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A study of chloroquine and desethylchloroquine plasma levels in patients infected with sensitive and resistant malaria parasites

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Abstract: This study was carried out on 63 patients in the town of Gadaref in eastern Sudan; each patient was given the standard therapeutic dose of chloroquine (CQ). Plasma levels of chloroquine and its major metabolite desethylchloroquine (DCQ) were measured by means of a high-performance liquid chromatographic method (HPLC) in patients infected with sensitive (sensitive group) and resistant (resistant groups) strains of *Plasmodium falciparum*. The ratios of chloroquine to desethylchloroquine (CQ/DCQ) in different groups were calculated and the results obtained were compared and correlated with the degree of parasitaemia. The statistical analysis of the results showed that the plasma content of CQ and the CQ/DCQ ratio in the majority of the patients fall within the normal mode of distribution. A small group of patients showed a deviation from the normal mode by having a rather high CQ plasma level and a high ratio of CQ/DCQ. The mean plasma levels of CQ and the CQ/DCQ ratio in the sensitive group was found to be higher than that in the resistant groups. However these differences were found to be not significant. Correlation tests showed that the levels of CQ and the CQ/DCQ ratios increase with the increase of the degree of parasitaemia in the sensitive group but decrease with the increase of parasitaemia in resistant groups.

Keywords: *HPLC; chloroquine; resistance; malaria.*

Introduction

Chloroquine (CQ) is still the drug of choice and the most extensively used anti-malarial in the Sudan. Malaria is predominantly caused by *Plasmodium falciparum* which accounts for about 90% of positive cases of the disease [1]. Recently, some cases of resistance of *P.falciparum* to treatment with CQ have been reported [2]. CQ-resistant malaria is claimed to be on the increase in certain areas in the country [3]. The possibility that CQ in different brands and dosage forms is decomposed or is present in sub-dosage has been investigated in the authors' laboratories. Almost all preparations were found to contain the stated amounts of the drug and no decomposition products were detected. Bioavailability studies on CQ [4] had also indicated satisfactory results. Factors causing CQ-resistant malaria are to date not fully accounted for [5]. Since good grades of CQ preparations with high bioavailability are used by the patients, it was thought necessary to study the plasma levels of the drug and its major metabolite in patients infected with sensitive and resistant groups of the *P. falciparum* malaria parasite. A comparison between the results obtained for the two groups is believed to indicate whether or not resistance is in some way related to changes in the metabolic pattern of the drug by patients not responding to treatment.

Materials and Methods

Materials

Chloroquine (CQ), desethylchloroquine (DCQ) and 6,8-dichloro-4-(1-methyl-4-diethylamino-butylamino)quinoline were obtained from Sterling Winthrop (UK); disodium hydrogen phosphate and phosphoric acid were Analar grade obtained from BDH (UK); heptanesulphonic acid, diethylamine and acetonitrile (HPLC) were obtained from Aldrich (UK).

Clinical study

Plasma samples were collected from patients

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in the town of Gadaref in eastern Sudan and frozen (−20°C) until analysis. All patients admitted to the study were infected with *P. falciparum* only and has an asexual parasite count of 1000–70,000 μl^{-1} blood. Sixty-three patients consented to take part in the study. The patients were classified (WHO criteria) into a sensitive group and resistant groups RI, RII and RIII. Each patient was given 25 mg kg^{-1} body wt of CQ base (Bayer) as tablets in three oral doses: 10 mg kg^{-1} body wt on each of day 0 and day 1 and 5 mg kg^{-1} body wt on day 2. The Dill–Glazko urine test for the detection of 4-aminoquinolines was carried out for all patients on day 0; for those who had a negative test, the test was repeated on day 1. Thick and thin blood smears were taken each morning and evening from all patients for 8 days (WHO 7-day test).

Chromatographic method

Venous blood (5 ml) was collected in lithium–heparin tubes from each patient, on day 0 (2–8 h after the first dose of CQ) and on days 3 and 7. The plasma was immediately separated and stored at −20°C until analysis. Samples were analysed for CQ and DCQ by a modification of the reversed-phase HPLC method described by Bergqvist and Frisk-Homberg [6]. The HPLC system used comprised an LDC Constametric R 300 pump (LDC Ltd, Stone, UK) with a Rheodyne (Model 7125) loop valve injector with a 20-μl loop. The detector was a single channel LDC Spectromonitor 3100 R variable wavelength UV detector connected to a BBC recorder. Measurements were made at 343 nm (max of CQ and DCQ). A stainless steel chromatographic column (250 × 4.6 mm i.d.) packed with Spherisorb S5 ODS1 (Anachem) was used. The mobile phase was phosphate buffer (0.1 M, pH 3.5)–acetonitrile (70:30, v/v). Heptanesulphonic acid (0.005 M) was used as an ion-pairing agent and diethylamine (1%, w/v) as a modifier base. The flow rate was 1 ml min^{-1}. All measurements were made at ambient temperature and solutions were protected from light.

