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Comparison of the partitioning *in vitro* of chloroquine and its desethyl metabolites between the erythrocytes and plasma of healthy subjects and those with falciparum malaria

FUNMILAYO O. AJAYI, L. A. SALAKO* AND J. O. KUYE
Department of Pharmacology & Therapeutics, University of Ibadan, Ibadan, Nigeria

Summary

The partitioning of chloroquine and its two desethyl metabolites between red blood cells (RBCs) and plasma was studied *in vitro*, using blood from healthy adults and from children with *Plasmodium falciparum* parasitaemia. Blood from the volunteers was incubated with varying concentrations of chloroquine (CQ), desethylchloroquine (DCQ) and bisdesethylchloroquine (BDCQ) for 15 min and the RBC/plasma concentration ratio determined.

Desethylchloroquine and BDCQ were concentrated in the red cells of uninfected blood to the same extent as chloroquine. On the other hand, DCQ and BDCQ were concentrated to a significantly lower extent than CQ in the red cells from malarial children.

The reduced ability of infected RBCs to concentrate DCQ and BDCQ may have an important bearing on the development of resistance to chloroquine by *P. falciparum*.

Résumé

Le partage de la chloroquine et ses deux métabolites désethyl en cellules de sang rouge (RBCs) et plasma est étudié *in vitro*: utilisation est faite de sang d'adultes bien portant et d'enfants ayant des parasites *Plasmodium falciparum*. Nous avons incubé le sang des volontaires avec des concentrations variantes de chloroquine (CQ), de déséthylchloroquine (DCQ), et de bidéséthylchloroquine (BDCQ) pendant 15 min et avons déterminé la proportion de concentration RBC/plasma.

La déséthylchloroquine et BDCQ ont été concentrées, dans la même proportion que la chloroquine, dans les cellules de sang rouge non infecté. D'autre part, la DCQ et la BDCQ ont été considérablement moins concentrées que la CQ dans les cellules rouges d'enfants atteints de paludisme.

La capacité réduite des RBCs atteintes de concentrer la DCQ et la BDCQ peut avoir un rapport important sur le développement de la résistance de la chloroquine par le *Plasmodium falciparum*.

*To whom correspondence should be addressed.

Introduction

The main metabolites of chloroquine (CQ) in man are desethylchloroquine (DCQ), which constitutes about 25% of total quinolines, and bisdesethylchloroquine (BDCQ) which forms 4–6% of total quinolines [1].

It has been shown that CQ, DCQ and BDCQ are almost equally active on CQ-sensitive *Plasmodium falciparum in vitro* [2–4], but the metabolites are less active than the parent compound against CQ-resistant strains [2]. These observations suggest that CQ metabolites, particularly DCQ, might play some part in the antimalarial effect of administered CQ, and also in its toxicity.

In the plasma, CQ and DCQ have a concentration–time profile which is almost parallel from about 6 h after an oral dose of CQ, up to about 30 days, with the concentration of the metabolite being 40–60% of that of the parent drug [5]. Although BDCQ is identifiable on a liquid chromatogram of a plasma sample after

an oral therapeutic dose of CQ, its concentration is too low for pharmacokinetic analysis [5].

Chloroquine is concentrated in human red blood cells (RBCs) to between 3.5 times and 10 times the concentration in plasma, and the accumulation is further increased in red cells infected by CQ-sensitive malaria parasites [6]. The distribution of DCQ and BDCQ between RBCs and plasma is not known. The antimalarial action of CQ might be related more to the concentration in RBCs than in plasma since the parasite is intra-erythrocytic for most of its existence in the blood. It is therefore desirable to investigate the partitioning of DCQ and BDCQ between RBCs and plasma in comparison with the partitioning of CQ.

Material and methods

Blood used for the studies was obtained from two sources:

(1) healthy adults of both sexes aged between 18 years and 36 years; and

(2) children of both sexes aged between 6 years and 10 years suffering from parasitologically proven acute falciparum malaria.

The adults were staff and students of the University, while the children were patients attending the Oni Memorial Children's Hospital, Ibadan. The objectives of the study and its procedure were clearly explained to the adults and to the parents or guardians of the children, and they all voluntarily agreed to participate in it. The study was approved by the College of Medicine Ethical Committee. None of the adults had taken chloroquine for at least 3 months. Amongst the children, recent ingestion of CQ was avoided by selecting only those children whose urine was negative for CQ using the Dill–Glazko test. The mean parasite density in the children was 6434 ± 263 per μl (range 3740–9660/μl), and their mean packed cell volume (PCV) was 33.9% (range 15–45%). The mean PCV in the adult volunteers was 45% (range 35–49%). The subjects were all homozygous for HbA.

