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Analytical and semi-preparative high-performance liquid chromatographic separation and assay of hydroxychloroquine enantiomers☆

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ABSTRACT

(±)-Hydroxychloroquine (HCQ) is an antimalarial and anti-arthritic drug which is administered as the racemate. An accurate, precise and sensitive high-performance liquid chromatographic assay was developed for the determination of HCQ enantiomers in samples from human plasma, serum, whole blood, and urine. After addition of (±)-chloroquine (internal standard), samples of blood component (0.5 ml) or urine (0.1 ml) were alkalinized and extracted with 5 ml of diethyl ether. After solvent evaporation the residues were derivatized with (+)-di-O-acetyl-L-tartaric anhydride at 45°C for 30 min. The resulting diastereomers were then resolved using a C_8 analytical column with a mobile phase consisting of 0.05 *M* KH_2PO_4 (pH 3)–methanol–ethanol–triethylamine (78:22:1:0.08). The ultraviolet detection wavelength was set at 343 nm. The derivatized HCQ enantiomers eluted in less than 40 min, free of interfering peaks. Excellent linear relationships ($r^2 > 0.997$) were obtained between the area ratios and the corresponding plasma concentrations over a range of 12.5–500 ng/ml. The diastereomers could be hydrolysed using microwave energy and neutral pH, which enabled us to resolve the enantiomers on a semi-preparative (C_{18} column) scale. The method was suitable for the analysis and semi-preparative separation of HCQ enantiomers.

INTRODUCTION

(±)-Hydroxychloroquine (HCQ) is an antimalarial drug which also possesses beneficial activity as a disease-modifying agent in arthritic conditions such as rheumatoid arthritis and systemic lupus erythematosus [1]. Like other antimalarial drugs, such as (±)-chloroquine (CQ), (±)-primaquine, and (±)-mefloquine, HCQ is chiral and marketed as the racemate. The relative anti-arthritic properties of the individual enantiomers of HCQ are not known, although it has been determined that the (+)-enantiomer of CQ, which is structurally very similar to HCQ, displays a

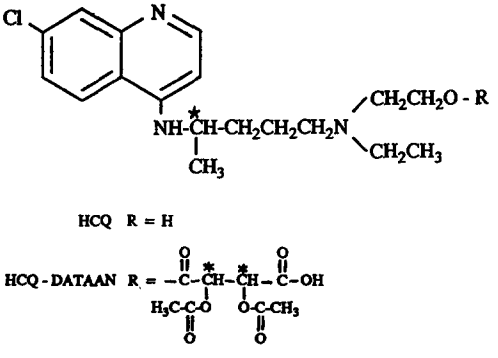

Fig. 1. Structure of underivatized (HCQ) and derivatized (HCQ-DATAAN) hydroxychloroquine.

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greater antimalarial activity than (−)-CQ in the mouse [2]. Despite the chiral nature of HCQ, pharmacokinetic data in humans have been generated using non-stereoselective assay methodologies [3–11], with the exception of a single report [12].

In the development of an assay designed to separate enantiomers, the most commonly utilized techniques involve either a chiral column or precolumn derivatization of the enantiomers to form diastereomers. Recently, two methods were published which described the separation of HCQ enantiomers using α_1-acid glycoprotein high-performance liquid chromatography (HPLC) columns [12,13]. With respect to diastereomer formation, there are three sites in the alkyl side-chain of HCQ where derivatization would be possible (Fig. 1): the aromatic nitrogen, the tertiary aliphatic nitrogen, and the hydroxyl group.

The use of (+)-di-O-acetyl-L-tartaric anhydride (DATAAN) as a derivatizing reagent was first reported by Lindner *et al.* [14], who successfully used it to separate the enantiomers of several β-adrenoceptor antagonists, including propranolol [15]. Rather than derivatizing the amino function, DATAAN preferentially reacts with the hydroxyl group of β-blockers to form esters. It was suggested that an intramolecular interaction between the free carboxylate moiety contributed by DATAAN, and the secondary amine of the β-blocker, permitted HPLC separation of the diastereomers. Because β-blockers, and HCQ possess structurally similar alkylamino and hydroxyl groups, it was reasonable to believe that DATAAN would also react at the latter site. Furthermore, although the hydroxyl group was rather far removed from the chiral center of HCQ, it was anticipated that DATAAN might interact with the side-chain nitrogens, thus facilitating enantiomer separation.

