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ANALYSIS OF BLOOD AND URINE SAMPLES FOR HYDROXYCHLOROQUINE AND THREE MAJOR METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A high-performance liquid chromatographic (HPLC) method using fluorescence detection is described for the quantification of hydroxychloroquine (HCQ) and three of its metabolites in blood and urine samples. The method is selective, permitting quantification of analytes without interferences from chloroquine or quinine in the sample. Detection limits for HCQ, desethylhydroxychloroquine, desethylchloroquine, and bisdesethylchloroquine are 10, 30, 5, and 5 ppb, respectively, for a 100-μl blood or urine sample. The internally standardized method requires only one extraction step and utilizes normal-phase HPLC conditions including an amine modifier in the mobile phase. These conditions facilitate fluorescence detection, selective separation, and acceptable peak shapes. A mobile phase of 0.5% *n*-butylamine in methanol–hexane–methyl *tert* butyl ether (1:1:1) is used in the analysis. Analysis of blood and urine samples from two healthy volunteers given 400 mg of Plaquenil (310 mg of HCQ base) weekly for four weeks provided data on HCQ metabolism for the two persons during the recommended chemoprophylactic regimen for malaria.

INTRODUCTION

Hydroxychloroquine (HCQ) is a 4-aminoquinoline compound whose properties as an antimalarial [1–3] and antirheumatic [4, 5] drug are similar to those of its structural analogue chloroquine (CQ). CQ is a relatively non-toxic drug, and HCQ is reportedly less toxic than CQ [6]. Several high-performance liquid chromatographic (HPLC) methods have been developed in recent years to sensitively and selectively assay HCQ and its metabolites desethylhydroxychloroquine (DHCQ), desethylchloroquine (DCQ), and bisdesethylchloroquine (BDCQ) [7–9] (Fig. 1). CQ is the internal standard for these methods. The

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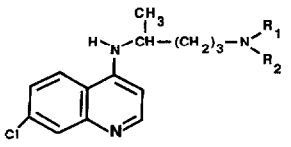

Compound (Abbreviations)	R_1	R_2
Hydroxychloroquine (HCQ)	CH_2CH_3	CH_2CH_2OH
Chloroquine (CQ)	CH_2CH_3	CH_2CH_3
Desethylhydroxychloroquine (DHCQ)	H	CH_2CH_2OH
Desethylchloroquine (DCQ)	CH_2CH_3	H
Bisdesethylchloroquine (BDCQ)	H	H
Internal Standard [IS (IPA)]	$CH(CH_3)_2$	H

Fig. 1. Structures of hydroxychloroquine and metabolites, chloroquine, and the internal standard (IPA).

method of Tett et al. [7] uses a poly(styrene divinylbenzene) column, an alkaline mobile phase, and fluorescence detection, while those of Morris [8] and of Brown et al. [9] use bonded silica columns, acidic mobile phases, and ultraviolet spectrophotometric detection.

Using CQ as internal standard in malaria studies is impractical because this antimalarial drug is widely used throughout the world. The presence of CQ in samples yields incorrectly low values for HCQ and metabolites using the above-mentioned assays, so identification of an alternative internal standard was indicated. Conventional silica HPLC columns with organic mobile phases containing less than 1% of amine modifiers provide efficient separation of CQ and its metabolites while permitting fluorescence detection [10, 11]. We applied this same approach to quantification of HCQ and its metabolites in the presence of CQ.

In this report we describe a sensitive and selective normal-phase HPLC method with fluorescence detection for quantification of HCQ, CQ, and their metabolites, which uses an analogue of DCQ as internal standard (Fig. 1). Analyses of whole blood and urine samples from two healthy, male, adult volunteers during weekly antimalarial chemoprophylaxis demonstrated the usefulness of the method.

EXPERIMENTAL*

Standards

Hydroxychloroquine sulfate (Plaquenil), desethylhydroxychloroquine base, and desethylchloroquine base were supplied by Sterling-Winthrop Research Institute (Rensselaer, NY, U.S.A.). Bisdesethylchloroquine base and the internal standard compound, 7-chloro-4-(1′-methyl-4′-isopropylaminobutylamino)quinoline (IPA), were provided by Walter Reed Army Institute of Research (Washington, DC, U.S.A.). Chloroquine diphosphate was purchased from Sigma (St. Louis, MO, U.S.A.).

