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Rapid and simple method to determine chloroquine and its desethylated metabolites in human microsomes by high-performance liquid chromatography with fluorescence detection

Julie Ducharme[1], Robert Farinotti*

Faculté de Pharmacie, Université de Paris XI, 5 Rue Jean-Baptiste Clément, 92290 Chatenay-Malabry, France

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Abstract

A sensitive and selective method was developed for the simultaneous determination of chloroquine (CQ) and its desethylated metabolites monodesethylchloroquine (DCQ) and bisdesethylchloroquine (BDCQ) in human liver microsomes. Analytes were separated on a C_1 column using methanol–water (70:30, v/v) and triethylamine (0.1% v/v) as the mobile phase. The fluorescence detector was set at 250 (excitation) and 380 nm (emission). Following protein precipitation with ice-cold acetonitrile, microsomal incubation supernatants were directly injected into the HPLC system. Typically, 200 μl of incubate were diluted with 200 μl of acetonitrile and 15 μl were injected. The limit of quantitation was 78 n*M* of CQ or metabolite. Intra-day variability averaged 2.9% for CQ, 1.5% for DCQ and 2.5% for BDCQ. Inter-day variability was 3.1% for CQ, 3.5% for DCQ and 3.7% for BDCQ. Mean accuracies were 100% for CQ and BDCQ and 102% for DCQ. © 1997 Elsevier Science B.V.

Keywords: Chloroquine; Monodesethylchloroquine; Bisdesethylchloroquine

1. Introduction

Following oral administration of single and multiple doses to healthy volunteers or malaria patients, CQ is rapidly dealkylated into monodesethylchloroquine (DCQ) and bisdesethylchloroquine (BDCQ) (Fig. 1). DCQ and BDCQ concentrations may reach up to 48 and 13% of CQ levels, respectively [1–8]. Other metabolites include 7-chloroquinoline derivatives, CQ side chain N-oxide and CQ di-N-oxide, which were infrequently detected in plasma samples [5,9]. Other uncharacterized molecules may also be present in very low concentrations [5,9].

CQ pharmacokinetics have been recently reviewed [10,11]. Despite its extremely long half-life, CQ has a non-negligible total clearance [2,4,12,13], with the liver and the kidney contributing approximately equally to its elimination [4,5]. In urine, 50% of the given dose was recovered as unchanged CQ and 10% as unchanged DCQ [3,4,7,14,15]. It is estimated that 30 to 50% of an administered dose of CQ is transformed by the liver [16], presumably via cytochrome P450 enzymes (CYPs). However, this has never been rigorously tested. Investigations were therefore initiated in our laboratory to characterize

*Corresponding author.

[1] Present address: Astra Research Centre Montreal, Montreal, Quebec, Canada.

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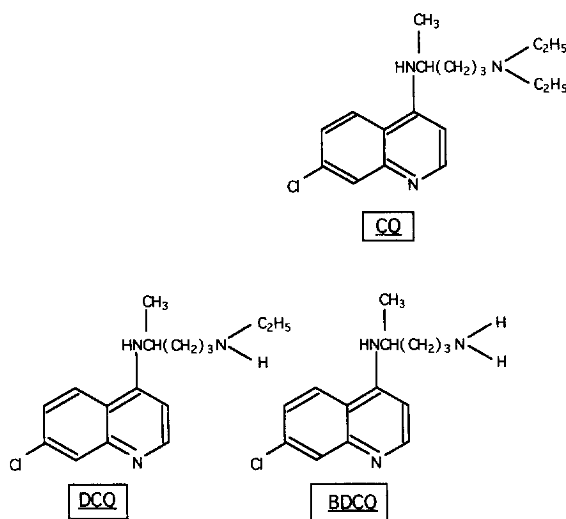

Fig. 1. Chemical structures of chloroquine (CQ) and its two main metabolites, desethylchloroquine (DCQ) and bisdesethylchloroquine (BDCQ).

CQ metabolism in human liver microsomes and to screen, in vitro, which enzymes are implicated in its metabolism [17].

