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Determination of hydroxychloroquine and its major metabolites in plasma using sequential achiral–chiral high-performance liquid chromatography

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ABSTRACT

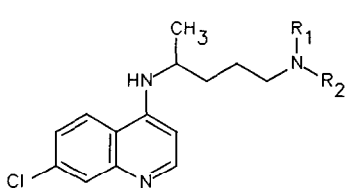
A sequential achiral–chiral high-performance liquid chromatographic system has been developed for the determination of the enantiomers of hydroxychloroquine, (+)-HCQ and (−)-HCQ, and the enantiomers of its three major metabolites, bisdesethylchloroquine, desethylhydroxychloroquine and desethylchloroquine, in plasma. The HCQ was separated from the metabolites and interfering components in the plasma and quantified on a cyano-bonded phase, and the enantiomeric composition determined using a Chiral-AGP chiral stationary phase. The assay was validated and applied to the analysis of a pilot study of the pharmacokinetics of (+)- and (−)-HCQ in rabbits.

INTRODUCTION

Drugs which have a stereogenic centre and exist as pairs of enantiomers are often administered as a racemic (50:50) mixture of the two isomers. This would be of no clinical significance except for the fact that there are often large pharmacodynamic and pharmacokinetic differences between enantiomers. As a consequence, it is possible that only one of the enantiomers is responsible for the desired pharmacological effect, while the other contributes to the adverse effects. In some cases, the pharmacological differences between the enantiomers are clear, leading to the clinical use of a single enantiomer. For example, D-penicillamine is administered as the pure enantiomer in the treatment of rheumatoid arthritis because its enantiomorph, L-penicillamine, causes unacceptable side-effects including optic neuritis [1]. In other cases, the differences in efficacy and toxicity between the stereoisomers are not clear or are unknown.

Hydroxychloroquine (HCQ, Fig. 1) is an example of a chiral compound used in the clinical treatment of rheumatoid arthritis [2] and lupus [3] where the pharmacology of the individual enantiomers is unknown. To be effective, HCQ must be taken daily for an extended period of time. The standard dose is 400 mg of the racemate per day and steady-state plasma levels of HCQ are reached in about a month [4].

HCQ undergoes extensive metabolism and after chronic administration, the plasma contains HCQ and significant levels of three metabolites,



	R_1	R_2
Hydroxychloroquine	CH_2CH_3	CH_2CH_2OH
Desethylhydroxychloroquine	H	CH_2CH_2OH
Desethylchloroquine	CH_2CH_3	H
Bisdesethylchloroquine	H	H
Chloroquine (internal std)	CH_2CH_3	CH_2CH_3

Fig. 1. Structures of hydroxychloroquine, its metabolites and chloroquine.

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desethylchloroquine (DCQ), desethylhydroxychloroquine (DHCQ) and bisdesethylhydroxychloroquine (BDCQ) (Fig. 1) [5]. Since HCQ, DCQ, DHCQ and BDCQ are all chiral, the steady-state plasma can contain up to eight distinct chemical entities. The pharmacokinetic and pharmacodynamic properties of these compounds are currently unknown.

An initial step in pharmacokinetic and pharmacodynamic studies of chiral compounds is the development of stereoselective assays to determine enantiomeric concentrations in biological matrices. High-performance liquid chromatographic (HPLC) chiral stationary phases (CSPs) have become key components of these assays and often can be used after the direct extraction of the matrix [6,7]. However, the coelution on the CSP of the stereoisomers of the parent and metabolite(s) is a problem which often makes it impossible to directly use a CSP [8,9]. This was the case for HCQ and its metabolites where each enantiomeric pair could be individually resolved on a CSP composed of α_1-acid glycoprotein (AGP-CSP) while the chromatography of the mixture yielded seven overlapping peaks rather than the expected eight.