In this report, we describe a stereospecific HPLC method for the resolution and semi-preparative isolation of HCQ enantiomers, using DATAAN as a pre-column chiral derivatizing reagent.

EXPERIMENTAL

Chemicals and reagents

(±)-Hydroxychloroquine sulfate and (±)-desethylchloroquine were gifts from Sterling-Winthrop (Rensselaer, NY, USA). (±)-Chloroquine diphosphate was purchased from Sigma (St. Louis, MO, USA). DATAAN (claimed purity >97%) was purchased from Fluka Chemika (Buchs, Switzerland). Methanol and water (both HPLC grade) were obtained from BDH (Edmonton, Canada), as were reagent grade diethyl ether, dichloromethane (DCM), trichloroacetic acid, triethylamine (TEA), NaOH, glacial acetic acid, and KH_2PO_4. Reagent ethyl alcohol (HPLC grade) and H_2SO_4 were purchased from Fisher Scientific (Edmonton, Canada). *S*-(+)-1-(1-Naphthyl)ethyl isocyanate, *S*-(+)-1-(1-phenyl)ethyl isocyanate, and (−)-menthyl chloroformate were purchased from Sigma.

Apparatus and chromatographic conditions

A Philips Model PU8740 UV-VIS scanning spectrophotometer (Philips, Cambridge, UK) was used to determine the optimal UV absorptions of derivatized and underivatized HCQ. The HPLC system was comprised of a Waters 590 pump and 481 variable-wavelength UV detector (Waters Scientific, Mississauga, Canada), and a Shimadzu SIL 9A autoinjector and CR501 integrator (Kyoto, Japan). Unless otherwise indicated, a Savant Speed Vac concentrator (Emerston Labs., Scarborough, Canada) was used to evaporate solvents.

The analytical separation of the HCQ diastereomers was accomplished using either a Partisil 5 C_8 (250 mm × 4.6 mm I.D.; 5 μm particle size) analytical column with a 30 mm × 4.6 mm I.D. guard column (Phenomenex, Torrance, CA, USA), or two 100 mm × 8 mm I.D. Nova-Pak C_8 analytical column cartridges (4 μm particle size), placed serially in an 100 mm × 8 mm radial compression module (RCM) with an extension kit, and a C_8 Guard-Pak precolumn insert (Waters Millipore, Milford, MA, USA). A 100 mm × 25 mm, 6 μm particle size Prep Nova-Pak HR C_{18} RCM column cartridge (Waters Millipore)

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF TWO C_8 COLUMNS FOR THE SEPARATION OF HCQ ENANTIOMERS FOLLOWING DERIVATIZATION WITH DATAAN

Column	Dimensions	Retention time (min)		Capacity factor		Resolution factor	α
		R	*S*	*R*	*S*		
Partisil, 5 μm	250 mm × 4.6 mm I.D.	39	41	8.5	9.0	1.0	1.1
Nova Pak, 4 μm	200 mm × 8 mm I.D.	35	38	8.6	9.3	1.9	1.1

was used for the semi-preparative HPLC of the diastereomers.

Unless otherwise stated, the mobile phase consisted of 0.05 *M* KH_2PO_4 (pH 3.5)–methanol–ethanol–TEA (78:22:1:0.08, v/v), pumped at a flow-rate of 0.75–0.8 ml/min through the Partisil column, or 1.5 ml/min through the Nova-Pak column. All mobile phases were degassed by filtering through a 0.45-μm membrane filter (Scientific Products & Equipment, Rexdale, Canada), and chromatography was carried out at room temperature. The UV detection wavelength was set at 343 nm. For the determination of extraction efficiency, a Model 980 programmable fluorescence detector (Applied Biosystems, Ramsey, NJ, USA) was used, with excitation and emission wavelengths set at 320 and 370 nm, respectively. All glassware used for sample preparation was either brand new or silanized with a 5% DCM solution of SurfaSil (Pierce, Rockford, IL, USA).

The optical rotation of the resolved enantiomers was established using a Perkin-Elmer 241 polarimeter (Überlingen, Germany) set at 589 nm, using a 10-cm cell.