Extraction procedure

The extraction procedure described by Ogunbona *et al.* [7] was adopted and modified. To 0.5 ml of plasma, the following solutions were added: sodium hydroxide (2 M, 0.5 ml); saturated sodium chloride solution (0.5 ml), *iso*propanol (0.2 ml); and a solution of the internal standard, 6,8-dichloro-4-(1-methyl-4-diethylamino-butylamino)-quinoline (D; 3 μg ml^{-1}, 10 μl). The mixture was shaken with hexane (3 ml) for 2 min and centrifuged at 8000 rpm for 10 min. The hexane layer (2 ml) was separated and extracted with the mobile phase (0.2 ml). The extract (50 μl) was analysed by the HPLC method.

Results and Discussion

Resistance to treatment of malaria with CQ is on the increase in the Sudan [3]. Some reports on CQ-resistant malaria suggested that resistance to CQ could be due to expulsion of the drug by the parasite [8, 9]. Verdier *et al.* [10] demonstrated that the uptake of CQ by red blood cells (RBCs) infected with resistant malaria parasites was lower than in those infected with sensitive parasites. This decrease in the uptake was found to give a higher concentration of CQ in the culture medium than in the RBCs. Fitch [11], using an *in vivo* method, reported results similar to those demonstrated by Verdier *et al.* [10].

In view of these findings it was thought necessary to measure the levels of CQ and its major metabolite DCQ in the plasma of patients infected with sensitive parasites and in the plasma of others infected with resistant parasites. A comparison between the results was believed to indicate the mechanism of resistance. The results were also thought to reveal whether there is some relationship between resistance and metabolism of the drug.

Measurement of CQ and DCQ plasma levels was accomplished by using a modification of the reversed-phase HPLC method used by Bergqvist and Frisk-Homberg [6]. Heptanesulphonic acid was used instead of potassium perchlorate as an ion-pairing agent, and diethylamine was used as a modifier base. This modification proved to give better chromatographic results. The internal standard (D) used, has a longer retention time than that of CQ which allowed for a more accurate measurement of the metabolite with no interference (Fig. 1). Calibration curves for CQ and DCQ were prepared using CQ-free plasma spiked with the internal standard (0.3 μg ml^{-1}) and a concentration range of 0.025–0.4 μg ml^{-1} of both the drug and the metabolite. The recovery of both CQ and DCQ was found to be

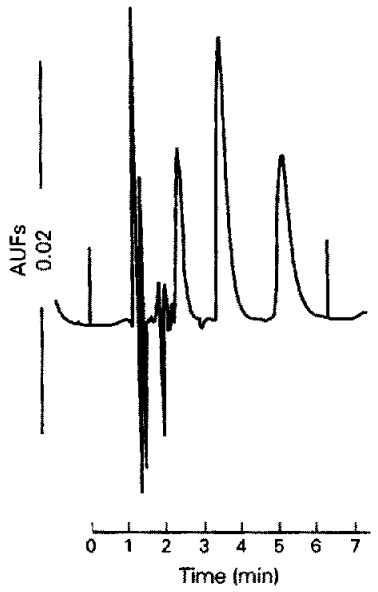

Figure 1
Chromatogram of a plasma extract from a patient, 3 days after intake of an oral dose of CQ (25 mg kg^{-1} body wt). Peaks in ascending retention time order: DCQ, CQ and the internal standard (D).

90% and their lower limit of detection was about 10 ng. The results obtained showed linear relationships over the concentration ranges used: the regression equation obtained for CQ was $y = 3.04x + 0.06$ ($r = 0.997$, $n = 40$) and that for DCQ was $y = 6.34x + 0.03$ ($r = 0.998$, $n = 40$). There was no significant difference in between determinations. The precision of repetitive assays at concentrations of 0.3 and 0.05 μg ml^{-1} was RSD = 0.9 and 1.5%, respectively for CQ, and 1.2 and 1.8% for DCQ.

The results obtained revealed that lower CQ plasma concentrations (0.235 ± 0.05 μmol l^{-1}) were attained in the resistant groups (combined RI, RII and RIII, thereafter referred to as Rall) compared with the sensitive group (0.275 ± 0.09 μmol l^{-1}). A large variation was observed in the concentration of CQ in the plasma of the different groups of patients (RSD = 20–35%). The distribution curves of the plasma CQ level (sampled on day 3) for all the groups showed positive skewness and platykurtic curves (Table 1). The results revealed that plasma CQ levels in the marjority of patients (83%) fall within the normal mode of distribution (0.21 ± 0.04 μmol l^{-1} for the resistant group and 0.339 ± 0.06 μmol l^{-1} for the sensitive group). A small group of patients (18%) deviates from the normal mode by

Table 1
The mean plasma levels* of CQ and normality test of its distribution in the sensitive and resistant groups on day 3

Type	Mean	SD	RSD (%)	Skewness	Kurtosis
S	0.275	0.098	35.6	0.357	0.953
RI	0.252	0.065	25.7	0.230	1.334
RII	0.223	0.045	20.1	0.066	1.480
RIII	0.225	0.073	32.4	0.780	1.587

*Units = μmol l^{-1}.

attaining rather a high plasma concentration (0.336 ± 0.04 μmol l^{-1} for the resistant group and 0.43 ± 0.04 μmol l^{-1} for the sensitive groups).