In this study, the problem was overcome by using a sequentially coupled achiral–chiral chromatographic technique [8,9]. In this system, the initial separation of HCQ and its metabolites from each other and from the matrix interferences was accomplished on an achiral HPLC column composed of cyano-bonded silica. The eluents containing the separated compounds were collected, concentrated and reinjected onto an AGP-CSP where the stereochemical resolutions were achieved. A similar approach has been reported for the determination of the enantiomers of chloroquine (CQ) and its desethyl metabolite in serum [10].

This paper reports the development and validation of the sequential achiral–chiral system described above and its application to a preliminary pharmacokinetic study in rabbits. The initial results indicate that after the subcutaneous administration of racemic HCQ, the absorption and distribution of the HCQ enantiomers are stereospecific leading to an apparent difference in the systemic bioavailability of the two isomers.

EXPERIMENTAL

Chemicals

CQ diphosphate was purchased from Sigma (St. Louis, MO, USA); HCQ, DCQ, DHCQ and BDCQ were kindly provided by Sterling Winthrop Research Group (New York, NY, USA).

Ammonium acetate, monobasic and dibasic sodium phosphate, sodium hydroxide and phosphoric acid were all obtained from BDH (Toronto, Canada); N,N-dimethyloctylamine (N,N-DMOA) was purchased from Aldrich (Milwaukee, WI, USA), and HPLC-grade ethanol and acetonitrile were purchased from Fisher Scientific (Ottawa, Canada).

Apparatus

HPLC was carried out using an ABI Kratos Spectroflow 400 pump (Applied Biosystems, Ramsey, NJ, USA), a Spectra-Physics SP8880 autosampler (Spectra-Physics, San Jose, CA, USA) equipped with a 50-μl loop. The solutes were detected using an ABI 783A programmable absorbance detector (Applied Biosystems) with λ = 320 nm. Data collection was carried out using a Spectra-Physics DataJet integrator connected to a Zeos 386SX IBM compatible computer (Zeos International, St. Paul, MN, USA) running Spectra-Physics WINner 286 autolab software.

The columns used for the achiral separations were an Ultremex cyano-bonded 3-μm phase (75 mm × 4.6 mm I.D.) (Phenomenex, Torrance, CA, USA) and a 5-μm cyano-bonded guard cartridge (Regis, Morton Grove, IL, USA). The enantioselective chromatography was carried out using a 150 mm × 4.6 mm I.D. column packed with CSP based upon immobilised AGP (Chiral-AGP) and a Chiral-AGP guard column (Regis).

Chromatographic procedures

The chromatographic separation of the enantiomers of HCQ and its metabolites consisted of two steps. The first step was chromatography on the Ultremex cyano column to separate HCQ and its metabolites from each other and from possible interfering plasma components. In the second step, the eluates containing HCQ and each metabolite were collected, concentrated in a Speed-Vac (Savant Instruments, Farmingdale,

NY, USA), reconstituted in mobile phase and injected onto the Chiral-AGP column in order to determine the enantiomeric ratios.

Step 1: achiral chromatography. The mobile phase consisted of 0.02 *M* N,N-DMOA phosphate (prepared by adding phosphoric acid to N,N-DMOA to precipitate the salt)–0.06 *M* ammonium acetate (40:60, v/v), pH adjusted to 4.5, at a flow-rate of 0.6 ml/min at ambient temperature.

Step 2: enantioselective chromatography. The mobile phase consisted of 0.03 *M* sodium phosphate buffer, pH 7.0–ethanol–acetonitrile (79:20:1, v/v/v) containing 0.005 *M* N,N-DMOA, at a flow-rate of 0.9 ml/min and at ambient temperature.

Sample preparation

Blood samples were collected in tubes containing sodium heparin as an anticoagulant, centrifuged at 1000 *g* for 15 min, and 1-ml aliquots of the plasma fraction were removed for extraction. To the plasma were added 0.5 ml of 5 *M* sodium hydroxide and 100 μl of internal standard solution (CQ, 0.01 mg/ml in methanol) followed by 5 ml of hexane–diethyl ether (1:1, v/v). The tubes were vortex-mixed for 1 min, centrifuged at 1000 *g* for 10 min, frozen in a slurry of dry ice and acetone, and the organic layer transferred to a clean tube. The aqueous phase was thawed, extracted again, and the two organic fractions were pooled and evaporated to dryness in a Speed-Vac concentrator. The residue was reconstituted in 100 μl of mobile phase and 50 μl were injected onto the achiral column.