Standard solutions

A stock solution of (±)-HCQ (100 μg/ml) was prepared by dissolving 12.86 mg of the sulfate salt in 100 ml of water. Dilutions of the stock solution of 1:10, 1:100 and 1:1000 were made using HPLC water on the day of sample preparation, and these were used for the preparation of standard curves. A stock solution of the internal standard, (±)-CQ (100 μg/ml), was made by dissolving 16.3 mg of the diphosphate salt in 100 ml of HPLC water. This solution was further diluted in water by a factor of 10 to provide the final CQ working stock solution. Solutions were stored at 4°C.

Sample preparation

Plasma and serum were separated and collected from whole blood by centrifugation at 1800 *g* for 10 min. To 0.5 ml of blood component (whole blood, plasma, or serum) or 0.1 ml of human urine, 125 μl of CQ working stock solution and 100 μl of 2 *M* NaOH were added. After vortex-mixing for 5 s, diethyl ether (5 ml) was added to the tubes, which were then vortex-mixed for 1 min and centrifuged at 1800 *g* for 5 min. The organic phase was transferred with pasteur pipettes to clean test tubes and evaporated to dryness.

The enantiomers of HCQ were derivatized by adding to each tube, in sequence, 200 μl of 0.01 *M* trichloroacetic acid in DCM and 100 μl of 0.25 *M* DATAAN in acetic acid–DCM (20:80, v/v). The tubes were vortex-mixed for 10 s after each addition, then capped and placed in an oven at 45°C. After 30 min, 1 ml of methanol was added, the tubes vortex-mixed for 5 s, and the contents evaporated to dryness. The residues were reconstituted with 200 μl of methanol–water (25:75, v/v) and injected into the HPLC columns.

Standard calibration curves

The enantiomers were quantified using sets of standard curves prepared by adding (±)-HCQ to 0.5 ml of drug-free human plasma or 0.1 ml of urine. Concentrations of each enantiomer in the

plasma samples were 12.5, 25, 50, 75, 125, 250, and 500 ng/ml. In urine, final concentrations of the enantiomers were 2, 4, and 8 μg/ml. The samples were analysed according to the procedure described above, using the Partisil column.

Extraction efficiency

The extraction efficiency was determined by adding known amounts of (±)-HCQ to drug-free plasma and extraction into diethyl ether as described above. Following evaporation of solvent the samples were reconstituted with 0.2 ml of acetonitrile and analysed using a non-stereospecific method. This procedure used a mobile phase of methanol–acetonitrile (20:80, v/v), pumped at 1.8 ml/min, and a 250 mm × 4.6 mm I.D. analytical column packed with 5-μm silica gel (Whatman Partisil 5; Whatman, Clifton, NJ, USA). The peak areas were compared to those obtained from equivalent volumes of standard solutions of HCQ which were evaporated to dryness, reconstituted with 0.2 ml of acetonitrile, and directly injected into the HPLC system. This method yielded linear calibration curves ($r^2 > 0.999$) from 12.5 to 1000 ng/ml racemate, using peak areas for quantitation.

The concentrations used to assess extraction efficiency were 12.5 and 125 ng/ml (±)-HCQ, with each determination being performed in quadruplicate.

Derivatization yield

Drug-free plasma samples ($n = 6$) spiked with 1 μg of (±)-HCQ were extracted with 5 ml of diethyl ether as described above. A 4-ml volume of the organic layer from each tube was then transferred to clean tubes and evaporated to dryness. Half of the samples were derivatized as described above, while the remaining samples underwent the same treatment except that the 100 μl of DATAAN solution were replaced with an equal volume of acetic acid in DCM. The ratio of molar excess for DATAAN to (±)-HCQ was approximately 8000:1. Prior to injection the dried samples were reconstituted with 200 μl of methanol in water (20:80, v/v) and analysed using the stereospecific method. The derivatization yield was estimated by noting the difference between the samples in the amount of underivatized HCQ present.

Other derivatization reactions

S-(+)-1-(1-Naphthyl)ethyl isocyanate and *S*-(+)-1-(1-phenyl)ethyl isocyanate were used individually to react with (±)-HCQ. For derivatization, the dried residues of the extracted samples were redissolved in 100 μl of chloroform, then derivatized with 100 μl of the isocyanate solution (1%, v/v, in chloroform). (−)-Menthyl chloroformate in acetonitrile (1:25, v/v) was also used as a derivatizing reagent. After adding 100 μl of acetonitrile to the tubes containing the sample residues, derivatization was accomplished by adding 100 μl of the (−)-menthyl chloroformate solution.