*Use of trade names and commercial sources is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

Reagents and materials

Spectroscopic-quality hexane, methyl *tert.*-butyl ether (MTBE), and methanol were glass-distilled (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). Ammonia in methanol solution was made by passing ammonia gas from a lecture bottle (Matheson) through a filter-flask trap and a gas-diffusion tube into a 400-ml quantity of methanol for 60 min. Titration with 1.0 *M* aqueous hydrochloric acid to a pH 4.5 end-point showed that the methanol contained 4.25% ammonia. This solution was added in appropriate amounts to the mobile phase to yield the final ammonia concentration desired for each chromatographic trial. The *n*-butylamine (Aldrich, Milwaukee, WI, U.S.A.) was of 99+% purity. Human blood used for standards was freshly collected with acid–citrate–dextrose (ACD) solution as an anticoagulant. All other chemicals used were of reagent grade or better.

Equipment

In preliminary work the HPLC apparatus consisted of a Waters Model QA-1 automated HPLC system coupled to a Waters Radial-PAK silica column (5 μm particle diameter; 100 mm×8 mm), a Perkin-Elmer Model 650 S fluorescence detector, and a Waters QA-1 data system (system I). Later work used an HPLC system containing a Waters Model M-6000A pump, a Waters Model U6K loop injector, a DuPont Zorbax-Sil column (5 μm particle diameter; 250 mm×4.6 mm I.D.), a Hewlett-Packard Model 1046A fluorescence detector, and a Hewlett-Packard Model 3390A integrator (system II). Both detectors were operated with an excitation wavelength of 320 nm and an emission wavelength of 380 nm.

Development of separation conditions

The Waters Radial-PAK silica-gel HPLC column in system I was used to evaluate the separation characteristics of various concentrations of ammonia and *n*-butylamine in methanol–MTBE–hexane mobile phases. The analyte mixture contained HCQ and its N-dealkylated metabolites, CQ, quinine (Q), and the IPA internal standard candidate. Final conditions for the method were developed with *n*-butylamine modifier in methanol–MTBE–hexane with system II incorporating the Zorbax-Sil column. This system was used to assay the samples.

Analysis of whole blood and urine samples

Standards were prepared by adding 100-μl quantities of blank blood, appropriate amounts of analyte standard solutions (ranging from 0 to 936, 985, 1030, 765, and 517 ng/ml for HCQ, CQ, DHCQ, BDCQ, and DCQ, respectively), and 20 μl of the internal standard solution (530 ng/ml in 0.002 *M* hydrochloric acid) to 15-ml centrifuge tubes. Blood samples (100 μl) and 20 μl of internal standard solution were also placed in 15-ml centrifuge tubes. Deionized water (2 ml) and 0.5 ml of 5 *M* sodium hydroxide were added to samples and standards alike to lyse the red cells. A 3-ml quantity of hexane–MTBE (1:1) was added to each, the tubes were capped with PTFE-lined screw caps and rotated for 30 min at 30 inversions per min to effect extraction (Glas-Col Model RD-250 rotator with a Model RD-60 rotator head; Terre Haute, IN, U.S.A.). Samples and standards

were centrifuged for 10 min at 900 *g*, and the organic layer of each sample was transferred to a clean centrifuge tube and evaporated to dryness under a gentle flow of nitrogen in a water bath held at 60°C. Samples and standards were reconstituted in 200-μl quantities of HPLC mobile phase, and 5–15 μl (depending on concentration) of each concentrated extract were injected onto HPLC system II.

For the determination of metabolites and CQ levels in urine, the procedure was similar to the whole blood method. However, due to its high concentration in urine, HCQ was determined separately using a 50-μl urine aliquot, 50 μl of internal standard (5.3 μg/ml), HCQ standards ranging from 0 to 37.4 μg/ml, and reconstitution to 500 μl with 5-μl injection volume.

The HPLC mobile phase for the assay was hexane–MTBE–methanol (1:1:1) containing 0.5% *n*-butylamine. Flow-rate was 1.0 ml/min. The fluorescence detector was set at an excitation wavelength of 320 nm and an emission wavelength of 380 nm. Fluorescence peak-height ratios were used to construct linear regression standard curves for each of the analytes, and samples were quantified with reference to these curves.

