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High-performance liquid chromatographic separation of the enantiomers of hydroxychloroquine and its major metabolites in biological fluids using an α_1-acid glycoprotein stationary phase

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ABSTRACT

An enantioselective two-stage off-line assay has been developed for the analysis of hydroxychloroquine and its three major metabolites in biological fluids. The first non-stereoselective stage of the assay (PRP-1 column) separates and quantitates parent drug and metabolites. Fractions containing hydroxychloroquine and each of the metabolites are collected manually, evaporated, reconstituted in mobile phase and re-injected onto an α_1-acid glycoprotein column to separate and determine proportions of individual enantiomers. Preliminary results from patient samples indicate that the disposition of hydroxychloroquine and its major metabolites is enantioselective.

INTRODUCTION

Hydroxychloroquine is an antimalarial and slow-acting antirheumatic drug that is used clinically as a racemate. No stereoselective assays or clinical studies of the disposition or activity of the enantiomers of hydroxychloroquine have been found in the published literature. However, an assay separating the stereoisomers of chloroquine, a structural analogue, has been published [1] and a number of studies have examined aspects of the stereoselective disposition of chloroquine [2,3].

The enantiomers of chloroquine were first partially separated using diastereomeric salt formation with *d*-bromocamphorsulfonic acid by Riegel and Sherwood [4]. Later Blaschke *et al.* [5] used polyamide liquid chromatography to obtain enantiomers of higher optical purity. Methods for the synthesis of chloroquine enantiomers have also been published by Blaschke *et al.* [5] and more recently by Craig *et al.* [6]. Craig *et al.* [6] also determined the absolute configuration of chloroquine, demonstrating that the levorotatory enantiomer has the *R* configuration. More recently the enantiomers of chloroquine have been separated by Okamoto *et al.* [7] using a cellulose tris(4-*tert.*-butyl phenylcarbamate) column and normal-phase chromatography, and also by Ibrahim and Fell [8] using an

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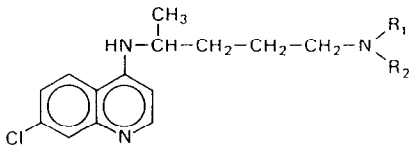

	R_1	R_2
Hydroxychloroquine (HCQ)	$-CH_2CH_3$	$-CH_2CH_2OH$
Chloroquine (CQ)	$-CH_2CH_3$	$-CH_2CH_3$
Desethylchloroquine (CQM)	$-CH_2CH_3$	$-H$
Desethylhydroxychloroquine (HCQM)	$-H$	$-CH_2CH_2OH$
Bisdesethylchloroquine (CQMM)	$-H$	$-H$

Fig. 1. Structures of hydroxychloroquine and its major metabolites.

α_1-acid glycoprotein column (Enantiopac) with UV detection. Ofori-Adjei *et al.* [1] developed an enantioselective high-performance liquid chromatographic (HPLC) assay for chloroquine also using the chiral stationary phase, Enantiopac, which consists of α_1-acid glycoprotein bonded to diethylaminoethyl-functionalised silica was developed by Hermansson [9].

There are an increasing number and range of chiral compounds that have been separated using the α_1-acid glycoprotein chiral stationary phase. This list includes neutral, acidic and basic compounds. The assay discussed in the present report allows for the quantitation of the enantiomers of hydroxychloroquine and its three major metabolites, desethylhydroxychloroquine, desethylchloroquine and bisdesethylchloroquine (Fig. 1). The assay uses a two-stage analysis which initially quantitates and separates parent and metabolites from whole blood, plasma or urine, then a second separation is used to measure the proportion of each enantiomer of each compound. The first stage of the assay is based on the hydroxychloroquine assay of Tett *et al.* [10]. The second stage of the analysis is a modification of the chloroquine enantiomer assay of Ofori-Adjei *et al.* [1].

The present enantioselective assay has been developed with the aim of studying the enantioselective disposition of hydroxychloroquine in humans.

