

## THE PHARMACOKINETICS OF CHLOROQUINE IN HEALTHY AND *PLASMODIUM CHABAUDI*-INFECTED MICE : IMPLICATIONS FOR CHRONOTHERAPY

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### Summary :

The schizogony of malarial parasite is a typical cyclic phenomenon where the different stages of parasite development appear at regular time intervals. Each of the stages is specifically sensitive to different antimalarial drugs. Knowledge of the details of the cycle, drug susceptibility and the pharmacokinetics of drugs, could allow the improvement of drug action by the chronotherapeutic approach: treatment at the time of appearance of the drug-sensitive stage with a drug that displays rapid pharmacokinetics. Since murine malarial mice serve as preferable models for *in vivo* drug testing, the pharmacokinetics of subcutaneously (sc) administered chloroquine (CQ) were tested in the whole blood of healthy mice and in animals slightly (1.5-3.5 % parasitemia) or heavily infected (21-25 % parasitemia) with *Plasmodium chabaudi chabaudi*. The half-time of absorption was around 5 min and almost independent of parasitemia. The apparent half-time of drug concentration decay was around 40 min in healthy animals, about 90 min at low parasitemia and about 410 min in heavy infection, indicating that the concentration of CQ is a typical spike, that is prolonged by asymptomatic disease, and considerably more by the active accumulation of CQ in infected cells. The latter is confirmed by the 3-fold higher peak blood [CQ] at the trophozoite stage and <1.5-fold increase during schizogony. In conjunction with our previous experiments which showed that a single sc injection of 5 mg/kg CQ is sufficient to eliminate the drug susceptible mid-term trophozoite stage, the present results seem to justify to propose the chronotherapeutic approach for the treatment of malaria.

**KEY WORDS :** *Plasmodium chabaudi*. mouse malaria. chloroquine. pharmacokinetics. chronotherapy.

**MOTS CLES :** *Plasmodium chabaudi*. paludisme murin. chloroquine. pharmacocinétique. chronothérapie.

### Résumé : PHARMACOCINÉTIQUE DE LA CHLOROQUINE CHEZ LA SOURIS SAINES ET INFECTÉE PAR *PLASMODIUM CHABAUDI* : APPROCHE CHRONOTHÉRAPEUTIQUE

La schizogonie du parasite responsable du paludisme est un phénomène cyclique typique au cours duquel les différents stades du développement parasitaire apparaissent à des intervalles de temps réguliers. Chacun des stades est sensible de manière spécifique aux différents antimalariques. La connaissance détaillée de ce cycle, de la sensibilité au médicament et de la pharmacocinétique de ces médicaments, pourrait permettre l'amélioration de l'action médicamenteuse par une approche chronothérapeutique : traitement, au moment de l'apparition du stade sensible au médicament dont l'absorption et l'élimination seraient rapides. Le paludisme murin est utilisé de manière préférentielle comme modèle pour les études médicamenteuses *in vivo*. C'est pourquoi la pharmacocinétique de la chloroquine (CQ), administrée par voie sous-cutanée (sc), a été étudiée dans le sang total de souris saines et faiblement (parasitemie : 1,5 à 3,5 %) ou fortement (parasitémie : 21 à 25 %) parasitées par *Plasmodium chabaudi chabaudi*. La demi-vie d'absorption était de 5 mn et pratiquement indépendante du niveau de la parasitémie. La demi-vie de décroissance apparente des concentrations de CQ était de 40 mn chez les souris saines, 90 mn chez celles faiblement parasitées et 410 mn chez celles fortement parasitées. Ces résultats montrent que la concentration de CQ, déjà prolongée par la maladie asymptomatique, l'est encore beaucoup plus, par l'accumulation de CQ dans les cellules infectées. Ceci est confirmé par un pic de concentration de CQ 3 fois plus élevé au stade trophozoite et moins d'une fois et demi, au cours des stades ultérieurs. Les résultats de cette étude confirment ceux de nos expériences précédentes qui montraient qu'une seule injection sc de 5 mg/kg de CQ suffit à éliminer le parasite au stade trophozoite moyen, et semblent donc pouvoir justifier la proposition d'une approche chronothérapeutique pour le traitement du paludisme.