To determine whether there is any difference between CQ plasma levels in the sensitive and resistant groups, significance tests were carried out (F and t-tests, $P = 0.05$). No significant difference was observed in CQ plasma levels between the sensitive and the resistant group (Rall). However, a significant difference was observed when the CQ plasma level in the sensitive group and the resistant group type RIII ($n = 8$) were compared (pooled variance of estimate, two-tailed probability test, 28 df, t-value = 2.19, tabulated t-value = 2.04). The resistant group type RIII showed lower CQ plasma levels than the sensitive group (0.225 ± 0.07 μmol l^{-1} cf. 0.275 ± 0.098 μmol l^{-1}). These results, which are not in agreement with those reported by Fitch [11] and Verdier *et al.* [10], suggest that the plasma CQ level decreases as as a result of the increase in the uptake of the drug by RBCs infected with resistant parasites. The increase in the uptake of CQ by RBCs infected with resistant strains of malaria parasites does not overrule the decrease in the uptake of the drug by the resistant parasite itself [7, 8].

For confirmation of the results obtained in the present study, the CQ plasma levels obtained were correlated with the degree of parasitaemia in the different groups of patients. A slight positive correlation was obtained for the sensitive group ($r = 0.014$) and the resistant group type RI ($r = 0.26$). On the other hand, a slight negative correlation was obtained for the resistant groups type RII ($r = -0.18$) and RIII ($r = -0.508$). However, when the test was carried out for the sensitive group and the resistant group with a negative urine test for CQ on day 0, a positive correlation was obtained for the sensitive group ($y = 7164x - 5002$, $r = 0.32$, $n = 6$) and a

negative correlation was obtained for the resistant group ($y = -435x + 110$, $r = -0.575$, $n = 5$). These results suggest that the concentration of CQ in the plasma of the resistant group is lower than in the sensitive group. The extent of this difference depends on the degree of parasitaemia.

For confirmation of these findings, measurement of CQ levels in both plasma and RBCs of both sensitive and resistant groups will be essential. Such a comparative study will reveal any differences in the uptake of CQ by RBCs infected with sensitive and resistant parasites.

To determine whether there is any difference in the metabolism of CQ in the sensitive and resistant groups, DCQ plasma levels were measured and the ratio CQ/DCQ was calculated for each group and compared (Table 2). The ratio CQ/DCQ has been used in this study to eliminate factors such as absorption, which might lead to changes in the concentration of the metabolite as a result of differences in the attained CQ plasma level.

The mean plasma CQ/DCQ ratio in the sensitive group was found to be higher than that in the resistant group RIII (3.139 ± 1.2 cf. 3.05 ± 1.2). However, the test of significance has shown that the difference in the ratio CQ/DCQ between the sensitive and the resistant group was not significant.

Correlation tests have shown that the plasma ratio CQ/DCQ increases with the parasite count in the sensitive group and only slightly decreases in the resistant group. The slight decrease of the ratio in the latter case could be attributed to increased drug uptake by the RBCs infected with resistant parasites or to increased CQ metabolism.

The distribution of the ratio in the sensitive and resistant groups showed positive skewness and platykurtic curves (Table 2). The results showed that the majority of the patients (78%, Fig. 2) had a CQ/DCQ ratio which falls within the normal mode of distribution (2.68 ± 0.56 for the resistant group and 2.72 ± 0.58 for the sensitive group). A small group of patients (22%) deviated from the normal mode by having rather a high plasma ratio of CQ/DCQ (5.12 ± 0.66 for the sensitive group and 4.9 ± 0.7 for the resistant group). The high plasma CQ/DCQ obtained in a small group of patients could be attributed to some metabolic differences in the de-ethylation of CQ and/or the CQ elimination rate.

Table 2
The mean CQ to DCQ ratio (CQ/DCQ) and normality test of its distribution in the sensitive and resistant groups on day 3

Type	Mean	SD	RSD (%)	Skewness	Kurtosis
S	3.139	1.202	38.2	0.995	0.953
RI	3.214	0.951	20.2	0.517	1.334
RII	3.506	1.460	41.6	0.916	1.400
RIII	3.057	1.207	39.5	0.986	1.481

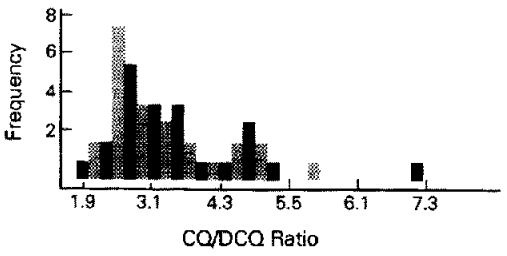

Figure 2
Frequency distribution of CQ/DCQ plasma levels of patients infected with malaria parasites.

For a further in-depth look into these differences pharmacokinetic studies will be more appropriate and it is suggested that such studies be carried out in a large group of patients.

Although the plasma level of CQ decreases with the increase of the resistant parasite count, therapeutic levels were found to be attained in almost all patients. From the results of this study it thus seems reasonable to suggest that insufficient amounts of the drug could reach and/or penetrate the resistant parasite.

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