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Enantioselective analysis of the metabolites of hydroxychloroquine and application to an in vitro metabolic study

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Abstract

A one-step chiral method for the quantification of the enantiomers of two hydroxychloroquine (HCQ) metabolites, desethylchloroquine (DCQ) and desethylhydroxychloroquine (DHCQ) by HPLC is described, in addition to its application to the in vitro study of HCQ metabolism in rat liver microsomes. Liquid–liquid extraction was used to extract the enantiomers from microsome samples and the separation was performed on a Chiralpak AD-RH column protected with an RP-8 guard column using hexane:isopropanol (92:8, v/v) plus 0.1% diethylamine as the mobile phase, at a flow rate of 1.0 mL min^{-1}. The detection was carried out at 343 nm. The method proved to be linear in the range of 50–5000 ng mL^{-1} for DCQ enantiomers and 125–2500 ng mL^{-1} for DHCQ enantiomers, with a quantification limit of 50 and 125 ng mL^{-1}, respectively. Precision and accuracy, demonstrated by within-day and between-day assays, were lower than 15%. The metabolic study demonstrated that metabolism is stereoselective for HCQ. The major metabolites formed in the incubation of racemic HCQ were (−)-(*R*)-DCQ and (−)-(*R*)-DHCQ with *R*/*S* ratios of 2.2 and 3.3, respectively.

Keywords: Hydroxychloroquine; Desethylchloroquine; Desethylhydroxychloroquine; Enantioselective separation; In vitro metabolism

1. Introduction

Hydroxychloroquine (HCQ) is used in the treatment of rheumatoid arthritis and systemic lupus erythematosus and as an antimalarial [1]. Like other antimalarial drugs, such as (±)-chloroquine, (±)-primaquine and (±)-mefloquine [2], HCQ is administered as a racemic mixture [*rac*-HCQ] of two enantiomers, *R*(−)-HCQ and *S*(+)-HCQ. The hepatic metabolism of HCQ generates three active metabolites, desethylchloroquine (DCQ), desethylhydroxychloroquine (DHCQ) and bisdesethylchloroquine (BDCQ), which are also chiral molecules [3,4] (Fig. 1). The relative antiarthritic and antimalarial properties of the individual enantiomers of HCQ are not known, although it has been determined that the (+)-(*S*)-enantiomer of CQ, which is structurally very similar to HCQ, displays a higher antimalarial activity than (−)-(*R*)-CQ in the mouse [5].

The pharmacokinetics and metabolism of HCQ are not totally known. Stereoselectivity in the disposition of HCQ was investigated in healthy volunteers [3] and in patients with rheumatoid arthritis [6,7]. The blood concentrations of the (−)-(*R*)-enantiomer of HCQ were found higher than those of (+)-(*S*) suggesting that the *N*-deethylation of HCQ is stereoselective. The blood concentrations of (+)-(*S*)-DCQ and (+)-(*S*)-DHCQ exceeded those of the (−)-(*R*)-metabolites [6]. In rabbits, the *R*/*S* ratios of HCQ blood concentrations are similar to those found in humans [8].

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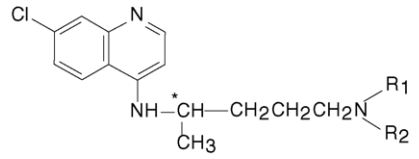

	R1	R2
Hydroxychloroquine (HCQ)	—CH_2CH_3	—CH_2CH_2OH
Desetylhydroxychloroquine (DHCQ)	—H	—CH_2CH_2OH
Desetylchloroquine (DCQ)	—CH_2CH_3	—H
Bisdesetylchloroquine (BDCQ)	—H	—H

Fig. 1. Hydroxychloroquine and its metabolites.

Wei et al. [4] investigated the stereoselective disposition of HCQ and its metabolites in blood and tissues of rats. The proportion of (*R*)-HCQ in blood was higher than that of (*S*)-HCQ, and the *S*-configuration predominated in the metabolites. In tissues, the proportions of the HCQ enantiomers were analogous to the blood ratios, but the tissue *R*/*S* ratios of HCQ and its metabolites were higher than those found in blood, suggesting that the transport of the compounds or tissue binding may also exhibit stereoselectivity and that the *R*-enantiomers may demonstrate a higher affinity towards tissue. The liver's *R*/*S* ratio of HCQ was the highest among the tissues but its metabolite ratios were among the lowest, showing that the liver is the major site for HCQ metabolism [4].