## INTRODUCTION

The recent worldwide resurgence of malaria stems mostly from the inadvertent evolution of parasite resistance to currently used drugs

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(Wernsdorfer, 1991). While new drugs are under development and clinical testing, it seems worthwhile to consider ways and means to improve the use of existing drugs. The asexual development of the parasite inside the erythrocytes of their vertebrate host is the cause of the pathological symptoms of malaria. Most currently used antimalarial drugs, including chloroquine (CQ), are schizontocides, i.e., they act on this phase of the cell cycle. While the precise mode of action of CQ is not yet known, the alluded selective toxicity of CQ to the different stages of the erythrocytic cycle, could, in principle, be used in conjunction with the features of the drug's pharmacokinetics, to improve the therapeutic treatment of the disease.

Consider the following reasoning (Landau *et al.*, 1991) : a) CQ injected (in man) either sc or im, rapidly appears in the blood, reaching maximal concentration within 30 min. This is followed by a polyexponential decline of drug concentration in the blood. The maximal concentration reached with parenteral administration is at least 5-fold higher than the mean drug level obtained by oral administration and during the slow terminal exponential elimination of the drug, and the duration of the concentration spike at halfmaximal peak concentration is ca. 80 min (White *et al.*, 1987). b) CQ is demonstrably cytotoxic. Temporary exposure of parasites to CQ is sufficient to elicit an irreversible and full inhibitory effect, at least on *Plasmodium falciparum* in culture (Krugliak and Ginsburg, 1991). c) The trophozoite stage is the one most susceptible to the drug both in murine malaras (Cambie *et al.*, 1991) and in falciparum malaria *in vivo* (Landau *et al.*, 1992) and in culture (Yayon *et al.*, 1983; Krugliak and Ginsburg, 1991), while the mature schizont and the merozoite stages (Langreth *et al.*, 1978) are probably not and the ring stage is only partially susceptible (Cambie *et al.*, 1991). Since different species of malaria parasites, including those affecting humans, develop in a synchronous fashion, timing of drug treatment to the appearance of the trophozoite stage, could result in a much more efficient therapeutic outcome. Such chronotherapeutic approach would seem to allow the rational treatment of CQ-resistant malaria since sub-toxic drug injections would nevertheless result in temporary high blood drug levels that could be sufficient to eliminate the parasites. Although it has been shown that parenteral administration of CQ in man, aimed to achieve a desired steady-state blood concentration, may result in transiently high CQ concentrations that may cause acute toxicity, the effect of lower single doses on the resolution of infection was not evaluated, and the protocol of these experiments did not take into account the stage of parasite development during injection.

Although murine malaras serve as preferable *in vivo* models for drug testing, the pharmacokinetics of CQ in the mouse has not been investigated hitherto. In the present work, the pharmacokinetics of CQ in healthy and *P. chabaudi*-infected mice has been studied with the aim of developing an animal model that could be used for investigating the paradigm of malaria chronotherapy outlined above.

## MATERIALS AND METHODS

**M**ale Swiss white mice, SWISS OFQI (Charles River) weighing 20-25 grs were used throughout. For day drug dosing, mice were

born and grown at a normal day/night regime (light from 8 am to 8 pm). For night drug dosing, mice born and grown at a day/night cycle that has been shifted by 12 hrs (light from 8 pm to 8 am) were used. Mice were infected with *P. chabaudi chabaudi* (strain 262YL) by intraperitoneal injection of 0.1 ml of thawed cryopreserved infected blood. The parasitemia was determined by microscopic inspection of Giemsa-stained thin blood smears made from a drop of caudal vein blood. CQ diphosphate (Sigma) solutions were prepared in sterile saline and injected subcutaneously into uninfected (5, 10 and 50 mg/kg) and infected mice (5 mg/kg). Day treatment and blood sampling was performed when the majority of the parasites were at the mid-trophozoite stage, whereas night treatment and sampling was done at the time of schizogony, i.e. when parasites were at the last schizont or ring stages. Eight whole blood 50  $\mu$ l samples were taken through the retro-optical sinus by means of a calibrated and heparinized glass pipette, at different time intervals starting 5 minutes after drug injection and up to 120 or 240 min. Zero-time (base level) samples were taken immediately prior to drug injection. Samples were held on ice till the end of collection and then frozen to -80°C. CQ concentration in the whole blood samples was determined by HPLC as previously described (Pussard *et al.*, 1986).