In each instance, after the addition of the derivatizating reagent, the samples were evaporated to dryness, reconstituted in mobile phase, and chromatographed under the conditions used for the determination of the extraction efficiency.

Semi-preparative isolation of individual enantiomers

(±)-Hydroxychloroquine sulfate (53 mg) was dissolved in 3 ml of HPLC-grade water. After basification with 2 *M* NaOH, the precipitated HCQ base was collected by filtering the solution through a 0.45-μm membrane filter. The filter was then placed in a beaker containing acetone, to dissolve the HCQ base, then rinsed with acetone and discarded. The acetone solution was transferred to a clean test tube, and the solvent evaporated; the residue was dried in a vacuum oven at 50°C for 16 h. To the residue were added 3 ml of 0.01 *M* trichloroacetic acid in DCM, and 3 ml of 0.25 *M* DATAAN in acetic acid–DCM (20:80, v/v). After incubation for 4 h at 45°C, 1 ml of methanol was added to the tube to react with excess DATAAN, and the solvent was evaporated. The residue was reconstituted in 2.4 ml of mobile phase as described under *Apparatus and chromatographic conditions*, except that the mobile phase contained methanol and KH_2PO_4 solution in a ratio of 18:82 (v/v).

Volumes of 60 μl of the derivatized mixture, containing approximately 200–300 μg, were injected into the HPLC apparatus and chromatographed using the Prep Nova Pak HR column with a flow-rate of 7.5 ml/min. The eluent fraction corresponding to the first-eluted HCQ diastereomer was collected until it had declined to approximately 60% of the peak height; the second-eluted peak was collected from approximately 80% of the incline in the peak height until its completion. The solvent from each eluted fraction was evaporated *in vacuo*, and the pH was adjusted to neutral by the addition of 2 *M* NaOH. To hydrolyze the HCQ-DATAAN derivatives each solution was placed in a commercial microwave oven, irradiated at a high setting for 5 min, and immediately placed in an ice bath. The solutions were then acidified by the addition of 1 ml of concentrated HCl, and approximately 75% of the solvent was removed *in vacuo* to permit precipitation of most of the phosphate and tartaric acid salts. The precipitated salts were removed by filtration through cellulose filter paper (Whatman, Maidstone, UK). The remaining solvent was transferred to a 250-ml separatory funnel, and solid sodium carbonate (5–10 g) was added until the pH of the solution was approximately 10.5. The enantiomers were then extracted twice with 100 ml of ethyl acetate, followed by another extraction with acetone (100 ml). The combined ethyl acetate–acetone extracts were filtered to remove any remaining salt and then transferred to a clean round-bottom flask. After evaporation *in vacuo* each remaining residue was dissolved in 10 ml of HPLC-grade methanol, and the optical rotation determined.

In vivo studies

After informed, signed consent, a 34-year-old healthy male volunteer (weight 80 kg) took a single oral dose of 400 mg of HCQ sulfate. Serial blood samples were collected for 24 h from a fore-arm vein in which a butterfly needle was inserted. Total urine output was also collected for 8 h; additional urine samples were collected at six days, ten days, and seven weeks after the dose. Plasma was separated from the blood by centrifugation immediately after collection in order to minimize hemolysis of the blood cells; samples were frozen at −20°C and stored until they were analysed.

Steady-state trough serum samples from five patients with rheumatoid arthritis receiving chronic therapy with HCQ were also collected and analysed. Single samples of plasma, whole blood, and a 24-h cumulative urine sample were obtained from a patient with systemic lupus erythematosus. Each of the patients received 400 mg of HCQ daily. The whole blood sample (0.5 ml) was lysed by adding 50 μl of 0.6 *M* H_2SO_4, vortex-mixed, and left standing for 5 min. Alkali (150 μl of 2 *M* NaOH) was then added, and extraction performed as described above.

Data are expressed as mean ± S.D.