The previous studies [3,6,7] were carried out using in vivo models but in vitro studies are also interesting. In vitro methods are particularly useful for the rational selection of animal species for toxicology studies and for metabolite profile comparison, assessment of species differences in metabolism, and metabolite generation for bioanalytical assay development. They also provide support for the clinical development of a drug in pharmacokinetic studies [9].

Nevertheless, to our knowledge, no stereoselective study has been performed on the in vitro metabolism of HCQ. Human liver microsomes provide the most convenient way to study CYP450 metabolism, but animal CYP450 isoforms have also been compared with those found in humans in order to improve the extrapolation of findings from animals studies to humans [9].

In this paper, we report the development and validation of a one-step chiral HPLC method and its application to an in vitro metabolism study of HCQ in rat liver microsomes. Despite the multi-step HPLC methods for the determination of the enantiomers of HCQ and its metabolites [3,10–13] the application of using one-step chiral HPLC method for metabolism study of HCQ has not been previously described.

2. Experimental

2.1. Chemicals and reagents

Racemic hydroxychloroquine sulphate, desethylchloroquine and desethylhydroxychloroquine were supplied by Sanofi/Wintropi Pharmaceuticals (New York, NY, USA). HPLC grade methanol, isopropanol and chloroform were purchased from EM Science (Gibbstown, NJ, USA), hexane was from Mallinckrodt (Paris, Kentucky, USA) and acetonitrile was from J.T. Baker (Phillipsburg, NJ, USA). Diethylamine (DEA) P.A. grade was supplied by Fluka (St. Gallen, Switzerland). β-Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Sigma (St. Louis, MO, USA). Water was purified with a Milli-Q plus system (Millipore, Bedford, MA, USA).

2.2. Standard solutions

Stock solutions of HCQ and its DCQ and DHCQ metabolites were prepared at the concentrations of 1000 μg mL^{-1} in methanol:water (50:50, v/v). Working solutions of metabolites at concentrations of 2, 5, 10, 20, 50, 100 and 200 μg mL^{-1} were obtained by appropriate dilutions in the same solvent system. All these solutions were stored at −20 °C and protected from direct light.

2.3. Instrumentation and chromatographic conditions

The HPLC system used consisted of an LC-10AT VP solvent pump, a 7725 Rheodyne injector with a 50 μL loop, a SPD-10A UV–vis detector and a C-R6A Chromatopac integrator (Shimadzu, Kyoto, Japan). The enantiomers were resolved on a Chiralpak AD-RH column (150 mm × 4.6 mm, 5 μm particle size) used in normal elution mode [14]. The chiral column was purchased from Chiral Technologies (Exton, Pennsylvania, USA). An RP-8 guard column (5 μm particle size, Merck, Germany) was used to protect the chiral column during the analyses. The wavelength for detection was adjusted to 343 nm. The analyses were carried out in a climatized room (22 ± 2 °C). The mobile phase, prepared by mixing all the components, consisted of hexane:isopropanol (92:8, v/v) plus 0.1% DEA and the flow rate was 1.0 mL min^{-1}.

2.4. Sample preparation

Drug-free plasma obtained from healthy human volunteers was used for the construction of calibration curves and for method validation. Plasma was diluted with potassium phosphate buffer (pH 7.4, 100 mmol L^{-1}), in order to achieve protein concentrations comparable to those of the microsomal incubation mixtures [15]. In amber tubes, diluted plasma or microsome preparation (500 μL) was spiked with 25 μL working solutions (only for the calibration curves),

and supplemented with 250 μL potassium phosphate (pH 7.4; 100 mmol L^{-1}), 250 μL 2% sodium bicarbonate (these solutions were added to have similar conditions as that used in the incubation experiments), 1 mL acetonitrile (used to stop the incubation reaction), and 100 μL sodium hydroxide (5 mol L^{-1}). The mixture was vortex mixed for 1 min and 2 mL of chloroform were then added, followed by mechanical shaking for 20 min. The extracted sample was centrifuged at 1800 × *g* for 5 min and the organic layer was separated and evaporated under a stream of air. The residue was dissolved in 100 μL of hexane:isopropanol (92:8, v/v) plus 0.5% DEA.