Recovery studies

Individual standard solutions of HCQ, CQ, DHCQ, DCQ, BDCQ, and internal standard were made up in mobile phase [0.5% butylamine in methanol–MTBE–hexane (1:1:1)]. Concentrations were all accurately known and about 1 μg/ml. Triplicate 100-μl quantities of blank blood were fortified with each of the analyte solutions in turn; concentrated whole blood extracts were injected and compared with the unextracted solutions after appropriate adjustments for aliquoting in the extraction procedure.

Dosing of volunteers, collection of samples, and stability studies

Volunteers I and II, weighing 84 and 88 kg, respectively, each ingested 400 mg of Plaquenil (hydroxychloroquine sulfate), 310 mg as base, weekly for four weeks. This dosage is the recommended antimalarial chemoprophylactic regimen [12]. Blood samples were collected by venipuncture (in heparinized tubes) at selected times during and after the four-week dosing period. Additionally, urine samples were taken at times corresponding to certain of the blood sampling times. All samples were stored at 4°C. Initial analyses were completed within two weeks after collection and certain samples were reanalyzed eight months later to ascertain the stability of HCQ and metabolites in whole blood.

RESULTS AND DISCUSSION

Development and characterization of the method

Both DHCQ and BDCQ yield broad, tailing peaks under the conditions described previously [11] for quantification of CQ and metabolites by normal-phase HPLC using 0.5% diethylamine (DEA) as a mobile phase modifier. We investigated the use of ammonia and of *n*-butylamine, less sterically hindered bases than DEA, as mobile-phase modifiers.

A mobile phase of methanol–MTBE–hexane (40:30:30) with 0.2% ammonia

gave peaks for HCQ, CQ, and dealkylated metabolites (Fig. 2) using HPLC system I. The peaks in the chromatogram are sharp except for that due to DHCQ, which is broadened to nearly twice the width that would have been predicted from its retention time and the peak widths of the CQ and internal standard peaks. Even so, the separation is good for analytes and internal standard. However, using this mobile phase routinely is impractical because unavoidable evaporation of the ammonia yields changing retention characteristics for the analytes.

A second mobile phase, 0.5% *n*-butylamine in methanol–MTBE–hexane (33:33:33), was tested with system I. The analytes were separated effectively with retention times for HCQ, Q, CQ, DHCQ, BDCQ, and DCQ of 3.04, 3.43, 3.93, 4.76, 5.80, and 7.06 min, respectively; however, under these conditions the IPA, proposed as internal standard, had a retention time of 4.60 min and was not separated from the DHCQ peak.

One approach to achieving complete separation of analytes and internal standard is to use the *n*-butylamine mobile phase and Radial-PAK silica column and a late-eluting internal standard. Such an internal standard would be a polar compound with similar fluorescence properties to the analytes, and in any event would lengthen analysis time. It was decided instead to identify a column and a mobile phase composition that would adequately separate analytes and the IPA internal standard candidate. The substitution of the DuPont Zorbax-Sil column incorporated in system II yielded sufficient separation of DHCQ from IPA to permit peak-height quantification of all analytes with good linearity and precision. The

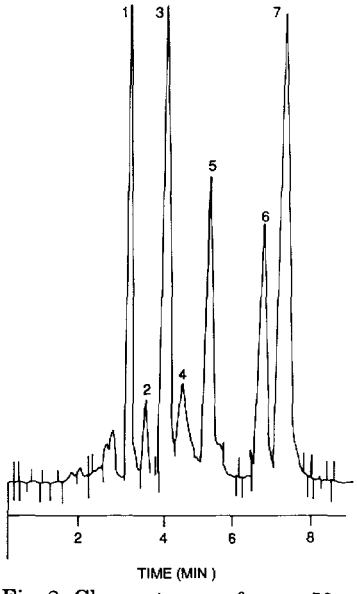

Fig. 2. Chromatogram from a 50-μl injection of standards dissolved in mobile phase solvent using HPLC system I with mobile phase methanol–MTBE–hexane (40:30:30) containing 0.2% ammonia, flow-rate 1.0 ml/min. Peaks: 1 = HCQ; 2 = Q; 3 = CQ; 4 = DHCQ; 5 = internal standard; 6 = BDCQ; 7 = DCQ. The amounts of the standards injected for the above analytes to give the peaks shown in the chromatogram are 11.7, 10.0, 12.3, 10.3, 6.38, 7.65, and 12.5 ng, respectively.

chromatograms seen for extracts of blood and urine standards and samples using system II are shown in Fig. 3 with an analysis time of 8 min.