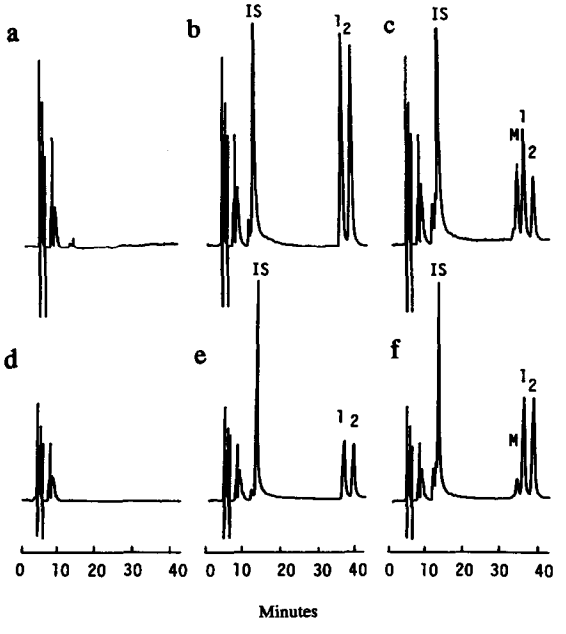

Fig. 2. Representative chromatograms of DATAAN-derivatized serum and urine obtained using the Nova Pak C_8 column. (a) Blank serum; (b) spiked serum sample (0.5 ml) containing 500 ng/ml HCQ enantiomer; (c) steady-state serum sample (0.5 ml) from a rheumatoid arthritis patient; (d) blank urine; (e) spiked urine sample (25 μl) containing 5 μg/ml HCQ enantiomer; (f) steady-state urine sample from a patient with systemic lupus erythematosus. Peaks: 1 = *R*-(−)-HCQ diastereomer derivative; 2 = *S*-(+)-HCQ diastereomer derivative; IS = (±)-chloroquine; M = metabolite.

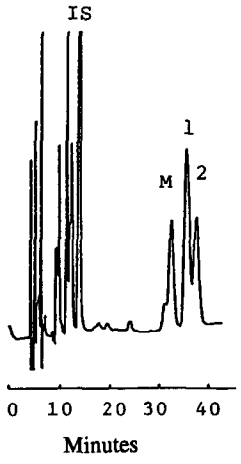

Fig. 3. Chromatogram of HCQ-DATAAN diastereomers in serum of a patient with rheumatoid arthritis obtained using a Partisil C_8 column. See Fig. 2 for key.

RESULTS

The retention time of the (±)-HCQ chromatographic peak was increased following reaction with each of the enantiopure reagents. This suggested that HCQ was indeed being derivatized. However, efforts to separate the isocyanate-HCQ and (−)-menthyl chloroformate-HCQ diastereomers were unsuccessful, despite the use of various combinations of reversed-phase solvents with C_{18} columns or normal-phase solvents with silica-packed columns. Only the DATAAN diastereomers of HCQ were successfully separated. The retention times of CQ and desethyl-CQ were not affected by DATAAN, indicating that the alcohol functional group of HCQ is the site of derivatization.

Representative chromatograms are depicted in Figs. 2 and 3. Interfering peaks were not present in the chromatograms of blank plasma or urine (Fig. 2) at the positions where the HCQ diastereomers eluted. Both analytical columns were capable of separating the derivatized HCQ enantiomers (Table I), although each column provided a specific advantage. The Nova Pak column gave baseline separation of the diastereomers (Fig. 2), but the Partisil column provided more symmetrical peaks with reduced tailing, particularly for CQ (Fig. 3). However, regardless of the column used, linear standard calibration curves could be generated for the quantitation of HCQ enantiomers. In addition, C_8 packing material provided a slightly higher resolution factor compared to C_{18} columns.

In the semi-preparative isolation of the HCQ enantiomers, we initially attempted to hydrolyse the chromatographically separated HCQ-DATAAN diastereomers using NaOH and mild heat (45°C). Although the derivatives were hydrolysed, several unidentified peaks also appeared in the HPLC traces; these were presumed to be degradative products formed during the procedure. To overcome this problem, we utilized microwave energy and neutral pH. Under these conditions degradative products were not visible using HPLC, and essentially almost complete hydrolysis of the derivatives was achieved.

The Prep Nova Pak HR column also was capable of separating the diastereomers (Fig. 4). The maximum load of (±)-HCQ as the DATAAN derivative which was separable by the column was approximately 400 μg. Analytical HPLC of the eluent fractions after microwave processing indicated that the derivatives had been almost completely hydrolysed. From approximately thirty injections of derivatized HCQ into the HPLC system, 2.3 and 3.4 mg of the first- and second-eluted enantiomer were isolated, respectively, after hydrolysis and extraction of the constituents present in the eluent fractions. The puri-

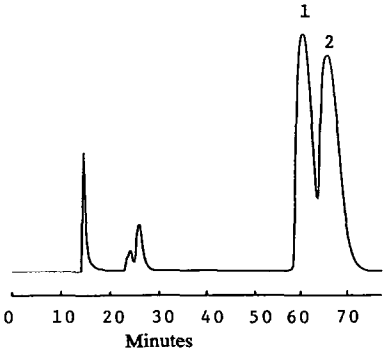

Fig. 4. Chromatogram of HCQ-DATAAN diastereomers injected into a Prep Nova Pak column. See Fig. 2 for key.