Even if CQ plasma concentrations rarely exceed the micromolar range, liver concentrations may be several hundred times higher (10^{-4} *M*) [18,19], prompting the use of micromolar to hundreds of micromolar concentrations for in vitro studies. These high concentrations of the parent compound compared to the expected low levels of the metabolites required an analytical method with extremely high specificity and sensitivity.

Among the many analytical methods available [20–28], only the most recent ones combined greater specificity and sensitivity [22–28]. Earlier assays were unable to differentiate the parent from the metabolites or the monodesethylated from the bisdesethylated metabolite and lacked sensitivity [20,21]. HPLC methods coupled with fluorescence detection achieved the highest sensitivity [22–25]. Although the HPLC methods developed by Augustijns and Verbeke [23], Tett et al. [24], Brown et al. [26] and Houze et al. [27] appeared well-suited for pharmacokinetic studies, they could not be used for incubation experiments since high CQ concentrations could not be optimally separated from low metabolite concentrations. In addition, all published methods relied on solid-phase [25] or liquid–liquid [22–24,26–28] extraction procedures, leading to lengthy sample preparations when large series of microsomal incubates had to be assayed. This prompted us to develop a new HPLC assay with extreme conditions of separation between CQ and its metabolites.

2. Experimental

2.1. Chemicals

Chloroquine sulphate, desethylchloroquine and bisdesethylchloroquine were kindly supplied by Rhone Poulenc Rorer (France). HPLC grade methanol was purchased from J.T. Baker (Deventer, The Netherlands) and acetonitrile from Merck (Darmstadt, Germany). Triethylamine was obtained from Prolabo (France).

2.2. Solutions

To prevent in vitro degradation of CQ and its metabolites, stock solutions were prepared in methanol–water (50:50 v/v). Diluted solutions were freshly prepared in distilled water. Due to the glass-adsorption properties of CQ and its metabolites [29], all solutions and biological samples were stored in polypropylene tubes.

2.3. HPLC instrumentation

The HPLC system consisted of a Shimadzu LC-6A pump (Touzart et Matignon, France), a Waters 717 autosampler (Millipore, France), a Shimadzu RF-535 fluorescence detector (Touzart et Matignon). Chromatograms were integrated on a Shimadzu CR6A integrator (Touzart et Matignon).

2.4. HPLC conditions

The mobile phase was made of methanol and distilled water (70:30, v/v) containing 7 m*M* (0.1% v/v) of triethylamine (TEA). The solution was degassed under vacuum and filtered through 0.45-μm membranes (Millipore). The mobile phase was prepared daily and never recirculated. The flow-rate was 1 ml/min. Analytes were separated on a 5 μm

Hypersil C_{18} guard column and on a 5 μm Spherisorb C_1 column (150×4.6 mm) (Life Sciences International PLC, HPLC division, UK). Excitation and emission wavelengths were set at 250 and 380 nm, respectively.

2.5. Sample preparation

Human livers were obtained from transplant donors and microsomes were prepared by homogenization and differential centrifugations of snap-frozen liver pieces [30]. Human liver microsomes were incubated with varying concentrations of CQ and Sorensen buffer in a total incubation volume of 200 μl. Reactions were initiated by adding reduced NADPH and stopped by an equal volume (200 μl) of ice-cold acetonitrile. Samples were vortex-mixed for 1 min and kept at 4°C for 10–15 min. Following centrifugation (10 min×600 *g*, at 4°C), supernatants were stored at −80°C until analysis within 2 weeks of the incubation experiments. Typically, 15 μl were injected onto the column.

2.6. Calibration curves

Drug-free plasma was diluted 70 times with distilled water in order to achieve comparable protein concentrations as those of the microsomal incubation mixtures. Diluted plasma was spiked with known amounts of CQ, DCQ and BDCQ (78 to 20 000 n*M*, $n=9$ concentrations). Standard curves were prepared separately for each analyte. Samples were diluted with an equal volume of ice-cold acetonitrile, vortex-mixed and centrifuged. Aliquots (15 μl) were directly injected onto the column. For each analyte, two calibration curves were constructed by linear regression of the peak areas vs. concentration curves, one for lower concentrations (78–1250 n*M*) and one for higher concentrations (1250–20 000 n*M*).