2.5. Method validation

Calibration curves were prepared by analyzing 500 μL diluted plasma samples spiked with standard *rac*-DCQ and *rac*-DHCQ solutions ($n = 2$ for each concentration) resulting in concentrations of 50, 125, 250, 500, 1250, 2500 and 5000 ng mL^{-1} for each enantiomer. Sample preparation and chromatographic conditions were as described before. Plots of metabolite concentrations versus peak area (DCQ) or height (DHCQ) were constructed and the linear regression lines were used for the determination of enantiomer concentration in the samples.

The linearity of the analytical method was assessed in the concentration range of 50–5000 ng mL^{-1} for DCQ and DHCQ enantiomers.

To determine absolute recovery, plasma samples spiked with 250, 500, 1250 and 2500 ng mL^{-1} for each enantiomer of the two metabolites were extracted in triplicate by the procedure proposed. The concentrations of the samples were determined on the basis of a calibration curve obtained with the data for the analyte not submitted to extraction. Recovery was expressed as percentage of the amount extracted.

The precision and accuracy of the method were evaluated by within-day ($n = 5$) and between-day ($n = 3$) assays using plasma samples spiked with DCQ and DHCQ at the concentration of 250, 1250 and 2500 ng mL^{-1} of each enantiomer. The results obtained were expressed as relative standard deviations and relative error.

The quantitation limit was assayed by analyzing aliquots of plasma ($n = 5$) spiked at concentrations of 50 and 125 ng mL^{-1} of each enantiomer against calibration curves with a concentration range of 125–5000 ng mL^{-1} and 125–2500 ng mL^{-1}, respectively.

2.6. In vitro metabolism studies

Adult male Winstar rats weighing 180–200 g were obtained from the Specific Patogen Free (SPF) Animal Facility of the Faculty of Pharmaceutical Sciences of Ribeirão Preto.

Animals were housed in a temperature-controlled room (25 °C) with a 12 h light cycle and fasted overnight before the experiments. In the morning they were anesthetized with ether and their livers were immediately perfused [16] with cold 0.9% (w/v) sodium chloride, excised and minced in cold potassium chloride (pH 7.4, 0.154 mol L^{-1}). Tissues were homogenized with three strokes in a homogenizer (Potter type) with temperature control (MA 181, Marconi, Brazil) and in a mechanical shaker (MA 039, Marconi, Brazil). The homogenate was filtered through two layers of gauze to remove connective tissue and the volume was adjusted with the cold potassium chloride solution to obtain a 20% (w/v) suspension. For microsome isolation, the homogenate was sedimented at 9000 × *g* for 15 min at 4 °C and the ensuing supernatant was sedimented at 100,000 × *g* for 60 min at 4 °C. The pellet was suspended in Tris–HCl (pH 7.6, 0.15 mol L^{-1}) and sedimented at 100,000 × *g* for 60 min at 4 °C to remove nonmembranous proteins such as hemoglobin. Microsomes were stored as suspension in 1.15% (w/v) KCl–Hepes (pH 7.6, 1.25 mmol L^{-1}), at −70 °C [17,18]. Protein concentration was determined as described by Cain and Skilleter [19].