EXPERIMENTAL

Materials

Racemic hydroxychloroquine sulphate, desethylhydroxychloroquine and desethylchloroquine were supplied by Sterling Pharmaceuticals (Sydney, Australia), bisdesethylchloroquine was provided by the Army Malaria Research Unit (Ingleburn, Australia), and chloroquine phosphate was obtained from Sigma (St. Louis, MO, USA). The *S*(+)-hydroxychloroquine diphosphate enantiomer was a gift from Sterling Drug (New York, NY, USA).

Water and diethyl ether were freshly distilled. Propan-2-ol, triethylamine, con-

centrated aqueous ammonia solution and sodium dihydrogen orthophosphate ($NaH_2PO_4 \cdot 2H_2O$) were all analytical reagent grade and obtained from Ajax Chemicals (Sydney, Australia). N,N-Dimethyloctylamine (95%) was purchased from Aldrich (Milwaukee, WI, USA). Methanol and acetonitrile were HPLC grade and supplied by Mallinckrodt (Sydney, Australia). Sodium hydroxide was analytical reagent grade and purchased from May & Baker (Footscray, Australia). All reagents were used as purchased.

Chromatography

System I: non-stereoselective quantification of hydroxychloroquine and metabolites. The system for the quantitation and separation of parent drug and metabolites [10] consisted of an Altex 110A HPLC pump, a Waters Assoc. autoinjector (WISP 710B) and a 5-μm reversed-phase 150 mm × 4.1 mm poly(styrene-divinylbenzene) (PRP-1, Hamilton, Reno, NV, USA) HPLC column. The mobile phase consisted of methanol–water (70:30, v/v) and 100 m*M* triethylamine adjusted to pH 11. Flow-rate was 1.0 ml/min. Injection volume varied between 50 and 150 μl depending on sample concentration.

System II: determination of the enantiomeric composition of hydroxychloroquine and metabolites. System II was used to determine the proportion of *R* and *S* enantiomers of hydroxychloroquine and metabolites. An Altex 110A pump with a Rheodyne 7125 manual injector (100-μl injection loop) was attached to a 10-μm, 100 mm × 4.0 mm silica DEAE bonded α_1-acid glycoprotein HPLC column (Enantiopac, Pharmacia LKB Biotechnology, Bromma, Sweden). The mobile phase consisted of 16 m*M* $NaH_2PO_4 \cdot 2H_2O$, with 5.5% (v/v) propan-2-ol and 0.02% N,N-dimethyloctylamine, adjusted to pH 7 (apparent pH of the mobile phase) using 1 *M* NaOH. Flow-rate was 0.4 ml/min. The injection volume was 100 μl. Enantiomer separations using system II were carried out at room temperature with post-column alkalinisation. This was achieved using a second Altex 110A HPLC pump adding triethylamine–methanol–water (10:20:70, v/v) to the column eluent at a flow-rate of 0.1 ml/min. The final mobile phase pH was greater than 11 to increase the sensitivity of detection. Hydroxychloroquine and its metabolites have a maximum fluorescence in this alkaline pH range.

Both mobile phases were filtered using a 10-μm filtering system (Millipore) and degassed prior to use. An Activon 208 pH meter and an Activon RU342 pH electrode were used to measure pH.

Detection

In both systems analytes were detected using a Shimadzu RF-535 fluorescence HPLC detector and chromatograms were recorded using a Sekonic SS-250F chart recorder. Fluorescence detection was achieved at an excitation wavelength of 337 nm and an emission wavelength of 405 nm.

Sample preparation

Samples were prepared as previously described [10], except that each sample was extracted twice with diethyl ether to increase the recovery for all compounds to greater than 90% from all fluids. Hydroxychloroquine and metabolites were extracted with diethyl ether from diluted heparinised whole blood (1:1 with water), or undiluted urine, after alkalinisation with 300 μl of concentrated aqueous ammonia solution, with chloroquine added as internal standard. The organic phase was decanted after separation by centrifugation (1200 *g* for 5 min) and freezing of the aqueous phase in a dry ice–acetone slurry. The diethyl ether was evaporated under a stream of nitrogen at room temperature. Samples were prepared for injection onto system I by reconsitution in 250 μl of a 1:1 mixture of 0.1 *M* H_2SO_4 and acetonitrile.