Statistical parameters for the method are shown in Table I. The standard curves were linear for all the analytes from 0 to 1000 ng/ml for extraction of 0.1 ml whole blood or urine. Detection limits were low (5–10 ng/ml) for all analytes except DHCQ. This compound was not as effectively extracted and gave a broader peak shape than the other compounds, with a detection limit of 30 ng/ml. The DHCQ peak shape also caused occasional peak-area integration problems which made

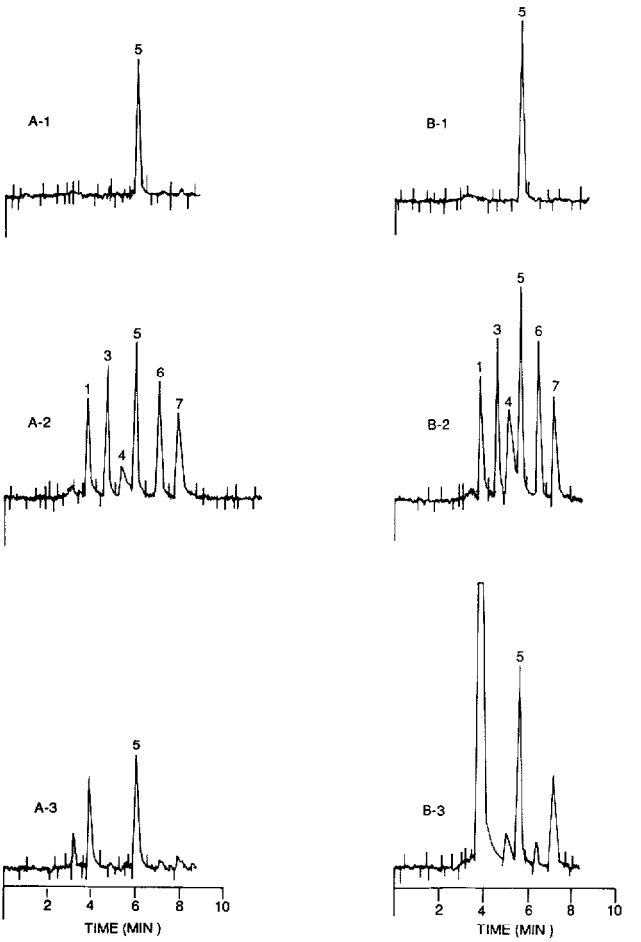

Fig. 3. Chromatograms from application of the method, HPLC system II, to (A) 100-μl quantities of whole blood and (B) 100-μl quantities of urine. A-1 resulted from injection of a blood blank containing internal standard alone; A-2 resulted from injection of HCQ, CQ, DHCQ, BDCQ, and DCQ (1.76, 1.85, 1.94, 1.43, and 0.97 ng, respectively) from the concentrated extract of a blood standard; A-3 resulted from injection of a corresponding extract of a 100-μl sample of the three-day, week 4, blood drawn from volunteer II. Peak numbering is as for Fig. 2. The chromatograms of urine extracts are analogous, except that 3.88 ng of DHCQ was in the injection, not 1 94 ng. These conditions give an off-scale peak for HCQ, but permit quantification of metabolites. Blood and urine blanks showed no peaks at the retention time corresponding to that of the internal standard.