Preparation of blood samples

Venous blood was collected into heparinized containers and centrifuged for 5 min at 2500 r.p.m. (> 1000 *g*). The buffy coat was carefully aspirated to remove the white blood cells and platelets which are known to concentrate CQ to an even greater extent than erythrocytes [7]. The blood, minus the buffy coat, was then mixed gently by inversion and its PCV determined using a microhaematocrit.

Time course for the uptake of CQ and DCQ by erythrocytes in vitro

The aim of this preliminary series of experiments was to determine how long it took for red blood cell CQ and DCQ concentration to reach equilibrium when whole blood was incubated with these drugs. Three millilitres of blood were put in stoppered 10 ml glass tubes and spiked with 60 μl of a 10 μg/ml solution of CQ or DCQ, to give a concentration of 200 ng/ml of blood. The tubes were left to stand at room temperature for up to 30 min. They were gently mixed by inversion at intervals of 2–3 min during this period. At 5, 10, 15 and 30 min, four tubes were removed and centrifuged at 2500 r.p.m. for 5 min, and 1 ml RBC and 1 ml plasma were withdrawn for CQ or DCQ analysis.

Partitioning of CQ, DCQ and BDCQ between plasma and RBCs in vitro

Four millilitres of blood from adult volunteers were put in a 10 ml stoppered glass tube and spiked with 20, 40, 60, 80, 100 or 120 μl of a 10 μg/ml solution of CQ, DCQ or BDCQ, to give a concentration of 50, 100, 150, 200, 250 or 300 ng/ml of blood. Two tubes at each concentration were set up for each of the three compounds. The tubes were left to stand at room temperature for 15 min. During this period, the blood was gently mixed by inversion at intervals of 2–3 min. At the end of the 15 min incubation period, 1 ml of blood was taken from each tube for the determination of the concentration of the drugs in whole blood. The remaining 3 ml of blood were centrifuged for 5 min at 2500 r.p.m. One millilitre of plasma and 1 ml of RBCs was taken for the determination of the drugs in these fractions.

A similar study was performed using blood obtained from the malaria-infected children. Three millilitres of blood from each child were put in a tube and spiked with 30, 60 or 120 μl of a 10 μg/ml solution of CQ, DCQ or BDCQ to

give a concentration of 100, 200 or 400 ng/ml of the drug in whole blood. The tubes were left to stand at room temperature, with periodic mixing, for 15 min. Thereafter they were centrifuged and 1 ml of plasma and 1 ml of RBCs withdrawn for the analysis of CQ, DCQ or BDCQ.

Drug analysis

Chloroquine (CQ) and its metabolites were analysed in plasma, whole blood and RBCs using a modification of the fluorimetric method of Adelusi and Salako [8]. One millilitre samples were made alkaline with 1.0 M NaOH in 10% NaCl and extracted with dichloromethane. The organic layer was shaken with 0.1 M HCl, and the aqueous layer was then buffered to pH 9.8 using phosphate borate buffer. Fluorescence was read at an excitation wavelength of 335 nm and emission wavelength of 390 nm using a Perkin–Elmer 240 fluorescence spectrophotometer. The lower limit of sensitivity for each compound was 10 ng/ml, and the coefficient of variation of repeated analysis of the same sample was 15% at 20 ng/ml.

Reagents and chemicals

Working solutions of CQ and its metabolites were made in a 10 μg/ml solution of 0.9% NaCl when needed, from a stock solution of 1 mg/ml, which was kept in glass flasks at 4°C. Under these conditions, adsorption of CQ to glass was insignificant. All reagents were of analar grade, and water was deionized and double glass-distilled.

Data analysis

The partitioning of CQ and its metabolites between plasma and RBCs was determined from the RBC/plasma concentration ratio of the drugs.

Comparisons were made between different values using Student's *t*-test and *P* values of less than 0.05 were regarded as significant.

Results

The results of the preliminary incubation experiments showed that, for both CQ and DCQ, equilibrium was reached between RBCs and plasma after 15 min. An incubation period of 15 min was therefore used in subsequent studies, on the partitioning of the drugs between plasma and RBCs, in which equilibrium conditions were required. Total drug concentration at the end of the incubation was the same as at the beginning, showing that there was no instability of CQ and its metabolites during the incubation, and in particular, that adsorption to glass was negligible.