2.7. Sensitivity and selectivity

The limit of quantitation (LOQ) was determined as the minimum concentration which could be accurately and precisely quantified (lowest data point of the standard curves). The limit of detection (on column) was defined as the amount which could be detected with a signal-to-noise ratio of 3.

Putative interferences from cytochromes commonly used P-450 substrates and/or inhibitors [31], such as α-naphtoflavone, coumarin, sulphaphenazole, quinidine, diethyldithiocarbamate, ketoconazole, tolbutamide and mephenytoin, were evaluated by injecting pure standards at 10 times the concentration used in incubation experiments.

2.8. Accuracy and precision

Intra-day precision was assessed by replicate calibration standards (156, 625, 1205 and 5000 n*M*, $n=3$ per concentration) analyzed on the same day. Inter-day precision was assessed by the same calibration standards analyzed on 4 different days. Accuracy was evaluated by comparing estimated amounts with the amount added.

3. Results and discussion

Since investigations of microsomal metabolism imply the analysis of numerous samples, the selected assay had to combine accuracy and precision with speed and simplicity of execution. With the present method, following a simple protein precipitation, small volumes of incubates (15 μl) could be directly injected into the system. Both DCQ and BDCQ could be well separated from very high concentrations of the parent.

Following protein precipitation with acetonitrile, constant volumes (15 μl) of supernatants were injected onto the column. Acetonitrile provided a more efficient precipitation than an equal volume of methanol, and, contrarily to phosphoric acid, did not adversely affect mobile phase pH. We could not find an internal standard with an appropriate retention time and suitable fluorescence properties. However, as long as acetonitrile was precisely measured and supernatant volumes precisely injected, the use of an internal standard was not essential.

CQ fluorescence being extremely pH-dependent, only an alkaline mobile phase allowed adequate sensitivity. Augustijns and Verbeke [21] had to rely on post-column alkalinization to achieve sufficient sensitivity. Our use of low percentages of triethylamine (TEA, 0.1%) served the double purpose of adjusting the apparent pH of the mobile phase to 10

and improving peak shape. Other research teams have used significantly higher concentrations of TEA in the mobile phase. Tett et al. [24] used methanol–water combinations with 100 m*M* of TEA, which required the use of a special pH-resistant column. Lowering TEA concentrations (0.0625%) increased retention times and improved the BDCQ–DCQ resolution (Fig. 2A vs. Fig. 2B).

This paper describes the first separation of CQ and its desethylated metabolites on a C_1 column. Using binary combinations of organic and aqueous solvents and varying TEA proportions, neither C_8 nor C_{18} columns allowed the two metabolites to be adequately separated. Indeed, Brown et al. [26], Augustijns and Verbeke [23] and Houze et al. [27] used a C_{18} column and all three analytes, CQ, DCQ and BDCQ, were eluted within 4 min. In normal-phase conditions, Si or CN columns allowed DCQ and BDCQ to be separated but the resolution was short-lived. After several days of injections, in spite of rigorous rinsing procedures, resolution was completely lost. Alvan et al. [21] used an all-organic mobile phase with a silica column and measured the unresolved summation of BDCQ and DCQ. Chaulet et al. [25]

