed in the same manner. (Quantities of Cq, DECq, and IPA standards were five-fold those used with blood spots.)

For the blood-spot storage time study, multiple 100-μl pipet applications of the two whole-blood samples were spotted on each of two sheets of filter paper. These were analyzed in duplicate immediately after spotting and at 1, 4, 8, and 12 weeks thereafter; samples were stored at room temperature.

The paired venipuncture and finger-stick samples were analyzed as outlined above and the values compared.

HPLC separation of a mixture of antimalarials

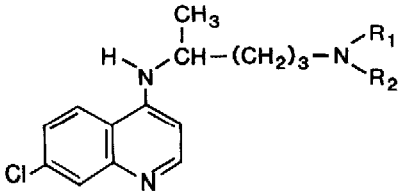
The HPLC system described above was applied to the separation of a mixture of antimalarials containing amodiaquine, pyrimethamine, chloroquine, and quinine in the following respective concentrations in ng/μl: 156, 150, 82, and 398. A 4-μl quantity of the mixture in mobile phase was injected. The mobile phase was hexane—MTBE—methanol—diethylamine (46:46:7.5:0.5). Detection was by UV absorbance at 254 nm at a sensitivity of 0.2 a.u.f.s.

RESULTS AND DISCUSSION

The structures of chloroquine, desethylchloroquine, and the internal-standard compound are shown in Fig. 1. The internal standard is the isopropyl analogue of desethylchloroquine, designated IPA.

Characterization of the method

The filter paper used in this study and recommended for field collections has been used for many years for collecting of blood samples for serological testing [4]. The paper is available in sheets and is generally cut into rectangles of the same dimension as microscope slides (75 × 25 mm). It is sufficiently thick and absorbent that a 100-μl quantity of blood spreads to form a circle of only about 12 mm. Filter papers should be handled by their edges only, and field personnel should take precautions to avoid contamination of the papers with traces of chloroquine from the fingers of persons who may have handled tablets — i.e., patients or the person taking blood samples.



Compound	Abbreviation	R_1	R_2
Chloroquine	Cq	CH_2CH_3	CH_2CH_3
Desethylchloroquine	DECq	CH_2CH_3	H
Internal standard	IPA	$CH(CH_3)_2$	H

Fig. 1. Structures of chloroquine, desethylchloroquine, and the internal-standard compound.

The 100-μl pipets were found to deliver 95 μl of whole blood under the conditions of sampling, i.e., delivery of blood with no subsequent rinsing of the pipet. All blood-spot values were calculated accordingly.

The extraction solvent of MTBE—hexane (1 : 1) provided a good compromise in polarity, effectively extracting the analytes and internal standard while minimizing the coextraction of potential interfering materials from blood. The use of diethyl ether—hexane mixtures was first explored, but under certain conditions resulted in the transformation of a portion of the chloroquine into a compound giving a peak eluting after chloroquine. The extent of transformation could be minimized by using freshly distilled diethyl ether (lower peroxide levels) and expediting the processing of samples. Since the retention time (t_R) of the transformation product was found to be near that of desethylchloroquine and quite variable depending on methanol concentration and column activity, methyl-*tert.*-butyl ether, with low propensity for peroxide formation, was substituted for diethyl ether. Even though the use of MTBE has eliminated the appearance in the chromatogram of the peak due to the chloroquine transformation product, we now routinely inject a solution containing the product to assure that there is no possibility of its significant formation during sample analysis with subsequent coelution with desethylchloroquine.

A methanol concentration of 25% in the solvent yields a t_R for the transformation product which falls between that of the internal standard and desethylchloroquine. A methanol concentration of 15% results in a t_R for the transformation product which is substantially longer than that for desethylchloroquine. The oxidation product has been identified as the aliphatic amine oxide of chloroquine by independent synthesis, chromatography, and proton nuclear magnetic resonance (NMR) spectrometry [5].

The extraction of the spots using 0.2 *M* hydrochloric acid was found to predispose the samples to the formation of an intractable emulsion when vigorous shaking was used for partitioning from aqueous base into the organic phase. A slow rotation of the samples at 30 inversions per min for a period of 15 min resulted in efficient partitioning and the formation of only a light emulsion which was readily spun down by centrifugation.