Validation studies

Standard curves were prepared by spiking drug-free plasma with known amounts of HCQ and its three metabolites prior to the extraction procedure described above. The concentrations used were 50, 100, 150, 200, 400 600 and 800 ng/ml. The standard curves were run in duplicate.

Inter-day and intra-day studies were performed by spiking drug-free plasma with HCQ and its metabolites at concentrations of 80 and 600 ng/ml.

Recoveries were estimated by comparing the mean peak areas of extracted spiked plasma samples to the mean peak areas of aqueous standard solutions of the same concentration.

In vivo metabolism and pharmacokinetic study

Male New Zealand White rabbits ($n = 2$) weighing approximately 2 kg were administered a single subcutaneous dose of racemic HCQ (13 mg HCQ per kg body weight) dissolved in sterile saline. Blood samples were removed by catheterisation of the central auricular artery in the ear and were collected at 0, 0.25, 0.5, 1.0, 2.0, 3.0, 6.0, 8.0 and 24.0 h.

RESULTS AND DISCUSSION

Achiral chromatography

The results from the chromatography of racemic HCQ and its metabolites are presented in Table I. The detection limits were 10 ng/ml for HCQ and its metabolites in this system. Representative chromatograms of a 1.0-ml blank plasma sample and a 1.0-ml plasma sample spiked with 200 ng each of HCQ and its metabolites are presented in Fig. 2A and B, respectively. The chromatogram resulting from the analysis of a plasma sample

TABLE I

CHROMATOGRAPHIC PARAMETERS OF HYDROXYCHLOROQUINE AND ITS METABOLITES ON THE ACHIRAL AND CHIRAL STATIONARY PHASES

Compound	Ultremex cyano column[a]	Chiral-AGP column[b]		
	k'[c]	k'_1[d]	α_{RS}[e]	R_{RS}[f]
BDCQ	1.61	12.11	1.25	1.29
DHCQ	2.56	10.19	1.32	1.39
DCQ	3.93	11.44	1.39	1.97
HCQ	5.33	8.67	1.39	2.08
CQ (I.S.)	9.34	N.A.[g]	N.A.[g]	N.A.[g]

[a] Chromatographic conditions: mobile phase, 0.02 *M* N,N-DMOA phosphate–0.06 *M* ammonium acetate (40:60, v/v) pH adjusted to 4.5; flow-rate, 0.6 ml/min; temperature ambient.

[b] Chromatographic conditions: mobile phase, 0.03 *M* phosphate buffer (pH 7.0)–ethanol–acetonitrile (79:20:1, v/v/v); flow-rate, 0.9 ml/min; temperature, ambient.

[c] Capacity factor.

[d] Capacity factor of first-eluted enantiomer.

[e] Enantioselectivity factor.

[f] Stereochemical resolution.

[g] N.A. = not applicable.

TABLE II

INTRA-DAY VALIDATION OF THE ACHIRAL ASSAY FOR HYDROXYCHLOROQUINE AND ITS METABOLITES

See Table I for chromatographic conditions.

Level (ng/ml)	Mean observed concentration (ng/ml)	Accuracy (%)	C.V. (%)
Bisdesethylchloroquine			
80	77.1	96.4	12.2
600	585.8	97.6	6.8
Desethylhydroxychloroquine			
80	80.2	97.5	11.7
600	593.9	98.9	5.3
Desethylchloroquine			
80	82.4	97.0	9.9
600	580.9	96.8	5.4
Hydroxychloroquine			
80	76.7	95.9	10.4
600	604.0	99.3	2.9

from one of the rabbits in the initial pharmacokinetic study is presented in Fig. 2C.