TABLE I

STANDARD CURVE (PEAK HEIGHT) DATA FOR 100 μl WHOLE BLOOD ANALYSIS

Compound	Recovery (%)	Concentration (ng/ml)	Coefficient of variation (%)		r^{2***}	Concentration range (ng/ml)	Detection limit (ng/ml)
			Inter-standard*	Intra-standard**			
HCQ	87.4	234	4.9	2.3	0.999667	0-936	10
		702	3.6	5.1			
CQ	91.2	246	3.4	1.9	0.994825	0-985	8
		739	3.7	3.5			
DHCQ	34.9	258	9 6	3.7	0 983724	0-1030	30
		733	6.9	3.4			
DCQ	84.5	129	2 6	1.9	0.999541	0-517	5
		388	4.5	5.7			
BDCQ	65.2	191	7.5	4.9	0 999209	0-765	5
		574	5.3	3.3			

*Multiple extractions of standard, $n = 8$.
**Multiple injections of standard, $n = 8$
***Square of correlation coefficient, five-point standard curve.

peak-height calculations necessary for this compound. For consistency all calculations were made by peak-height comparisons, although there was good correlation between peak-area and peak-height analytical results and statistical parameters for all other analytes. Assay reproducibility, both intra-standard and inter-standard, was determined using peak-height comparisons for low-to-mid and mid-to-high range concentrations of all the analytes and was below 10% in all cases.

A method designed to quantify HCQ and metabolites in malaria patients must be able to separate CQ from other eluting compounds and, if desired, quantify it, because of the widespread use of this compound in malaria chemoprophylaxis and chemotherapy. Three published methods for the quantification of HCQ and metabolites in patients with rheumatoid diseases [7–9] use CQ as internal standard and are therefore inappropriate for use in assays of body fluids from malaria patients. Q, if present, would coelute with CQ by the method described herein using system II. A peak at this retention time would indicate previous use of an antimalarial, most likely CQ. Under the conditions of separation BDCQ elutes before the less polar DCQ.

The present method employs a single extraction with hexane–MTBE (1:1). MTBE is much less prone to peroxide formation than diethyl ether, reducing the possibility of aliphatic N-oxide formation during sample preparation [11]. The sensitivity afforded by fluorescence detection permits quantification of HCQ and metabolites in a 100-μl blood sample so that sample collection may be by finger stick.

Volunteer studies

The concentrations of HCQ and metabolites found in blood at various times during the four-week dosing of volunteers are given in Table II. Concentration–time profiles for HCQ in whole blood during weeks 1 and 4 of chemoprophylaxis are displayed in Fig. 4. Results for urine samples are given in Table III. The concentrations of metabolites are small compared to those of HCQ for all of the samples. Reanalysis after eight months of four whole blood samples (two time points from each volunteer) stored at 4°C showed no loss of HCQ and other analytes compared to levels seen in the initial analyses.

HCQ concentrations in the blood of volunteer II for week 4 sampling are less than half of the corresponding values for CQ in a completely analogous dosing regimen administered to the same volunteer in an earlier study [13]. In this earlier study the volunteer is identified by the initials F.C., and the data are given in Table II. HCQ data from the present study should be converted to nmol/l for direct comparison with Table II in ref. 13.

The small levels of CQ seen in the urine of volunteer II are due to CQ taken prophylactically several months previously for travel to a malarious area (Table III). This observation demonstrates the importance of having a method in which CQ is separated from the analytes and the internal standard. Levels of CQ in the blood of volunteer II were below detection limits.

TABLE II

CONCENTRATIONS OF HCQ AND METABOLITES IN THE BLOOD OF TWO VOLUNTEERS RECEIVING HCQ SULFATE (310 mg AS BASE) WEEKLY

Values are for volunteer I; values in parentheses are for volunteer II.

Dosing week	Time after dosing (h)	Concentration of analyte (ng/ml)			
		HCQ	DHCQ	DCQ	BDCQ
	Predose	<10 (<10)	<30 (<30)	<5 (<5)	<5 (<5)
1	4	250 (261)	<30 (<50)	14 (20)	<5 (15)
	8	140 (184)	<30 (<30)	9 (13)	<5 (<5)
	24	72 (69)	<30 (<30)	8 (11)	<5 (6)
	72	36 (29)	<30 (<30)	12 (<5)	13 (<5)
	168	20 (19)	<30 (<30)	<5 (8)	<5 (6)
2	4	178 (516)	<30 (<30)	14 (31)	<5 (<5)
3	4	298 (518)	33 (<30)	18 (30)	<5 (<5)
	168	66 (44)	<30 (<30)	<5 (10)	<5 (<5)
4	4	432 (315)	45 (<30)	30 (30)	<5 (<5)
	8	315 (–)	<30 (–)	30 (–)	9 (–)
	24	– (156)	– (<30)	– (30)	– (<5)
	30	150 (–)	<30 (–)	17 (–)	6 (–)
	72	87 (108)	50 (<30)	18 (27)	15 (<5)
	7 days	– (78)	– (<30)	– (14)	– (<5)
	9 days	68 (63)	<30 (<30)	12 (12)	6 (5)
	14 days	45 (–)	<30 (–)	16 (–)	19 (–)
	21 days	31 (37)	<30 (<30)	8 (11)	<5 (17)