The in vitro metabolism study was performed using standard solutions of HCQ racemate (500 μg mL^{-1} of each enantiomer) and also with its separate enantiomers (420 μg mL^{-1} of the first enantiomer and 630 μg mL^{-1} of the second enantiomer, approximately). Each sample (in amber tubes) consisted of 500 μL of microsome homogenate, 240 μL potassium phosphate (pH 7.4, 100 mmol L^{-1}) and 10 μL of HCQ standard solutions. The incubation solution (NRS) consisted of 1.7 mg mL^{-1} NADP, 7.8 mg mL^{-1} glucose-6-phosphate, and 1.5 units mL^{-1} glucose-6-phosphate dehydrogenase in a 2% (w/v) $NaHCO_3$ solution. The samples and the NRS (incubation solution) were warmed in a 37 °C shaking water bath for 5 min. After 5 min, 250 μL of the warmed NRS solution

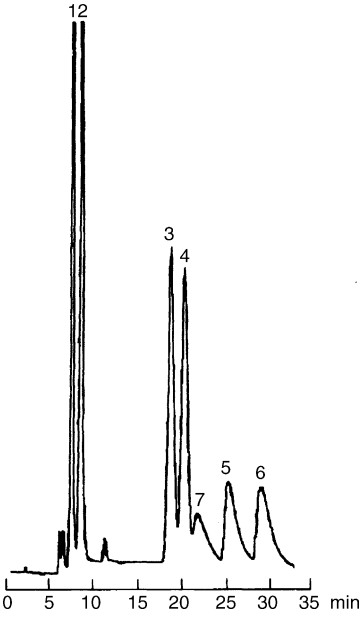

Fig. 2. Chromatogram showing the resolution of HCQ and its metabolites DCQ and DHCQ in diluted plasma spiked with 50 μg mL^{-1} of the enantiomers: (−)-(*R*)-DCQ (1), (+)-(*S*)-DCQ (2), (−)-(*R*)-HCQ (3), (+)-(*S*)-HCQ (4), (−)-(*R*)-DHCQ (5), (+)-(*S*)-DHCQ (6) and *rac*-BDCQ (7). Chromatographic conditions: Chiralpak AD-RH (150 mm × 4.6 mm, 5 μm particle size); hexane:isopropanol (92:8, v/v) plus 0.1% DEA as the mobile phase at a flow rate of 1.0 mL min^{-1}; detection at 343 nm.

Table 1
Recovery and linearity of the method for the analysis of DCQ and DHCQ enantiomers

	Recovery		Linearity	
	%	R.S.D. (%)	Range (ng mL^{-1})	r
(−)-(*R*)-DCQ	67.5	6.7	50–5000	0.9985
(+)-(*S*)-DCQ	67.6	7.4	50–5000	0.9986
(−)-(*R*)-DHCQ	64.3	9.7	125–2500	0.9966
(+)-(*S*)-DHCQ	63.5	13.6	125–2500	0.9978

R.S.D.: relative standard deviation; *r*: correlation coefficient.

was added to the samples to start the reaction. After 180 min, the incubation was stopped by adding 1 mL of acetonitrile [20], and the samples were submitted to extraction (Section 2.4) and chromatographic analysis.

3. Results and discussion

3.1. Screen of columns

HCQ and its DCQ and DHCQ metabolites were previously resolved in our laboratory using a Chiralpak AD column and hexane:isopropanol (90:10, v/v) plus 0.1% DEA as the mobile phase [21], but this separation could not be reproduced after using the column for the analyses of other drugs under different conditions. The separation of HCQ, DCQ and DHCQ enantiomers through this column was only possible using hexane:methanol (96:4, v/v) plus 0.4% DEA as the mobile phase. However, we observed changes over 5 min in retention times during routine use.

Several other polysaccharide-based columns [22,23] were also investigated, in reversed (Chiralpak AD-RH and Chiralcel OD-H) and normal-phase elution modes (Chiralpak AD, Chiralpak AS, Chiralpak AD-RH, Chiralcel OG, Chiralcel OF and Chiralcel OD-H). The successful chiral resolution of HCQ and its metabolites was obtained using the same amilose tris-(3,5-dimethylphenylcarbamate) derived chiral stationary phase but with a lower particle size and surface treated silica gel support [24], i.e. a Chiralpak AD-RH column. It is recommended to use this column in the reverse-phase elution mode, but in the present study we used it in the normal elution mode, after conversion using the method of Cass et al. [14], with a mobile phase of hexane:isopropanol (92:8, v/v) plus 0.1% DEA. The six peaks of HCQ, DCQ and DHCQ enantiomers eluted with a total retention time of approximately 35 min (Fig. 2).