Recovery from plasma was greater than 90% for HCQ and DCQ and 85–90% for DHCQ, BDCQ and CQ. Small daily fluctuations in recoveries were reflected in all of the compounds. Calibration curves for all of the compounds were linear over the range 50–800 ng/ml and all curves had correlation coefficients greater than 0.99. The intra-day and inter-day validation studies were done with plasma samples spiked with 80 and 600 ng/ml racemic HCQ and racemic mixtures of its metabolites. The results are presented in Tables II and III. In all cases, the coefficients of variation (C.V.) were less than 15%.

TABLE III

INTER-DAY VALIDATION OF THE ACHIRAL ASSAY FOR HYDROXYCHLOROQUINE AND ITS METABOLITES

See Table I for chromatographic conditions.

Level (ng/ml)	Mean observed concentration (ng/ml)	Accuracy (%)	C.V. (%)
Bisdesethylchloroquine			
80	78.6	98.2	12.1
600	622.6	96.2	4.9
Desethylhydroxychloroquine			
80	81.8	97.7	4.2
600	622.6	96.2	3.8
Desethylchloroquine			
80	73.6	91.9	11.8
600	595.5	99.3	1.8
Hydroxychloroquine			
80	78.4	98.0	12.0
600	610.1	98.3	4.9

Enantioselective chromatography

The results from the chromatography of racemic mixtures of HCQ and its metabolites on the Chiral-AGP CSP are presented in Table I and Fig. 3A–D, respectively. The elution order of the enantiomers of HCQ was determined by the independent chromatography of the resolved enantiomers whose optical rotation had been determined using a Jasco DIP-140 digital polarimeter (Jasco, Easton, MD, USA). Under the chromatographic conditions used in this study (−)-HCQ eluted first. Sufficient quantities of the resolved metabolites were not available for the determination of their optical rotation and, therefore, the enantiomeric elution order of the metabolites is unknown. However, they can be assumed to have the same elution order as HCQ as they differ only in the alkyl groups on the amino moiety of the side-chain. The limit of detection for HCQ in this system was less than 5 ng per enantiomer. The resulting chromatogram from the enantioselective analysis of HCQ taken from a rabbit plasma sample 1 h following subcutaneous administration of 13 mg/kg racemic HCQ is shown in Fig. 4.

Plasma samples

The results from the single subcutaneous dose studies using this sequential achiral–chiral chromatographic technique are shown in Figs. 5 and 6. The mean time to peak plasma concentration of total HCQ was 0.5 h (Fig. 5) and the best fit for the total HCQ data was to a two-compartment model. These results are in agreement with those of a previously published paper on the pharmacokinetics of other antimalarial agents [11]. The time course of the individual HCQ enantiomers