Fractions of eluent from system I were collected manually at the retention times of hydroxychloroquine and metabolites and were evaporated under nitrogen in a heated water bath. Fraction collection times were validated by re-injection of evaporated fractions onto system I. Times were adjusted until pure fractions were obtained. Residues were reconstituted in 100 μl of system II mobile phase and injected onto system II for subsequent enantiomer analysis.

Collection of biological fluids

Blood samples were collected using silanised Vacutainers (Mallinckrodt) that were heparinised with 100 I.U. lithium heparin. Urine was collected in a silanised vessel, the time and volume of collection were recorded, and a 10-ml aliquot was kept for analysis. Samples were frozen at −22°C until the time of analysis. Ethics approval from St. Vincents Hospital Research and Ethics Committee was obtained for collection of blood and urine samples from a rheumatoid arthritis patient at St. Vincents Hospital (Darlinghurst, Sydney, Australië).

Data analysis

The concentrations of hydroxychloroquine and metabolites were determined using peak-height ratios (using chloroquine as the internal standard) and a standard curve using system I [10]. The reproducibility of system I has been previously reported in plasma and whole blood [10]. Enantiomeric ratios were determined using peak-area ratios from system II. To assess assay variability four replicate samples at each of three different concentrations were analysed. Four samples were analysed at two concentrations in plasma, 20 and 200 ng/ml, and four samples at one concentration in blood, 1000 ng/ml. These samples and racemic concentrations were chosen to reflect the concentrations expected in patients. Peak-height ratios from system I and peak-area ratios from sysstem II were calculated for each compound at the different concentrations. Using these data the relative standard deviation for each enantiomer was determined. The resolution factor was determined by injecting a standard solution of each analyte on to

system II. The mean of at least three injections was used. Resolution factor (R_s) was calculated using the equation;

$$R_s = \frac{2}{1.66}\left[\frac{t_R(+) - t_R(-)}{W_{\frac{1}{2}h}(+) + W_{\frac{1}{2}h}(-)}\right]$$

where $t_R(+)$ and $t_R(-)$ are the retention (in mm) of the $S(+)$ and $R(-)$ enantiomers, respectively, and $W_{\frac{1}{2}h}(+)$ and $W_{\frac{1}{2}h}(-)$ are the width of the peak at half the height (in mm) for the $S(+)$ and $R(-)$ isomer, respectively.

RESULTS

Chromatograms for system I have previously been published [10]. Fig. 2 shows the chromatograms for the enantiomers of hydroxychloroquine and the three major metabolites on system II using fractions collected from system I. Samples were extracted from whole blood. The retention times for each enantiomer (*R* and *S*, respectively) are as follows: 29 and 40 min for hydroxychloroquine, 37 and 47 min for desethylhydroxychloroquine, 38 and 49 min for desethylchloroquine, and 43 and 52 min for bisdesethylchloroquine. Recovery for each compound from urine was previously found to be the same as from plasma and blood [10]. The reproducibility of peak-height ratio for system I was found in this study to be the same as previously reported [10]. Table I shows the relative standard deviation for the determination of each enantiomer at three different concentrations. Typical racemic concentrations in plasma and blood were chosen to determine the assay reproducibility. In multiple-dose patient studies blood, plasma and urine

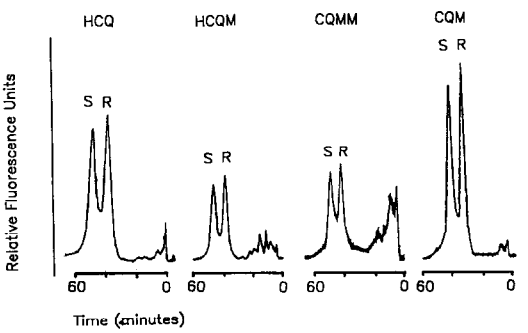

Fig. 2. Chromatograms of resolved enantiomers of hydroxychloroquine (HCQ), desethylhydroxychloroquine (HCQM), desethylchloroquine (CQM) and bisdesethylchloroquine (CQMM) on system II. Whole blood samples contained 500 ng/ml of each enantiomer of HCQ, HCQM and CQM, and 100 ng/ml of CQMM enantiomers. Each sample was reconstituted in 100 μl of system II mobile phase and injected. The fluorescence detection range was 128 for HCQ, HCQM and CQM, and 64 for CQMM. For other chromatographic conditions see Experimental.