3.2. Optimization of sample preparation

The liquid–liquid extraction method was applied to isolate DCQ and DHCQ from the microsome homogenate and the plasma matrix. Due to the basic character of HCQ and its metabolites, the samples were alkalinized (pH 10.0) by the addition of 100 μL NaOH (5 mol L^{-1}). The extraction procedure resulted in efficient recoveries and eliminated the possible interferents from the matrix. As we did not use an internal standard for the quantitation of DCQ and DHCQ enantiomers, special care was followed during sample treatment and injection to maintain the errors within the values recommended in the literature. The quantitation of HCQ enantiomers was not performed because only a small percentage (10–20%) of them were metabolized.

3.3. Method validation

The developed method was validated by evaluating linearity, recovery, precision, accuracy and limit of quantitation. Coefficients of variation and relative errors of less than 15% were considered acceptable, except for the quantitation limit for which these values were established at 20%, as recommended in the literature [25–27]. The data obtained are

Table 2
Precision and accuracy of the method for the analysis of DCQ and DHCQ enantiomers

	Within-day (n = 5)			Between-day (n = 3)		
	Concentration (ng mL^{-1})	R.S.D. (%)	E (%)	Concentration (ng mL^{-1})	R.S.D. (%)	E (%)
250 ng mL^{-1}						
(−)-(*R*)-DCQ	237	4.9	−5.1	266	9.1	6.2
(+)-(*S*)-DCQ	238	5.2	−4.9	265	8.6	5.9
(−)-(*R*)-DHCQ	215	2.9	−13.9	253	13.5	1.1
(+)-(*S*)-DHCQ	215	3.6	−13.9	261	14.6	4.5
1250 ng mL^{-1}						
(−)-(*R*)-DCQ	1111	6.6	−11.1	1182	6.0	−5.4
(+)-(*S*)-DCQ	1109	6.9	−11.3	1184	6.3	−5.3
(−)-(*R*)-DHCQ	1151	6.9	−7.9	1295	10.9	3.6
(+)-(*S*)-DHCQ	1092	5.4	−12.6	1226	10.1	−1.9
2500 ng mL^{-1}						
(−)-(*R*)-DCQ	2386	2.1	−4.6	2530	6.7	1.2
(+)-(*S*)-DCQ	2384	2.0	−4.6	2535	6.5	1.4
(−)-(*R*)-DHCQ	2523	3.9	0.9	2738	9.2	9.5
(+)-(*S*)-DHCQ	2375	4.3	−5.0	2568	9.6	2.7

n: Number of determinations; R.S.D.: relative standard deviation; *E*: relative error.

Table 3
Limit of quantitation of the method for the analysis of DCQ and DHCQ enantiomers

Enantiomers	Theoretical concentration (ng mL^{-1})	Obtained concentration (ng mL^{-1})	Precision (R.S.D., %)	Accuracy (*E*, %)
(−)-(*R*)-DCQ	50	44	8.2	−11.6
(+)-(*S*)-DCQ	50	45	8.5	−10.0
(−)-(*R*)-DHCQ	125	105	1.3	−16.4
(+)-(*S*)-DHCQ	125	107	1.3	−14.3

R.S.D.: relative standard deviation; *E*: relative error.

Table 4
Results of the incubation of HCQ with rat liver microsomes

	Metabolites			
	(−)-(*R*)-DCQ	(+)-(*S*)-DCQ	(−)-(*R*)-DHCQ	(+)-(*S*)-DHCQ
rac-HCQ				
Concentration (ng mL^{-1})	273	125	667	201
R.S.D. (%)	3.2	10.9	9.6	5.6
R/*S* ratio	2.2		3.3	
(−)-(*R*)-HCQ				
Concentration (ng mL^{-1})	127		240	
R.S.D. (%)	10.9		1.5	
(+)-(*S*)-HCQ				
Concentration (ng mL^{-1})		61		85
R.S.D. (%)		13.2		14.5

$n = 3$ for each incubation; R.S.D.: relative standard deviation.

summarized in Tables 1–3. The calibration curves (Table 1), obtained by least-squares linear regression, were linear in the concentration range of 50–5000 ng mL^{-1} for DCQ enantiomers and of 125–2500 ng mL^{-1} for DHCQ enantiomers and the correlation coefficients observed were 0.9966 or better. Table 1 also shows reproducible recovery of the enantiomers using the proposed procedure.