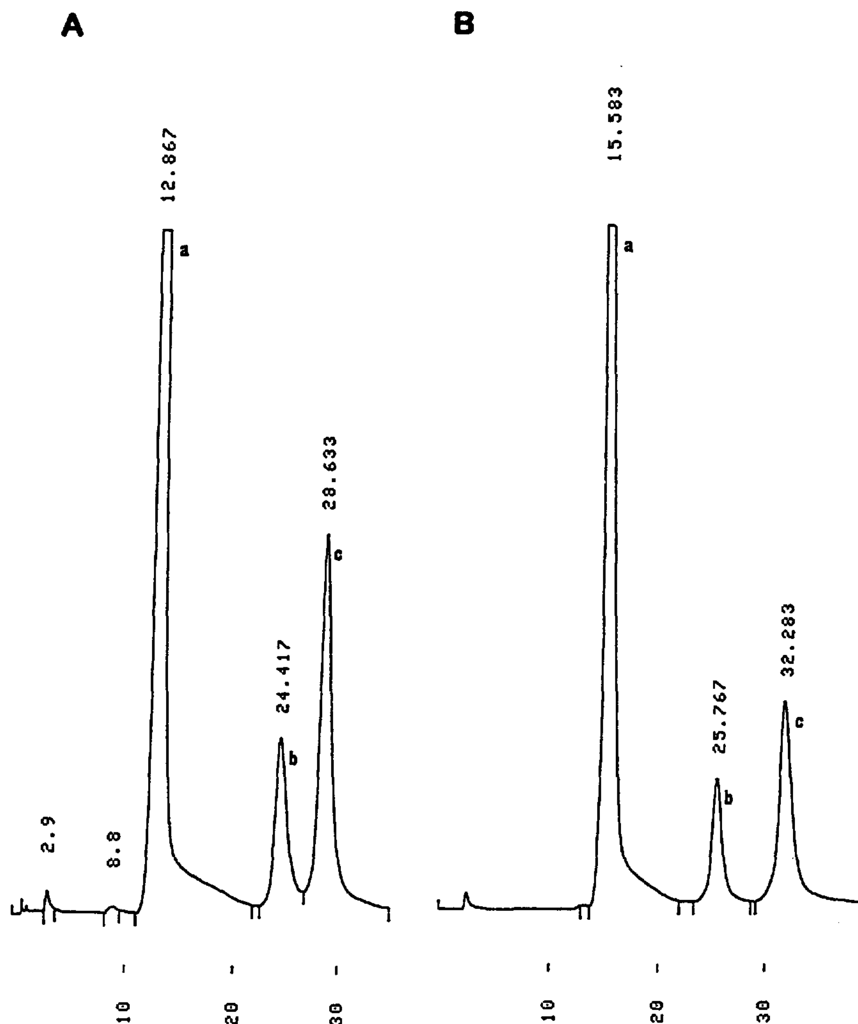

Fig. 2. Chromatograms of 15 μl of a solution containing 25 μ*M* of CQ, 2500 n*M* of BDCQ and 5000 n*M* of DCQ. The mobile phase contained 7m*M* (0.1%) (A) or 4.375 m*M* (0.0625%) (B) of triethylamine. a=CQ, b=BDCQ and c=DCQ.

also used an inert silica column but analytes had to be separated by gradient elution. Only the C_1 column combined optimum resolution with stable injection conditions.

Chromatograms of spiked solutions are presented in Fig. 2. Before each day, 15 μl of a solution containing 25 000 n*M* of CQ, 2500 n*M* of BDCQ and 5000 n*M* of DCQ was injected onto the column to verify that all analytes were adequately separated. Non-equal concentrations allowed the visual recognition of each analyte. They were chosen to be representative of incubation conditions, with an excess of the parent compound and higher concentrations of DCQ compared to BDCQ. CQ and its monodesethylated metabolite were separated by more than 15 min, allowing sufficient time for extremely high concentrations of the parent to be eluted. Solutions containing CQ:DCQ concentration ratios up to 1000:1 were successfully chromatographed.

Typical chromatograms of human liver microsomes incubated with or without CQ are presented in Fig. 3. Blank human liver microsomes were free from interfering peaks. When CQ was incubated, only DCQ was formed. Since BDCQ was formed when DCQ was incubated, its absence from CQ

