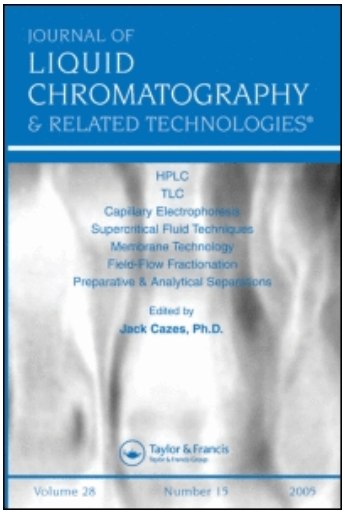

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A HPLC Method for the Separation and Quantification of the Enantiomers of Hydroxychloroquine and Its Three Major Metabolites

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A HPLC METHOD FOR THE SEPARATION AND QUANTIFICATION OF THE ENANTIOMERS OF HYDROXYCHLOROQUINE AND ITS THREE MAJOR METABOLITES

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ABSTRACT

A two step HPLC method for analysis of the enantiomers of Hydroxychloroquine (HCQ) and its three major metabolites, Desethylhydroxychloroquine (DHCQ), Desethylchloroquine (DCQ) and Bisdesethylchloroquine (BDCQ), was developed. Fluorescence detection was used at λ_{ex}=230nm and λ_{em}=385nm with a 370nm cut-off filter. This method has higher sensitivity and better resolution of the parent drug and its three metabolites when compared to published methods.

In the first step, a cyano column was used to separate and collect fractions containing HCQ and its three metabolites. The mobile phase was 20% pH 6.0 (0.015M K_2HPO_4) buffer, 30% methanol and 50% acetonitrile at a flow rate of 2 ml/min and 50°C. This method was linear over the concentration ranges of 2-20, 20-300 and 200-2000 ng/ml of blood (r>0.99) and was used for quantitation.

In a second step, chiral separation was performed on a chiral-AGP column using a mobile phase of 94% pH 7.0 (0.05M $NH_4H_2PO_4$, 0.005M dihexylamine) buffer, 5% isopropanol and 1% acetonitrile at a flow rate of 1 ml/min and 35°C. Baseline separation was obtained for the enantiomers of the parent drug and its metabolites.

Resolution of the enantiomers on the chiral column for HCQ, DHCQ, DCQ and BDCQ was 3.2, 3.2, 2.9 and 2.5, respectively. The limit of detection for HCQ and its metabolites was less than 1 ng for each enantiomer.

INTRODUCTION

Hydroxychloroquine (HCQ) is used widely as an antimalarial and in the treatment of rheumatoid arthritis and systemic lupus erythematosus. HCQ has three major metabolites, desethylhydroxychloroquine (DHCQ), desethylchloroquine (DCQ), and bisdesethylchloroquine (BDCQ). Two multistep HPLC methods for the determination of the enantiomers of HCQ and its metabolites have been published [1,2]. In both of these methods, the parent drug and its metabolites were separated and quantitated in the first stage, and then the ratios of the enantiomers were determined in the second stage. The methods require fractional collection of the separate eluting peaks (racemic drug or a metabolite) which are then injected onto another HPLC system for chiral separation of the enantiomers.

The first of these methods [1] uses the quantitative procedure of Tett, et al. [3] as its first stage. With fluorescence detection this method has good sensitivity but exhibits only minimal separation of HCQ from its metabolites. This poor resolution makes it difficult to collect pure fractions of drug or metabolite for use in the second stage of analysis. The chiral separation also exhibited several limitations: baseline separation of the enantiomers was not obtained, special equipment for postcolumn alkalinization was required, and an elution time of nearly one hour was needed to separate each pair of enantiomers. The second method [2] had good resolution

of compounds in both stages of the procedure, but obtained only limited sensitivity with the use of a UV detector. In addition, both methods have the disadvantage of using diethylether in sample preparation.

The method presented in this paper also uses a two-stage procedure, but incorporates several improvements over the other two methods. These include use of a less volatile extraction solvent and choices of chromatographic conditions leading to increased detector sensitivity and excellent resolution of the racemic compounds and enantiomeric pairs. This method is applicable to the study of the pharmacokinetic properties of HCQ and its three major metabolites (DHCQ, DCQ and BDCQ) and for the determination of enantiomer ratios of each of the compounds.