TABLE I

RELATIVE STANDARD DEVIATION OF THE COMPOSITE (SYSTEM I AND II) ASSAY FOR THE ENANTIOMERS OF HYDROXYCHLOROQUINE AND ITS METABOLITES

Sample	Concentration of racemate (ng/ml)	Relative standard deviation (%) (n = 4)							
		HCQ		HCQM		CQMM		CQM	
		R	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Plasma	20	3	5	6	4	7	7	7	9
Plasma	200	1	1	3	3	5	2	2	2
Blood	1000	3	2	3	2	2	2	2	2

levels are rarely below 100 ng/ml [11]. In single-dose studies hydroxychloroquine maintains plasma levels of above 20 ng/ml for some weeks [12]. Resolution factors for each analyte are 2.4 for hydroxychloroquine (HCQ), 2.5 for desethylhydroxychloroquine (HCQM), 2.1 for desethylchloroquine (CQM) and 1.8 for bisdesethylchloroquine (CQMM).

Peak identification

The elution order of hydroxychloroquine enantiomers on system II was determined by separate injection of the pure (*S*) enantiomer. The peak at 40 min after a racemic injection of hydroxychloroquine was identified as the (*S*) enantiomer. By exclusion the peak at 29 min was assumed to be the (*R*) enantiomer. Ofori-Adjei *et al.* [1] reported the same elution order for desethylchloroquine and chloroquine, with the (*R*) enantiomer eluting first. The enantiomer elution order for the other metabolites on system II was assumed to be the same as for chloroquine,

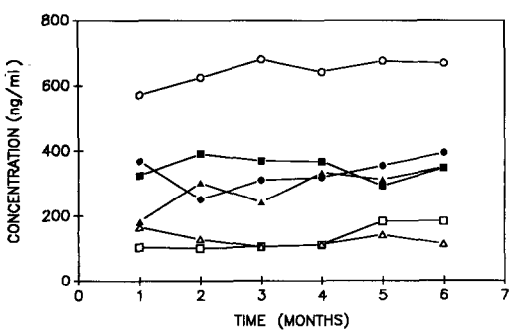

Fig. 3. Whole blood concentration–time profile of the enantiomers of hydroxychloroquine, desethylhydroxychloroquine and desethylchloroquine for a patient with rheumatoid arthritis receiving hydroxychloroquine therapy daily for six months. (○) *R*(−) and (●) *S*(+)-hydroxychloroquine; (△) *R*(−)- and (▲) *S*(+)-desethylhydroxychloroquine; (□) *R*(−)- and (■) *S*(+)-desethylchloroquine.

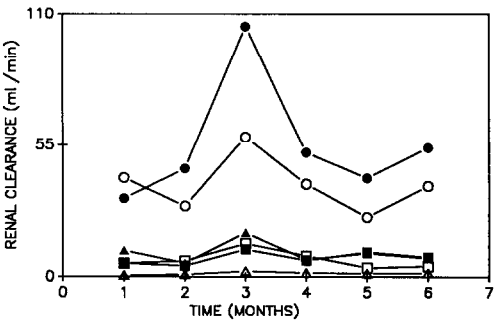

Fig. 4. Renal clearance data for the enantiomers of hydroxychloroquine, desethylhydroxychloroquine and desethylchloroquine for a patient with rheumatoid arthritis receiving hydroxychloroquine therapy daily for six months. (○) *R*(−)- and (●) *S*(+)-hydroxychloroquine; (△) *R*(−)- and (▲) S(+)-desethylhydroxychloroquine; (□) *R*(−)- and (■) *S*(+)-desethylchloroquine.

hydroxychloroquine and desethylchloroquine as all the compounds share the same chiral centre, with *R* as the first peak.