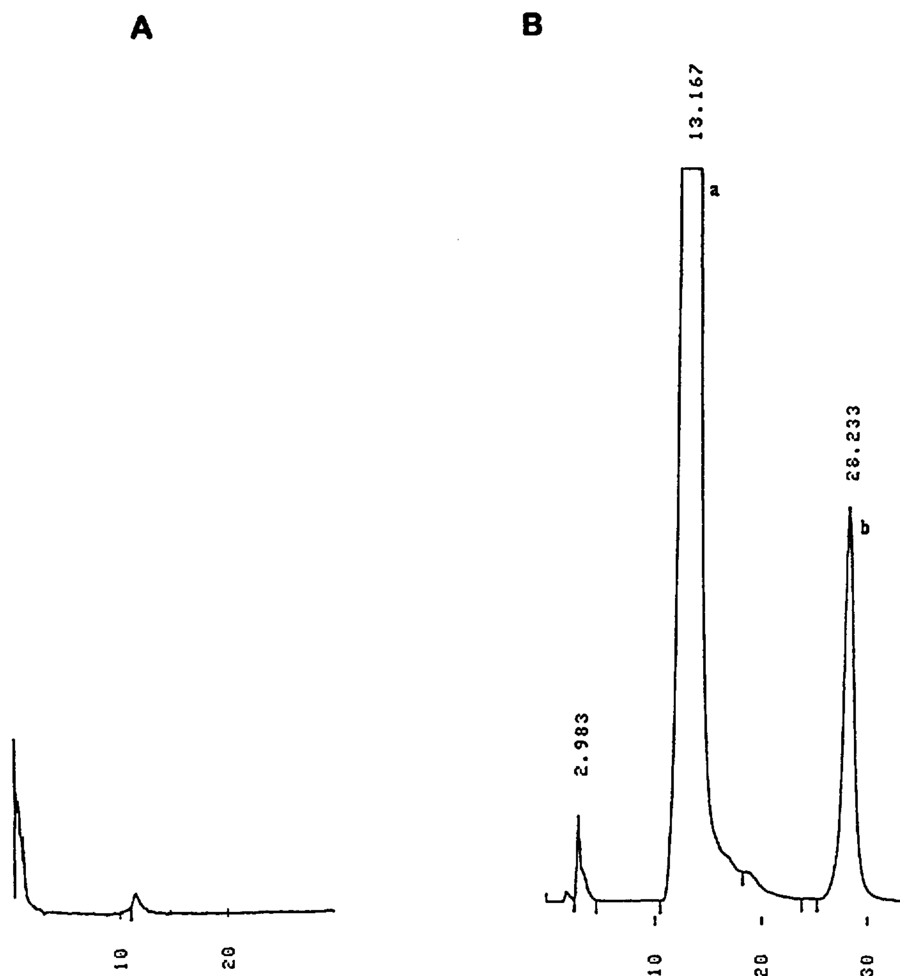

Fig. 3. Chromatograms of blank human liver microsomes (A) and of human liver microsomes incubated with 400 μ*M* of CQ (B). Incubates (200 μl) were diluted with ice-cold acetonitrile (200 μl) and 15 μl were injected directly onto the column. The mobile phase contained 7 m*M* (0.1%) of triethylamine. a=CQ and b=DCQ.

incubations might be due to the CYP-inhibiting properties of both CQ and DCQ [32–34]. Indeed, at CQ concentrations exceeding 3000 μ*M* product inhibition was observed in all tested livers (to be published). When samples from DCQ incubations were assayed, only 0.0625% of TEA (4.375 m*M*) was added to the mobile phase, to maximize the BDCQ–DCQ time interval. Since retention times were then considerably lengthened, and could not be shortened by increasing methanol proportions without losing resolution, these conditions were not chosen as routine. If, in a given sample, BDCQ could be detected, the complete series of microsomal incubates was reinjected with 0.0625% of TEA in the mobile phase.

Standard curves for DCQ and CQ were carried out with 0.1% of TEA in the mobile phase while those of BDCQ were assayed with 0.0625% of TEA. Also, calibration curves were prepared separately for each analyte, to avoid any bias from in vitro degradation. Since human liver microsomes are extremely valuable, standard curves were prepared in diluted plasma containing comparable protein concentrations. Blank plasma samples and blank human liver microsomes led to similar chromatograms, devoid of any interfering peaks. For all analytes, calibration curves were linear over the concentration range examined. To improve precision, two standard curves were constructed for each analyte. One for low concentrations (78–1250 n*M*, n=5 concentrations) and one for high concentrations (1250–20 000 n*M*, n=5 concentrations). For CQ, mean±S.D. correlation coefficients were 0.9967±0.0028 and 0.9996±0.0002, for the lower and higher range, respectively (n=4 standard curves). For DCQ, mean correlation coefficients were 0.9981±0.0019 and 0.9985±0.0027, for the lower and higher range, respectively (n=7 standard curves). For BDCQ, correlation coefficients averaged 0.9976±0.0008 and 0.9951±0.0079 (n=4 standard curves).