MATERIALS AND METHODS

Instrumentation

The analysis was performed on a Hewlett Packard model 1090 HPLC system fitted with a Hewlett Packard model 1046A fluorescence detector. The effluent was monitored at λ_{ex}=230nm and λ_{em}=385nm through a 370nm cut-off filter. The output was recorded on a Hewlett Packard model 3392A integrator. The column used for achiral assay was a cyanopropyl column (250 x 4.6mm I.D., 5μ) manufactured by Baxter/Burdick & Jackson with a cyano guard column (10 x 3mm I.D., 7μ) manufactured by Applied Biosystems. The column used for the enantiomeric separation was a Chiral-AGP (100 x 4.0mm I.D., 5μ) manufactured by ChromTech AB (distributed by Regis).

Reagents

HCQ-sulfate, R(-)-HCQ, DHCQ, BDCQ and DCQ were obtained from Sanofi/Winthrop Pharmaceuticals. Propranolol-HCL was obtained from Sigma. All organic solvents were HPLC grade. K_2HPO_4, $NH_4H_2PO_4$, H_3PO_4, dihexylamine and NaOH were analytical grade.

Solution Preparation

Drug solutions: Stock solutions containing HCQ, DHCQ, BDCQ and DCQ were prepared at concentrations of 100 μg/ml methanol. Working dilutions to 10, 1 and 0.1 μg/ml methanol were prepared from the stock solutions.

Internal standard (IS) solution: IS stock solution containing propranolol-HCL was prepared at 500 μg/ml methanol.

Blood lysing solution: Blood lysing solution was prepared by diluting 20 μl IS stock in 500 ml deionized distilled water.

Extraction solution: Ethyl acetate and isopropanol were mixed (90:10) for extraction solution.

Quality Control (QC) Samples

Drug free whole blood was spiked with known concentrations of HCQ and its metabolites. The QC samples were stored frozen and used to determine intraday and interday precision of the assay.

HPLC Conditions

Achiral chromatography: Mobile phase consisted of 20% buffer (0.015M K_2HPO_4, pH adjusted to 6.0 with

H_3PO_4), 30% methanol and 50% acetonitrile. Flow rate was 2.0 ml/min at 50°C.

Chiral chromatography: Mobile phase consisted of 94% buffer (0.05M $NH_4H_2PO_4$ containing 0.005M dihexylamine, pH adjusted to 7.0 with 3N NaOH), 5% isopropanol and 1% acetonitrile. Flow rate was 1.0 ml/min at 40°C.

Sample Preparation and Analysis

Whole blood was spiked to prepare standards containing 2-2000 ng each of the drug and metabolites/ml blood. All samples (standards and QCs) were processed by adding 2.0 ml of blood lysing solution to 1.0 ml of whole blood which had been placed in a polypropylene tube. They were then vortexed for 10 seconds, sonicated for 10 minutes and centrifuged at 3000 rpm for 20 minutes. Exactly 2.0 ml of the supernatant was transferred to a second tube where 1.0 ml of 0.1N NaOH and 8.0 ml of extraction solution were added. These mixtures were vortexed for 30 seconds and centrifuged for 15 minutes at 3000 rpm. The organic layer was transferred to a third tube using a disposable polyethylene pipet and evaporated to dryness at 35-40 degrees C under a gentle stream of air. The residue was reconstituted in 250 µl methanol by vortexing for 30 seconds. The reconstituted samples were transferred to plastic HPLC vials and injected (at volumes of 10-100 µl) onto the achiral column.

The peaks of HCQ and its three metabolites eluting from the achiral system were collected separately and evaporated to dryness. The individual dried fractions were then reconstituted in 200 µl of the chiral system mobile phase, and 10-100 µl was injected onto the chiral column to separate the enantiomers.

Absolute recovery of HCQ and the metabolites was estimated by comparing the achiral peak heights of spiked whole blood standards extracted in triplicate to methanol solutions of the compounds.

RESULTS AND DISCUSSION

Achiral Separation

A cyano HPLC column was used to separate and quantitate HCQ and its metabolites. The achiral system gave excellent resolution of the compounds and allowed easy collection of the pure single peaks (Table 1 and Figure 1). The limit of quantitation was less than 2ng/ml of blood for each of the four compounds. The standard curves were calculated using three concentration ranges: 2-20ng/ml, 20-300ng/ml and 200-2000ng/ml. All the correlation coefficients of the standard curves of HCQ, DHCQ, BDCQ and DCQ were greater than 0.99 indicating that this method can be used for a very large range of concentrations. The mean

TABLE 1

Capacity Factors and Retention Times of HCQ and Its Three Major Metabolites on the Achiral HPLC System.