TABLE II

PRECISION AND ACCURACY OF THE METHOD IN HUMAN PLASMA AND URINE

Each value represents a total of nine determinations.

Added concentration of HCQ enantiomer (ng/ml)	Mean measured concentration (ng/ml)		Mean absolute error (%)		Coefficient of variation (%)	
	R-(−)	*S*-(+)	*R*-(−)	*S*-(+)	*R*-(−)	*S*-(+)
Plasma						
12.5	11.9	11.9	8.4	8.4	8.4	11.9
25.0	25.5	24.7	2.0	1.2	10.0	9.2
50.0	50.4	49.2	1.2	1.6	5.1	11.1
75.0	74.8	73.1	0.27	2.5	8.6	7.4
125	123	120	1.6	4.2	2.7	5.8
250	258	257	3.2	2.8	3.4	6.8
500	498	497	0.40	0.60	0.54	1.0
Urine						
2000	2030	2040	1.5	2.0	5.7	4.7
4000	4030	4080	0.75	2.0	3.5	2.9
8000	8010	8130	0.13	1.6	0.45	2.3

ty of the enantiomers, assessed after diastereomer formation and analytical HPLC of the HCQ enantiomers in the final residues, were >99% and 97% for the first- and second-eluted peak, respectively. The optical rotations of the methanol-reconstituted residues derived from the first- and second-eluted fractions were −0.030 and +0.049, corresponding to the *R*-(−) and *S*-(+)-enantiomers [12], respectively.

There were two UV absorbance maxima for HCQ in the mobile phase, at 330 and 343 nm, for both underivatized and derivatized HCQ. Either wavelength could be used, although the absorbance maximum at 343 nm was slightly greater than that at 330 nm.

The efficiency of extraction for HCQ using diethyl ether was 83.2 ± 1.81 and 86.9 ± 0.419% at 12.5 and 125 ng/ml, respectively. The derivatization yield after 30 min incubation at 45°C was 97.8 ± 0.48%; extending the incubation time did not appreciably improve the yield. The time required to prepare twenty samples for injection into the HPLC column was less than 2 h.

The assay precision and accuracy are depicted in Table II. The method was validated to concentrations as low as 12.5 ng/ml of each enantiomer, based on 0.5 ml of plasma. Calibration curves, describing the relationship between peak-area ratio and concentration, for the *R*- and *S*-HCQ enantiomers were $y = 0.00657x + 0.00206$ and $y = 0.00679x - 0.00489$, respectively, where x represents HCQ enantiomer concentration and y represents the peak-area ratio of HCQ enantiomer to (±)-CQ. Standard calibration curves were linear in the range of 12.5–500 ng/ml in plasma, the regression coefficients (r^2) being over 0.997 for both enantiomers. In urine the calibration curves were also linear in the range 500–2000 ng/ml, with mean r^2 being over 0.999 for each of the HCQ enantiomers; the lines of regression in urine were described by $y = 0.356x + 0.0204$ and $y = 0.369x + 0.00957$ for *R*- and *S*-HCQ, respectively. The standard curves generated using HCQ in whole blood or serum were identical to those using plasma.

The plasma concentration–time profiles of the HCQ enantiomers in the healthy subject are depicted in Fig. 5. There was some difference between enantiomers in the absorption phase, although the plasma concentrations were low after

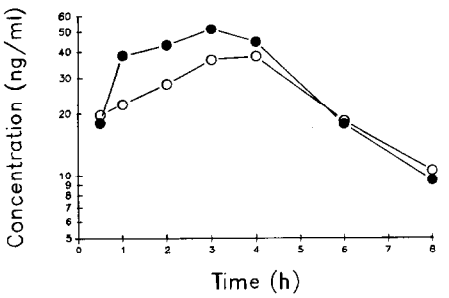

Fig. 5. Plasma concentration *versus* time curves from a healthy volunteer given a single 400-mg dose of HCQ sulfate orally. (○) *R*-(−)-HCQ; (●), *S*-(+)-HCQ.

a single dose and could only be followed for 8 h. The cumulative excretion in urine over 8 h was 0.51 and 0.73% of the given dose of the *R*- and *S*-HCQ, respectively, with an *R*/*S* concentration ratio of 0.69. Six and ten days after the dose the *R*-HCQ/*S*-HCQ concentration ratios in urine were 0.82 and 0.80, respectively; HCQ enantiomers were still detectable in urine seven weeks after the administration of the single dose (*R*-HCQ/*S*-HCQ = 1.02).