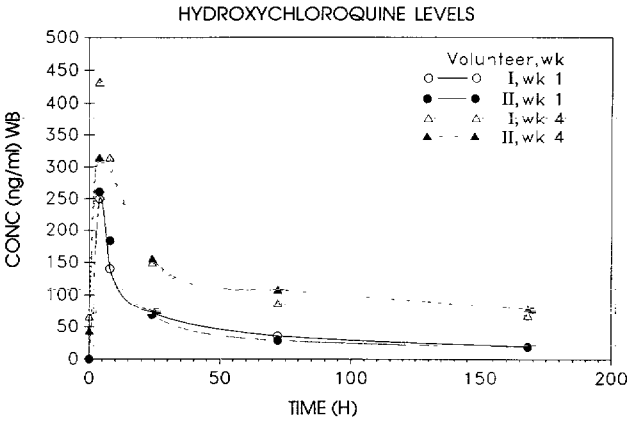

Fig. 4. Hydroxychloroquine levels in whole blood of volunteers I and II during week 1 and week 4 of weekly Plaquenil (310 mg as base) chemoprophylaxis.

TABLE III

CONCENTRATIONS OF HCQ AND METABOLITES IN THE URINE OF TWO VOLUNTEERS RECEIVING HCQ SULFATE (310 mg AS BASE) WEEKLY

Values are for volunteer I; values in parentheses are for volunteer II Detection limit is about 0.01 μg/ml for each analyte.

Dosing week	Time after dosing (h)	Concentration of analyte (μg/ml)				
		HCQ	DHCQ	DCQ	BDCQ	CQ
	Predose	– (0.00)*	– (0.00)	– (0.03)	– (0.02)	– (0.04)*
2	4	– (27.41)	– (0.51)	– (0.84)	– (0.15)	– (0.28)
	24	2.62 (3.95)	0.10 (0.06)	0.09 (0.29)	0.03 (0.04)	0.00 (0.18)
3	4	17.73 (15.66)	0.83 (0.21)	0.67 (0.42)	0.15 (0.08)	0.00 (0.14)
	24	3.24 (5.09)	0.16 (0.12)	0.17 (0.34)	0.04 (0.05)	0.00 (0.17)
4	4	10.63 (10.03)	0.55 (0.14)	0.37 (0.46)	0.12 (0.06)	0.00 (0.11)
	24	2.48 (3.10)	0.15 (0.08)	0.11 (0.21)	0.04 (0.04)	0.00 (0.07)

*Volunteer II had received CQ during chemoprophylaxis while traveling in Africa.

CONCLUSION

The published HPLC methods to assay HCQ and metabolites use reversed-phase conditions that yield narrow, early peaks for metabolites; such conditions have a salutary effect on the detection limits and peak shapes for these analytes. However, there are several advantages to using normal-phase conditions with an amine modifier in the mobile phase for separation of aminoquinoline and quinolinemethanol antimalarials. Sample preparation may be accomplished with one extraction step. By judicious choice of amine modifiers, the methanol–hexane–MTBE solvent system may be used to quite selectivity separate combinations of analytes, such as HCQ, CQ, and metabolites, as seen in the present work, and of

CQ and metabolites, Q, and quinidine [14]. In this HCQ study with normal-phase conditions, the metabolites emerge after the parent drug and certain of these analytes can show appreciably broadened peaks (e.g. DHCQ); however, the parent drug is first eluted which enhances detectability because of its narrow peak shape. Choice between the various published methods depends on the requirements for the application planned. The HPLC method described herein is specifically tailored to malaria studies since it is designed to avoid interference by CQ.

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