Fig. 2 depicts a chromatogram resulting from the analysis of filter-paper

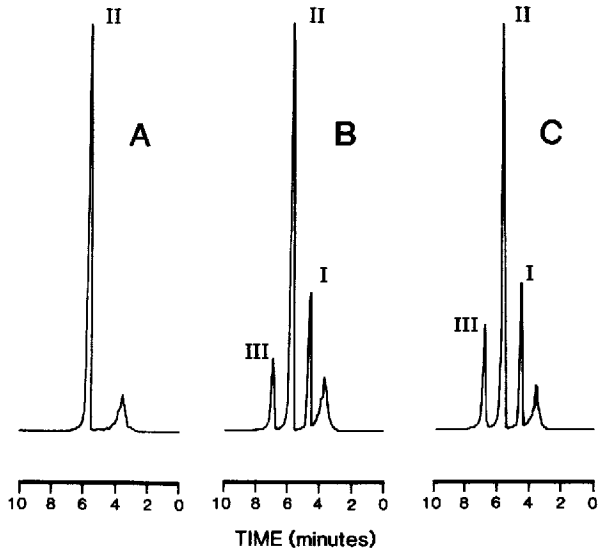


Fig. 2. Chromatograms resulting from the application of the method to: (A) a 100-μl blood-spot blank containing added internal standard alone; (B) a 100-μl blood-spot standard containing 24.6 ng of chloroquine and 12.5 ng of desethylchloroquine; (C) a 100-μl blood-spot sample taken 24 h subsequent to tablet ingestion in the 25th week of a 300-mg/week chemoprophylaxis regimen. Peaks: I = chloroquine, II = internal standard, III = desethylchloroquine.

blood spots. The composition of the mobile phase used was hexane—MTBE—methanol—diethylamine (37.25 : 37.25 : 25.0 : 0.5).

Statistical evaluation of the results of blood-spot filter-paper analysis for both chloroquine and its major metabolite showed that precision was excellent for both analytes (Tables I and II). That for the desethylchloroquine appeared to be somewhat better than for chloroquine, undoubtedly reflecting the former's closer structural similarity to the internal standard. The detection limits is 5 ng/ml for both analytes.

TABLE I

STANDARD-CURVE DATA FOR CHLOROQUINE

Cq added (x, ng)	Cq—internal standard peak-area ratio	Cq calculated* (x', ng)
0.00	0.0000	−0.36
9.85	0.1295	9.94
24.63	0.2986**	23.38
	0.3036	23.78
	0.3150	24.69
	0.3018	23.64
49.25	0.6534	51.60
73.88	0.9169	72.55

*Calculated from the least-squares straight line, $y = mx + b$ (m = 0.01258, b = 0.004516); r^2 = 0.9978; n = 5.

**R.S.D. (interstandard, n = 4) = 2.35%, R.S.D. (intrastandard, 3 injections) = 1.51%.

TABLE II

STANDARD-CURVE DATA FOR DESETHYLCHLOROQUINE

DECq added (x, ng)	DECq—internal standard peak-area ratio	DECq calculated* (x', ng)
0.00	0.0000	0.34
3.12	0.0467	2.83
6.23	0.1043	5.89
12.47	0.2379**	13.01
	0.2339	12.80
	0.2323	12.71
	0.2329	12.74
24.93	0.4609	24.89

*Calculated from the least-squares straight line, $y = mx + b$ ($m = 0.01878$, $b = -0.006367$); $r^2 = 0.9989$; $n = 5$.

**R.S.D. (interstandard, $n = 4$) = 1.07%, R.S.D. (intrastandard, 3 injections) = 0.36%.

To study stability of chloroquine and desethylchloroquine in filter-paper blood spots stored at room temperature, concentrations of both compounds were determined in stored blood samples that had been taken at 24 h and 10 days after administration of the final tablet in a chemoprophylactic regimen. The concentrations of chloroquine and desethylchloroquine at the end of the twelve weeks' period, the longest period tested, appear to be no different from those at the beginning of the storage study (Table III).