Uptake of CQ, DCQ and BDCQ by RBCs from healthy subjects

The partitioning of CQ and its metabolites between plasma and RBCs, at different concentrations of the drugs, in unparasitized whole blood is shown in Table 1. This shows that for all three compounds the RBC/plasma partition ratio was not dose-dependent. Similarly, Table 1 also shows that at all concentrations, and overall, the mean partition ratio for one drug was not significantly different from the value for the other drugs.

Uptake of CQ, DCQ and BDCQ by parasitized RBCs

Table 2 shows the RBC/plasma CQ, DCQ and BDCQ concentration ratios when blood from malaria-infected children was incubated with different concentrations of the drugs. For each drug, the partition ratio was not significantly different at the different doses. However, at each dose and also overall, the partition ratio for CQ was significantly higher than that of DCQ or BDCQ ($P < 0.01$). There was no significant difference between the partition ratios for DCQ and BDCQ at any concentration, or overall.

Discussion

All species of malaria parasites of the genus *Plasmodium* undergo the asexual phase of their life cycle inside the red blood cells. It is therefore to be expected that antimalarial drugs whose action is directed against the erythrocytic stage of the parasite should have the ability to enter red cells. Chloroquine is unique amongst

Table 1. Calculated partition ratios between normal RBCs and plasma for each concentration (n = 8), and for all the concentrations taken together (n = 48 or 32)

Compounds	Partition ratio* obtained at various concentrations (ng/ml)						All concentrations
	50	100	150	200	250	300	
CQ	4.09 ± 0.001	3.81 ± 0.003	3.67 ± 0.010	3.93 ± 0.010	3.87 ± 0.020	3.88 ± 0.005	3.87 ± 0.001
DCQ	3.66 ± 0.050	3.90 ± 0.100	4.16 ± 0.080	4.23 ± 0.200	4.18 ± 0.050	4.04 ± 0.007	4.03 ± 0.003
BDCQ	3.52 ± 0.400	3.17 ± 0.060	—	3.44 ± 0.040	—	3.61 ± 0.300	3.44 ± 0.003

*Values are given as mean ± s.e.

Table 2. Partition ratio of CQ, DCQ and BDCQ between parasitized erythrocytes and plasma

Compounds	Partition ratio* obtained at various concentrations (ng/ml)			
	100	200	400	All concentrations
CQ	28.30 ± 2.38 (n = 7)	30.09 ± 1.25 (n = 8)	29.23 ± 3.49 (n = 9)	29.21 ± 2.00 (n = 24)
DCQ	11.63 ± 1.12 (n = 8)	13.63 ± 2.25 (n = 7)	13.40 ± 1.00 (n = 8)	12.89 ± 1.2 (n = 23)
BDCQ	11.53 ± 1.88 (n = 8)	11.68 ± 2.43 (n = 7)	12.61 ± 1.62 (n = 8)	11.94 ± 1.40 (n = 23)

*Values are given as mean ± s.e.

blood schizontocidal antimalarial drugs in its high degree of concentration within erythrocytes. Other schizontocides, for example quinine (RBC/plasma concentration ratio (P) = 0.20) and mefloquine (P = 1.7), enter RBCs but are not concentrated to any great extent [9]. In man, parasitized erythrocytes concentrate CQ to a greater extent than erythrocytes which are free of malaria parasites [6]. This is also true of rodents in which it has been shown that red cells parasitized by CQ-sensitive *P. berghei* concentrate CQ more than those parasitized by CQ-resistant plasmodia [10,11]. Our present study has confirmed *in vitro* observations made *in vivo* of the concentration of CQ by human erythrocytes. The mean partition ratio between normal RBCs and plasma in this study (3.7 ± 0.4) is similar to the RBC/plasma CQ concentration ratio of 5.3 obtained by Adelusi *et al.* [6] in children recently recovered from acute falciparum malaria. During infection the RBC/plasma CQ concentration ratio of 21.0 was significantly higher than the value after recovery, but is similar to the value of 29.2 obtained in this study *in vitro* using blood with malaria parasite densities of between 0.07 and 0.19%.

The results of this study show that DCQ and BDCQ are concentrated in RBCs to the same extent as CQ when blood from healthy subjects is used. However, when the blood is obtained from patients with malaria parasitaemia, the extent to which DCQ and BDCQ are concentrated is less than that of CQ.

Salako and Ajayi [12] have shown that in rats, DCQ and BDCQ are concentrated in tissues like the liver, spleen, kidney, lungs and heart to approximately the same extent as CQ. The present observation that the three compounds are also similarly concentrated in human erythrocytes *in vitro* shows that chloroquine and its desethylmetabolites have similar pharmacokinetic characteristics.

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