Compound	K[a]	t_r[b] (min)
HCQ	9.1	9.72
DHCQ	13.9	14.34
BDCQ	18.6	18.90
DCQ	24.4	24.52

a. Capacity factors.
b. Retention times.

recoveries of HCQ, DHCQ, BDCQ and DCQ from blood were 75.2%, 86.7%, 47.0%, and 94.8%, respectively.

Quality control blood samples containing HCQ and the metabolites were analyzed in triplicate on three separate days versus freshly spiked standards. Tables 2 and 3 summarize the results of the QC analysis. Intraday and interday variation over a wide range of concentrations (4ng/ml to 1500ng/ml) indicate good reproducibility (CV% range 0.2-14.7%) and accuracy (90.6-115.4%) of the method.

Chiral Separation

A two-stage procedure was chosen because simultaneous separation of all the enantiomers of HCQ

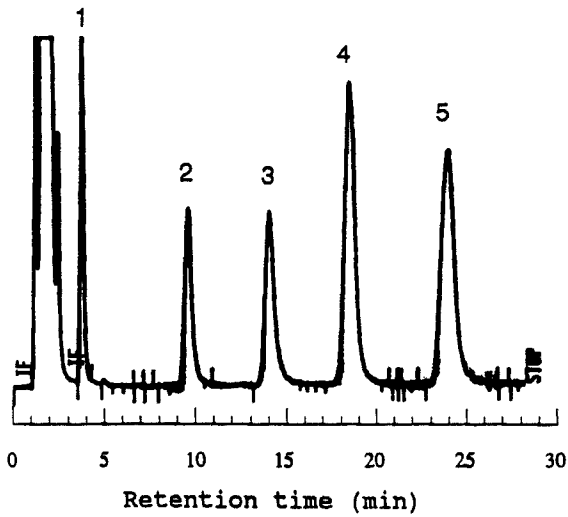

FIGURE 1. Chromatogram of achiral separation of spiked whole blood (50ng/ml). Peaks: 1 = IS, 2 = HCQ, 3 = DHCQ, 4 = BDCQ, 5 = DCQ.

TABLE 2

Accuracy and Reproducibility of Quantitation of HCQ and Its Metabolites--Intraday Quality Control Data (n=3).

	Blood Concentration (ng/ml)			
	Added	Measured	CV%	Accuracy%
HCQ	4.0	4.1	1.9	102.1
	250.0	252.5	3.8	101.0
	1500.0	1392.0	5.3	92.8
DHCQ	4.0	3.6	4.0	90.6
	250.0	255.3	0.2	102.1
	1500.0	1610.9	10.3	107.4
BDCQ	4.0	4.6	10.4	115.4
	250.0	244.3	7.0	97.7
	1500.0	1597.6	9.7	106.5
DCQ	4.0	3.8	6.0	95.4
	250.0	248.4	1.8	99.4
	1500.0	1611.0	10.4	107.4

TABLE 3

Accuracy and Reproducibility of Quantitation of HCQ and Its Metabolites--Interday Quality Control Data (n=3).

	Blood Concentration (ng/ml)			
	Added	Measured	CV%	Accuracy%
HCQ	4.0	4.1	4.9	102.8
	250.0	243.0	7.1	97.2
	1500.0	1377.4	2.9	91.8
DHCQ	4.0	3.7	2.4	92.9
	250.0	248.6	14.2	99.5
	1500.0	1496.8	1.0	99.8
BDCQ	4.0	3.9	14.7	98.7
	250.0	261.6	6.6	104.6
	1500.0	1586.9	8.3	105.8
DCQ	4.0	4.0	3.7	100.0
	250.0	251.6	9.5	100.6
	1500.0	1494.8	3.5	99.7