TABLE III

STORAGE STABILITY OF CHLOROQUINE AND DESETHYLCHLOROQUINE IN FILTER-PAPER-COLLECTED 100-µl BLOOD SAMPLES FROM A VOLUNTEER UNDERGOING CHEMOPROPHYLAXIS

Sample designation	Time (weeks)	Levels during storage (duplicate spots)	
		Cq (ng/ml)	DECq (ng/ml)
24 h*	Pre-trial analysis**	259 267	161 154
	0	277 263	170 173
	1	266 224	139 135
	4	292 286	165 179
	8	267 277	157 149
	12	265 249	170 178
10 days*	Pre-trial analysis**	65 67	43 45
	0	59 74	44 38
	1	71 63	47 47
	4	57 63	42 47
	8	69 66	42 45
	12	67 65	53 49

*The volunteer had been on chemoprophylaxis for 25 weeks. Sample designations refer to the time between administration of the final tablet and sampling.

**Determined from 500-µl whole-blood samples processed without absorption on filter paper.

TABLE IV

COMPARISON OF FILTER-PAPER-COLLECTED, FINGER-STICK BLOOD-SPOT VALUES FOR CHLOROQUINE AND DESETHYLCHLOROQUINE WITH THOSE FROM CORRESPONDING VENIPUNCTURE DETERMINATIONS

Sample designation	Cq (ng/ml)		DECq (ng/ml)	
	Venipuncture	Finger-stick	Venipuncture	Finger-stick
W-20, 7 days	123	127	58	64
W-21, 4 h	509	546	182	178
W-21, 24 h	311	282	121	124
W-22, 24 h	249	226	111	104

Venipuncture/finger-stick comparability study

There was close agreement between the analytical results from blood samples collected by venipuncture and corresponding finger-stick blood samples collected on filter papers (Table IV).

General approach to the separation and quantification of antimalarials

We used normal-phase chromatography in our approach to chloroquine analysis, as had Alván et al. [3] previously, because of the selectivity afforded. Our mobile-phase system allows facile adjustment of solvent polarity at lower values of solvent strength than that of Alván et al. [3], permitting application to the separation of less polar antimalarials such as pyrimethamine and amodiaquine. The reservoir for one pump contains hexane—MTBE—diethylamine (49.75:49.75:0.5) while that for the second pump contains methanol—diethylamine (99.50:0.5). The solvents are blended by a solvent programmer, and all analyses are run isocratically. Fig. 3 illustrates the utility of the chromatographic system, depicting a chromatogram run on a mixture of anti-

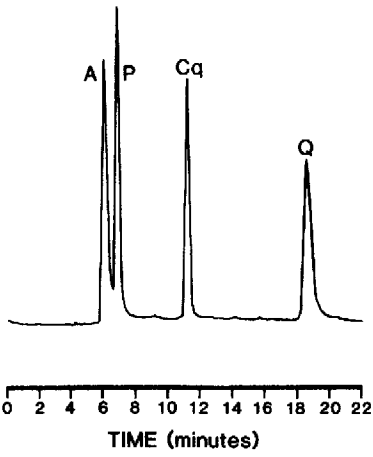


Fig. 3. Chromatogram resulting from the injection of a mixture containing 156 μg/ml of amodiaquine (A), 150 μg/ml of pyrimethamine (P), 82 μg/ml of chloroquine (Cq), and 398 μg/ml of quinine (Q). The mobile phase was hexane—MTBE—methanol—diethylamine (46:46:7.5:0.5), and UV detection was employed at 254 nm and a sensitivity of 0.2 a.u.f.s.

malarials at an intermediate methanol concentration with UV detection at 254 nm. Selection of methanol concentration and UV wavelength allows tailoring of the system to each sample for which the analysis of a specific mixture of antimalarials is to be run. The amodiaquine peak tails somewhat, probably reflecting incomplete attenuation of phenolic acidity by the intramolecular hydrogen bonding. Pyrimethamine is not completely separated from amodiaquine at this methanol concentration. Sulfadoxine, formulated with pyrimethamine in the antimalarial combination Fansidar®, elutes from the silica column at a methanol concentration of 33%.

Applications of the chloroquine filter-paper, blood-spot method

The method described above makes possible simple and convenient collection and transport of whole-blood samples from the field. Problems associated with collecting venipuncture samples and special handling requirements of such samples in transit are avoided. These advantages make possible the gathering of data from remote regions of malaria endemicity, allowing, in concert with in vitro anti-malarial susceptibility testing, efficient mapping of the prevalence and spread of chloroquine-resistant *Plasmodium falciparum* malaria.

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