Single-patient study

Whole blood and urine concentrations were determined in one 72-year-old male patient with rheumatoid arthritis who was receiving either 200 or 400 mg Plaquenil (hydroxychloroquine sulphate, Sterling Pharmaceuticals) daily as part of a double-blind trial. Samples were taken at the end of each month for six months. The blood concentration–time profiles for hydroxychloroquine, desethylhydroxychloroquine and desethylchloroquine, both *R* and *S* isomers are shown in Fig. 3. Enantiomer renal clearance data were calculated by dividing the urinary excretion rate of each enantiomer by the blood concentration of that enantiomer at the midpoint of the urine collection time. Fig. 4 shows the enantiomer renal clearance data for six months. The concentrations of bisdesethylchloroquine are not shown in Fig. 3 or 4 as this metabolite was present only in very low levels in this patient.

DISCUSSION

System II does not adequately separate parent and metabolites and hence system I, the non-enantioselective assay, is used to achieve this separation initially. The resultant assay procedure provides a sensitive and selective method for the separation and quantitation of hydroxychloroquine and its three major metabolites in whole blood, plasma and urine. Samples containing 20 ng/ml (10 ng/ml for each enantiomer) are readily detectable with a relative standard deviation for each enantiomer of about 5%.

Other methods of enantiomer resolution were initially investigated for possible

application for assay development for the enantiomers of hydroxychloroquine. These included derivatisation with the chiral acetylator *endo*-1,4,5,6,7,7-hexachlorobicyclo(2.2.1)hepta-5-ene-2 carboxylic acid, (−)-HCA, as the acid chloride with *p*-dimethylaminopyridine (as HCl scavenger) and also using the acetylation activator, 1,3-dicyclohexylcarbimide [13]. (−)-Menthoxyacetic acid was also investigated as a possible chiral derivatising agent. Hydroxychloroquine proved difficult to derivatise, probably due to the sterically hindered nature of the chiral centre. Chiral ion-pair formation was investigated using *d*-10-camphorsulfonic acid and amino acid–Cu^{2+} complexes (with L-tryptophan and L-phenylalanine) but similarly proved unsuccessful. Pirkle-type chiral stationary phases have successfully separated the structurally similar antimalarial, primaquine, using normal-phase chromatography [14]. This method was unsuccessful for hydroxychloroquine.

Ofori-Adjei *et al.* [1] have identified the order of elution of the chloroquine and desethylchloroquine enantiomers using the α_1-acid glycoprotein column (system II) by injecting the pure enantiomers. The same elution order (*R* elutes first) was seen for hydroxychloroquine enantiomers in the present assay. Due to the non-availability of the pure enantiomers of desethylhydroxychloroquine and bisdesethylchloroquine direct identification could not be achieved. However, it was assumed that because of structural similarities (same chiral centre) the elution order for the enantiomers of these metabolites is the same as for chloroquine, hydroxychloroquine and desethylchloroquine; that is, that the (*R*) enantiomer elutes first. Craig *et al.* [6] have determined the absolute configuration of the chloroquine enantiomers. In the present assay hydroxychloroquine and its metabolites are assumed to have the same absolute configuration that has been determined for chloroquine, that is, the (*R*) enantiomer is levorotatory and the (*S*) enantiomer is dextrorotatory.

With the advantage of good enantiomer resolution and sensitivity from whole blood, plasma and urine, the assay described in this report has been employed in the analysis of patient blood and urine sample data. Fig. 3 shows that after one month dosing the (*R*)-hydroxychloroquine blood concentrations exceed the blood concentrations of the (*S*) isomer. In contrast, the metabolite enantiomeric ratios are reversed with the concentrations of the (*S*) isomer being greater than the (*R*) isomer for both desethylhydroxychloroquine and desethylchloroquine. From the data in Fig. 4 it can be seen that the (*S*)-hydroxychloroquine isomer has a greater renal clearance then (*R*)-hydroxychloroquine, whereas renal clearance of the (*R*) and (*S*) metabolites were not different.

These preliminary data obtained from the one patient studied suggests that hydroxychloroquine exhibits stereoselective disposition. The enantiomer whole blood levels for the metabolites are consistent with stereoselective metabolism. The (*S*) enantiomer of the parent drug appears to be preferentially metabolised. Renal clearance data calculated from the urine concentrations for each enantiomer suggest that hydroxychloroquine undergoes stereoselective renal

elimination that favours (*S*)-hydroxychloroquine. Renal clearance of the enantiomers of the metabolites appears to be not stereoselective. These findings are in general agreement with the published studies for chloroquine [1–3].

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