The serum samples from the five patients with rheumatoid arthritis had mean *R*- and *S*-HCQ concentrations of 290 ± 59.0 and 160 ± 7.54 ng/ml, respectively; the mean *R*/*S* ratio was 1.8 ± 0.31. The plasma from the patient with lupus erythematosus had nearly identical concentrations of the enantiomers (*R*-HCQ, 96.3 ng/ml; *S*-HCQ, 94.9 ng/ml), although the enantiomer concentrations in whole blood were stereoselective and much higher (*R*-HCQ, 877 ng/ml; *S*-HCQ, 445 ng/ml). In the 24-h cumulative urine sample from the patient with lupus erythematosus, 30.0 and 32.9 mg of the *R*- and *S*-HCQ enantiomers were recovered, corresponding to 19.3 and 21.1% of the daily dose of the respective enantiomers.

In all of the samples from patients there were two closely spaced peaks in the chromatograms about 3 min prior to the first HCQ-DATAAN diastereomer peak (Figs. 2 and 3). These peaks were of negligible size in the chromatograms of the samples from the healthy subject given a single dose of HCQ sulfate.

The chromatographic peak of the desethyl-CQ metabolite appeared less than 1 min prior to the internal standard. Although it was not fully resolved by the Nova Pak column, in biological samples its maximal peak height was negligible (< 5%) compared to that of the internal standard peak. The desethyl-CQ peak was fully resolved from CQ using the Partisil column.

DISCUSSION

The enantiomers of HCQ were successfully derivatized within 30 min and separated; the method was accurate, precise and sensitive. In the first report describing the assay of HCQ enantiomers [12], a prior non-stereoselective HPLC separation of (±)-HCQ was required. The eluent fractions containing HCQ were collected, evaporated to dryness, and the residues were reconstituted and injected into a second HPLC system in which the enantiomers were separated using an α_1-acid glycoprotein column. The time required for complete elution of the HCQ enantiomers through the chiral column was over 60 min [12]. Furthermore in the paper by McLachlan *et al.* [12] the resolution factor was determined to be 2.4 for the HCQ enantiomers, even though they were not baseline-resolved in the presented chromatograms. Using the published chromatogram [12], and formula for calculation of resolution factor, we have calculated the resolution factor to be 1.0. The method presented in the present paper provides an enhanced separation of the enantiomers, particularly if the Nova Pak column is used.

After submission of this manuscript, a stereoselective assay for HCQ enantiomers in rabbit plasma (1 ml) was brought to our attention [13]. The procedure is very similar to that utilized by McLachlan *et al.* [12], in which a non-stereoselective separation is used to collect fractions containing HCQ and its metabolites, followed by chiral separation using an α_1-acid glycoprotein column. The HCQ enantiomers appeared to be well resolved. The non-stereoselective portion of the assay was validated to 80 ng/ml, and a sensitivity of 5 ng was claimed in the second portion of the assay, which used the α_1-acid glycoprotein column. However, the authors did not report validation data for the complete assay procedure

comprising of the non-stereoselective separation, fraction collection, solvent evaporation, and stereoselective chromatography. Our method has some advantages over the assays for HCQ enantiomers, which use the α_1-acid glycoprotein chiral columns [12,13], because it is less complex and requires a shorter sample preparation time.

Because stereochemically pure HCQ enantiomers were not available to us, their order of elution was determined after the semi-preparative isolation and subsequent polarimetry analysis as described above. We observed, as did McLachlan *et al.* [12], who had authentic *S*-(+)-HCQ, that the *in vivo* steady-state whole blood concentration ratio of *R*-HCQ/*S*-HCQ was about 2. The degree of stereoselectivity in HCQ concentration was less in plasma than in serum and blood. Blood cells are known to concentrate HCQ [16], which explains the 4.7- and 9.1-fold greater concentrations of the *S*- and *R*-enantiomers in whole blood than in the plasma of the patient with lupus erythematosus, respectively. The *R*-HCQ/*S*-HCQ ratio of 24-h urinary excretion in the patient with lupus erythematosus seemed to more closely reflect that of the steady-state plasma concentration than whole blood. The long terminal half-life of HCQ is reflected in the urinary recovery in the healthy subject, in which the HCQ enantiomers could be followed for weeks after a single dose.