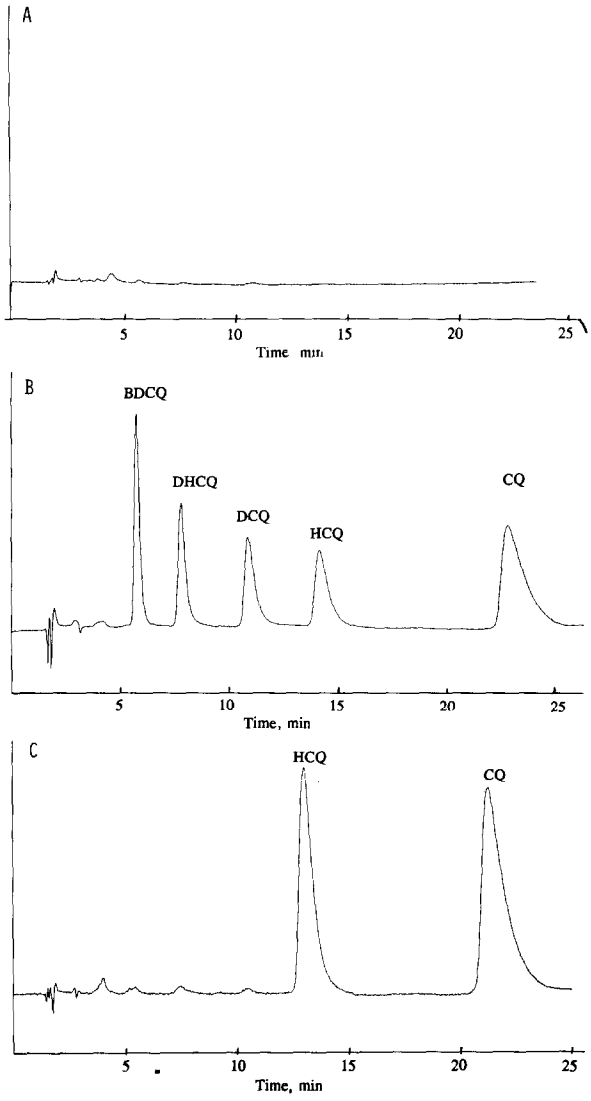

Fig. 2. Representative chromatograms of racemic HCQ and its metabolites on the Ultremex cyano-bonded HPLC column. (A) Blank plasma sample; (B) plasma sample spiked with 200 ng/ml HCQ and its metabolites; (C) plasma sample taken from a rabbit after the subcutaneous administration of 13 mg/kg racemic HCQ. See text for chromatographic conditions.

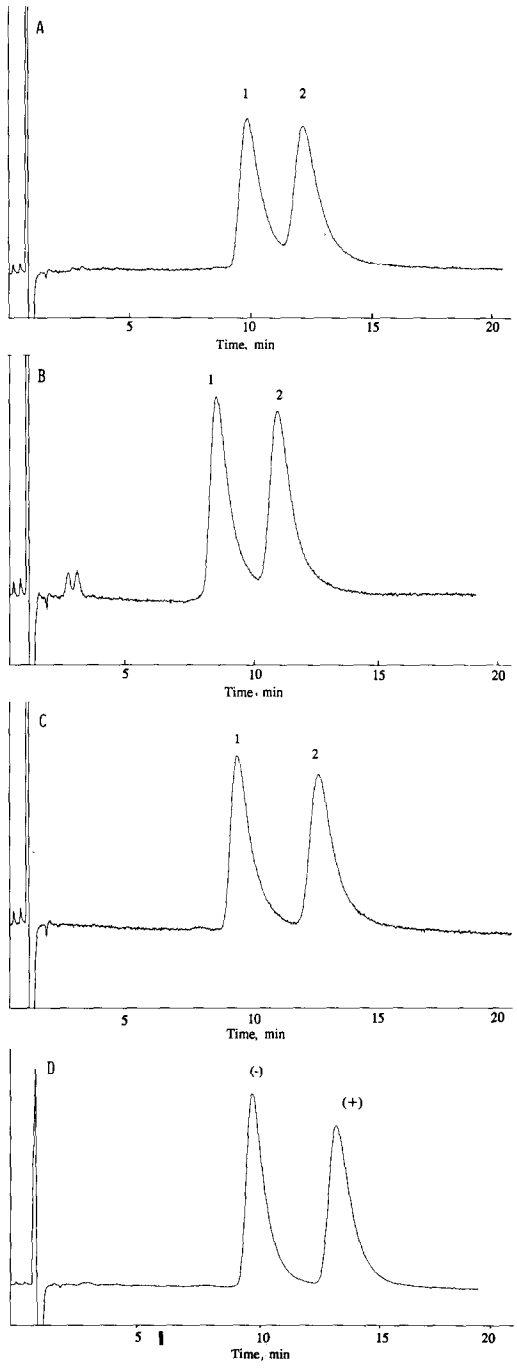

Fig. 3. Representative chromatograms of racemic HCQ and its metabolites on the Chiral AGP CSP. (A) BDCQ; (B) DHCQ; (C) DCQ; (D) HCQ. See text for chromatographic conditions. Peaks: 1 = first-eluted enantiomer; 2 = second-eluted enantiomer. Optical rotations of BDCQ, DHCQ and DCQ were not assigned due to unavailability of individual enantiomers.