For all three analytes, the method was found to be highly reproducible (Table 1). For all analytes, intra-day variability was lower than 5% while inter-day variability did not exceed 11%. Intra-day variability averaged 2.9% for CQ, 1.5% for DCQ and 2.5% for BDCQ. Mean inter-day variability was 3.1% for CQ, 3.5% for DCQ and 3.7% for BDCQ. Accuracy never deviated from 100% by more than 11% (Table 1).

Table 1
Intra- and inter-day validation for CQ, DCQ and BDCQ in diluted plasma samples

Theoretical concentration (n*M*)	Intra-day		Inter-day		
	Measured concentration (mean n*M*±S.D.)	C.V. (%)	Measured concentration (mean n*M*±S.D.)	C.V. (%)	Accuracy (%)
CQ					
156	154±6	4.1	164±11	7.0	105
625	571±23	4.1	590±21	3.5	94
1250	1268±32	2.5	1263±8	0.6	101
5000	4999±53	1.1	4913±64	1.3	98
DCQ					
156	165±4	2.6	173±13	7.3	111
625	631±1	0.2	617±15	2.4	99
1250	1249±11	0.9	1255±7	0.5	100
5000	4902±113	2.3	4852±175	3.6	97
BDCQ					
156	145±7	4.9	166±18	10.6	106
625	605±19	3.1	594±14	2.3	95
1250	1263±11	0.9	1258±5	0.4	101
5000	4891±51	1.0	4849±63	1.3	97

Mean accuracies were 100, 102 and 100% for CQ, DCQ and BDCQ, respectively (Table 1). To minimize analytical variability, analyte concentrations in biological samples were always derived according to the same day standard curve.

For all analytes, 1 nmol could be detected on column. Low volumes (15 μl out of 400) were purposely injected into the system to minimize the injection of proteins onto the column and the peak area of the very high concentrations of incubated CQ. Under these conditions, the LOQ of each analyte was 78 n*M*. Taking into account differences in peak shapes (e.g., CQ peaks were sharper), all three analytes showed comparable fluorescence. This is consistent with their chemical structures, differing only by the presence or absence of ethyl groups on the side chain.

In our incubation samples of CQ (or DCQ), DCQ (or BDCQ) concentrations were always well above the LOQ. There was no interference from any of the co-incubated chemicals, including specific cytochrome P-450 inhibitors or substrates. Only quinidine and α-naphtoflavone showed some low fluorescence at the selected wavelengths. However, peaks were too small and eluted immediately after the solvent front, well before CQ, thus not interfering with the analytes.

To protect the analytical column from direct injections, a C_{18} guard column was installed, without prejudice to the method selectivity. The guard column was changed every 50–100 injections of biological material, when back-pressure was elevated. Samples could be continuously injected over 48 h without any loss of selectivity or sensitivity. Also, all analytes were stable for 48 h in the automatic sampler without the need for sample refrigeration. This was ensured by replicate injections of standard curves. After 4 months at −20°C, aqueous solutions of CQ did not show any degradation (peak areas accounted for 102–104% of the peak areas of freshly prepared solutions).

Typically, samples were injected for 24 h. After each day of analysis, the column was rinsed overnight with 70% acetonitrile in water at 0.5 ml/min. This ensured good column performance and avoided the accumulation of TEA in the system. As a result, retention times and peak areas were extremely stable between runs. The assay was successfully applied to the investigation of the microsomal isoforms implicated in CQ metabolism in vitro [17] and results will be published in a separate paper.

Clinically, CQ is administered as a racemic mixture of two enantiomers, *S*-(+)-CQ and *R*-(−)-CQ [11]. In vivo, when both enantiomers were separately administered to healthy volunteers, *S*-(+)-CQ had a shorter half-life than *R*-(−)-CQ [35]. Since a faster elimination could result from a faster urinary excretion and/or an increased hepatic metabolism, in vitro metabolism experiments could prove useful in characterizing the source of the stereoselectivity in CQ disposition. On that account, chiral assays recently developed for hydroxychloroquine [28,36], a structural analogue of CQ, could be adapted to the chiral analysis of CQ and DCQ in microsomal incubates.

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