HCQ is biotransformed to three chiral metabolites, desethyl-HCQ, desethyl-CQ, and bis-desethyl-CQ [17]. The separation of the desethyl-CQ enantiomers was not possible because the requisite alcohol moiety is not present. The other availabe HPLC assays [12,13] which use α_1-acid glycoprotein columns are capable of separating the enantiomers of each of the chiral metabolites of HCQ. Unfortunately the bis-desethyl-CQ and desethyl-HCQ metabolites were not available to us. Nevertheless, the two peaks eluting 3 min before the *R*-HCQ chromatographic peak in the chromatograms from patients (Figs. 2 and 3) are presumed to correspond to the diastereomers of desethyl-HCQ, which possesses the hydroxyl group necessary for derivatization.

Although we could apparently derivatize (±)-HCQ with *S*-(+)-1-(1-phenyl)ethyl isocyanate, *S*-(+)-1-(1-naphthyl)ethyl isocyanate and (−)-menthyl chloroformate, we could not separate the HCQ-diastereomers. Prior efforts at pre-column derivatization of HCQ with (−)-menthoxyacetic acid or (−)-*endo*-1,4,5,6,7,7-hexachlorobicyclo(2.2.1)hepta-5-ene-2 carboxylic acid were reportedly unsuccessful [12].

The relative pharmacological potency of HCQ enantiomers has yet to be established. Nevertheless, stereochemical considerations may modify the previous conclusions reached regarding the relationship between concentrations of HCQ enantiomers blood components *versus* clinical effect [3]. Further pharmacokinetic and pharmacodynamic studies are warranted.

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REFERENCES

1 A. H. Mackenzie, *Am. J. Med.*, 75 (Suppl. 1A) (1983) 5.
2 A. Haberkorn, H. P. Kraft and G. Blaschke, *Tropenmed. Parasitol.*, 30 (1979) 308.
3 D. R. Miller, J. J. Fiechtner, J. R. Carpenter, R. R. Brown, R. M. Stroshane and V. J. Stecher, *Arthritis Rheum.*, 30 (1987) 567.
4 S. E. Tett, D. J. Cutler and K. F. Brown, *J. Chromatogr.*, 344 (1985) 241.
5 R. R. Brown, R. M. Stroshane and D. P. Benziger, *J. Chromatogr.*, 377 (1986) 454.
6 R. G. Morris, *J. Chromatogr.*, 338 (1985) 422.
7 S. B. Williams, L. C. Patchen and F. C. Churchill, *J. Chromatogr.*, 433 (1988) 197.
8 S. E. Tett, D. J. Cutler, R. O. Day and K. F. Brown, *Br. J. Clin. Pharmacol.*, 26 (1988) 303.
9 S. E. Tett, D. J. Cutler, R. O. Day and K. F. Brown, *Br. J. Clin. Pharmacol.*, 27 (1989) 771.
10 S. E. Tett, D. J. Cutler and R. O. Day, *J. Pharm. Sci.*, 81 (1992) 155.

11 D. R. Miller, S. K. W. Khalil and G. A. Nygard, *Drug Intell. Clin. Pharm. Ann. Pharmacother.*, 25 (1991) 1302.
12 A. McLachlan, S. E. Tett and D. J. Cutler, *J. Chromatogr.*, 570 (1991) 119.
13 I. Iredale and I. W. Wainer, *J. Chromatogr.*, 573 (1992) 253.
14 W. Lindner, Ch. Lindner and G. Uray, *J. Chromatogr.*, 316 (1984) 605.
15 W. Lindner, M. Rath, K. Stoschitzky and G. Uray, *J. Chromatogr.*, 487 (1989) 375.
16 J. K. French, N. P. Hurst, M. L. O'Donnell and W. H. Betts, *Ann. Rheum. Dis.*, 46 (1987) 42.
17 E. W. McChesney, *Am. J. Med.*, 75 (Suppl. 1A) (1983) 11.