in plasma (Fig. 6) demonstrated that the concentration of (+)-HCQ quickly exceeded that of (−)-HCQ. Pharmacokinetic modelling of these data indicated a difference in the rate of absorption yielding a higher C_{max} and earlier T_{max} for the (+)-enantiomer. However, the clearance (Cl) and half-lives ($t_{1/2}$) were similar for the two enantiomers: (+)-HCQ, Cl = 15.55 ml/min, $t_{1/2}$ = 1.127 h; (−)-HCQ, Cl = 23.2 ml/min, $t_{1/2}$ = 1.038 h. These results suggest that for HCQ the initial absorption and distribution phases are

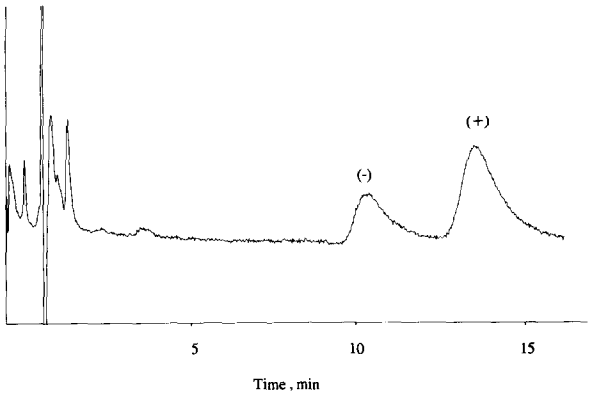

Fig. 4. Chromatogram resulting from the enantioselective analysis of HCQ from a plasma sample taken from a rabbit following subcutaneous administration of 13 mg/kg racemic HCQ. See text for chromatographic conditions.

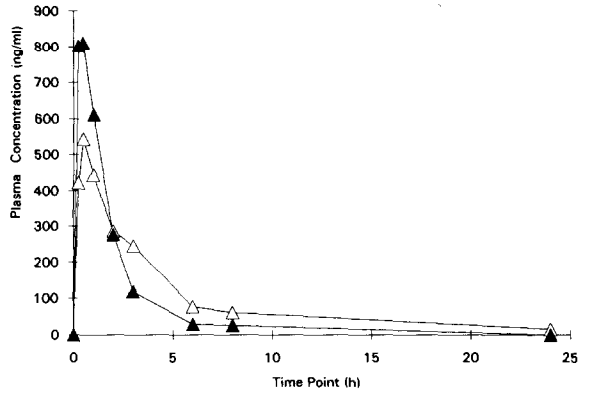

Fig. 5. Plasma concentrations *versus* time curves of total HCQ in rabbits following a single subcutaneous dose of 13 mg/kg. (△) Rabbit No. 7566; (▲) rabbit No. 7567.

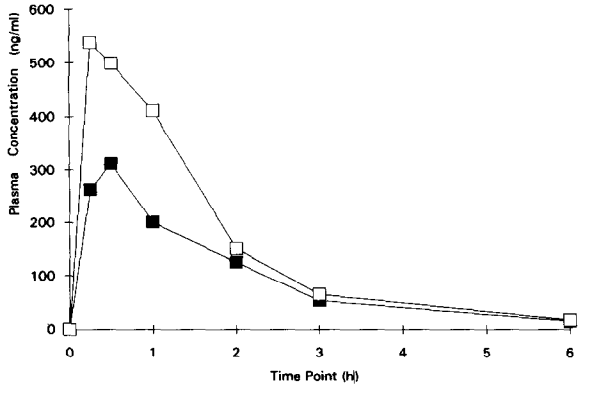

Fig. 6. Plasma concentrations *versus* time curves of the enantiomers of HCQ in rabbits following a single subcutaneous dose of 13 mg/kg. (□) (+)-HCQ; (■) (−)-HCQ.

stereoselective. A complete pharmacokinetic study as well as an investigation on the tissue distribution of the HCQ enantiomers is currently underway using the assay described in this manuscript.

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