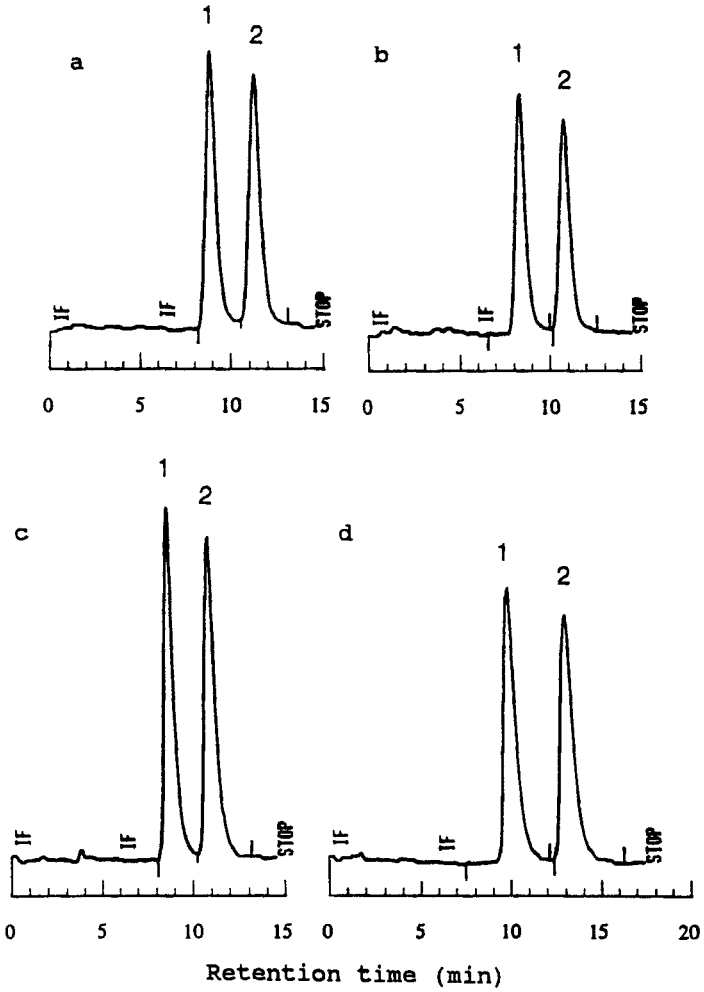

FIGURE 2. Chromatograms of chiral separation. a. HCQ, b. DHCQ, c. BDCQ and d. DCQ. Peak 1 = S(+)enantiomer, Peak 2 = R(-)enantiomer.

TABLE 4

Resolution and Retention Time Data for the Enantiomeric Separation of HCQ and Its Three Metabolites.

Compound	Resolution	t_r(S) (min)	t_r(R) (min)
HCQ	3.2	8.78	11.25
DHCQ	3.2	8.29	10.80
BDCQ	2.9	8.73	11.00
DCQ	2.5	10.04	13.30

t_r(S): S(+) enantiomer retention time
t_r(R): R(-) enantiomer retention time

and its three metabolites on a chiral column is not feasible. The first step involved an achiral separation to resolve and collect the racemates (Figure 1). This was followed by the chiral separation of each pair of drug or metabolite enantiomers (Figure 2). The fractions collected from the achiral column were validated as pure single peaks by being reinjected onto the achiral column. The ratio of each pair of enantiomers was determined by calculating the ratio of their peak areas.

The resolution and retention times of the enantiomer pairs are listed in Table 4. The detection limit for each enantiomer was less than 1 ng. Baseline separation of the compounds and sharp peaks contributed to high sensitivity of the analysis.

The elution order of the enantiomers of HCQ was determined by injecting a pure R(-)-HCQ enantiomer onto the chiral column (Figure 3). The enantiomers of the metabolites were assumed to have the same elution order as HCQ (S, then R) under the conditions of this method [1,2].

The choice of excitation and emission wavelengths depends on the absorbance and fluorescence

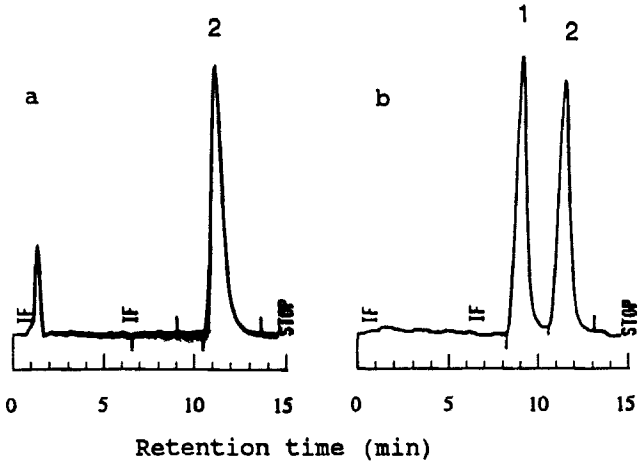

FIGURE 3. Chromatograms on chiral system. a. R(-)-HCQ enantiomer, b. HCQ racemate. Peak 1 = S(+)-HCQ, Peak 2 = R(-)-HCQ.

characteristics of the particular molecular structures, and also depends on the design of the detector. In the current work, the use of a lower excitation wavelength (230nm instead of 320-330nm, as has been commonly used in other work [1,4]) gave increased sensitivity of detection. Compared to the published methods [1,2], the current method has higher sensitivity as well as better resolution of the enantiomers. It is therefore well suited to the analysis of samples in pharmacokinetic and bioavailability studies of HCQ and its metabolites and in studies designed to determine the disposition of the enantiomers.

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