Precision and accuracy were assessed by performing replicate analysis of spiked samples against calibration curves. The within-day and between-day precision is reported in Table 2. R.S.D.s of less than 15% were obtained for all samples analyzed.

In the present study we used the limit of quantitation (Table 3) as a parameter for the measurement of the sensitivity of the method, defined as the lowest concentration which can be determined with acceptable accuracy and precision (percent of R.S.D. and systematic error lower than 20% [25]). The limit of quantitation determined from extracted spiked plasma was 50 ng mL^{-1} for DCQ enantiomers and 125 ng mL^{-1} for DHCQ enantiomers.

A complete stability text was not carried out but our results obtained in the between-day assay show that the samples could be stored at −20 °C for at least 10 days.

The elution order was determined by the analysis of individual enantiomers (previously separated and collected according to the proposed method) using a Chiral AGP column according to the method described by Iredale and Wainer [10]. The analysis demonstrated that the first peak analyzed corresponds to the (−)-(*R*)-enantiomer and the second to the (+)-(*S*)-enantiomer for all three pairs of enantiomers.

The metabolic study was carried out with rat liver microsomes with a protein concentration of 38.95 mg mL^{-1} for the microsome homogenate. The time of incubation, the amount of microsome homogenate and the concentration of HCQ were optimized. The present results show that HCQ is metabolized and two of its major metabolites, DCQ and DHCQ, were separated and quantified using the optimized conditions. Representative chromatograms of the rat microsomal incubation are shown in Fig. 3. To determine whether a metabolite was formed from the (−)-(*R*)- or the (+)-(*S*)-enantiomer, the

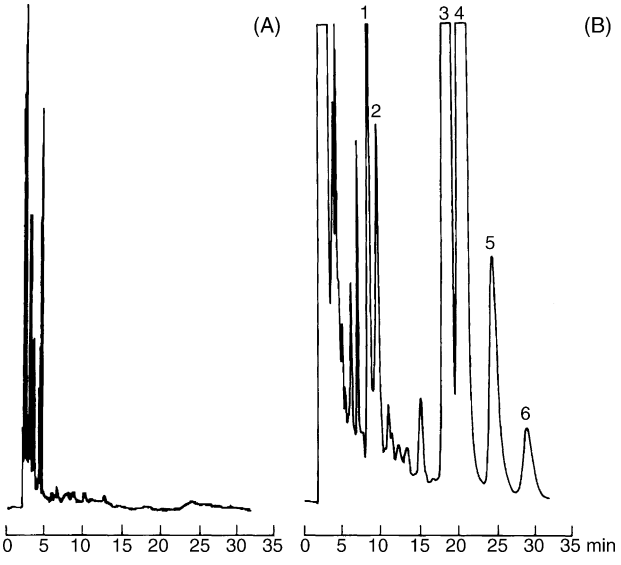

Fig. 3. Chromatograms of drug-free rat microsomes (A) and rat microsome incubation with *rac*-HCQ (B). (−)-(*R*)-DCQ (1) and (+)-(*S*)-DCQ (2), (−)-(*R*)-HCQ (3) and (+)-(*S*)-HCQ (4), (−)-(*R*)-DHCQ (5) and (+)-(*S*)-DHCQ (6). Chromatographic conditions were the same as described in the legend to Fig. 2.

two HCQ enantiomers were incubated separately and analyzed. Table 4 shows the concentrations for HCQ metabolites after incubation of racemic and isolated enantiomers. For the stereoselective metabolism of racemic HCQ, the ratio (−)-(*R*)/(+)-(*S*) obtained was 2.2 for DCQ and 3.3 for DHCQ metabolites. In addition, (−)-(*R*)-DHCQ represented the main metabolite.

4. Conclusion

The determination of enantiomers of HCQ metabolites in rat liver microsomes can be achieved by one-step chromatographic method. The method is superior in terms of the simplicity and stereoselectivity.

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