In order to study the effect of parasitemia and parasite stage on the pharmacokinetics of CQ, six groups of mice were constituted: Healthy day(HD), and healthy night-treated mice (HN); slightly infected (1.5-3.5 parasitemia) day- (LPD) and night-treated mice (LPN); heavily infected (20-25 % parasitemia) day- (HPD) and night-treated mice (HPN). Although 4-5 mice were in each group, in some groups data could not be calculated for all individuals of the group.

## DATA ANALYSIS

**D**ata analysis was performed by means of non-linear regression analysis of whole blood CQ concentration vs time, fitting the data to a one-compartment model by the equation  $C_p = F/V_d * D * K_a * (e^{-k_a * t} - e^{-k_e * t}) / (K_a - K_e)$ . F is the fraction absorbed into the blood compartment from the subcutaneous site of injection, V<sub>d</sub> is apparent the volume of distribution, K<sub>a</sub> and K<sub>e</sub> are the absorption and the elimination rate constants, and D is the dose injected. The half time of absorption and elimination were calculated as  $t_{1/2} = \ln 2 / K$ , where K is equal to K<sub>a</sub> and K<sub>e</sub>, respectively. The area under the curve (AUC) was calculated by the trapezoidal rule, and the time to peak drug concentration was calculated as  $t_{max} = \ln(K_e / K_a) / (K_e - K_a)$ .

Results were expressed by mean  $\pm$  s.d. Dose effect was studied by linear regression analysis. Variance analysis by the Newman-Keuls test was done for each parameter in order to find significant differences between results obtained from healthy day- and night-treated mice, whether healthy, slightly or heavily parasitized.

## RESULTS

Representative single experiments are depicted in Figure 1 with the respective best-fit curves. The results of the pharmacokinetic analyses are compiled in Table I, and simulated curves were generated with the means of the relevant parameters and shown in Figs. 2 and 3. The effect of 3 doses (5, 10 and 50 mg/kg) was studied in day-treated healthy mice. Analysis was performed on data taken from 0 to 120 min after drug injection. No differences were observed in  $K_a$  (or  $t_{1/2a}$ , around 5 min),  $t_{max}$  (15-19 min), or  $K_e$  ( $t_{1/2e}$ , 36-61 min) for the 3 doses. The peak blood concentrations ( $C_{max}$ , Fig. 4) and the  $AUC_{0-120}$  increased linearly with the dose ( $136.5 \pm 55.3$ ,  $222 \pm 114$  and  $1,045 \pm 130$   $\mu\text{mol min/l}$ ,  $p < 10^{-5}$ , for 5, 10 and 50 mg/kg, respectively).

The time of drug injection to healthy mice had no effect on pharmacokinetics in as much as the parameters at day and at night were indistinguishable (Table I).

As shown in Figs. 2 (day-treated) and 3 (night-treated), already a slight infection has a tendency to

change the concentration time-profile ( $K_e$  decreased significantly,  $p < 0.05$ ), though no differences could be observed between day and night treatment. All other parameters were similar when comparing healthy and slightly parasitized animals.

High parasitemias had a much more pronounced effect on the pharmacokinetics of CQ : There was a marked and significant increase in  $t_{1/2e}$  ( $p < 0.05$ ), the time to peak drug concentration ( $t_{max}$ ) and in the values of AUC and  $C_{max}$  ( $p < 0.01$ ). The AUC in the day-treated mice was more than twice higher than in night-treated mice ( $p < 0.01$ ).

## DISCUSSION

The pharmacokinetics of CQ has been studied beforehand in a variety of animals, including healthy and *P. falciparum*-infected humans. In general, the half-life of drug elimination decreases with the size of the animal, being in the range of days in man (Frisk-Holmberg *et al.*, 1984), and in the scope of hours for the dog (Aderounmu & Fleckenstein, 1983) or the rabbit (Salako & Adelus, 1983). To the best of our knowledge, only in one publication the blood levels of CQ in mice injected sc with 30 mg/kg daily for 7 days, were reported (Thomson *et al.*, 1967), but the kinetic analysis is incomplete. The relationship between elimination time and body size has also been observed with ampyroquine. We have studied the kinetics of ampyroquin, a 4-aminoquinoline antimalarial drug

Group	Dose	Parasitemia	F/Vd	$K_a$	$t_{1/2a}$	$K_e$	$t_{1/2e}$	$t_{max}$	$C_{max}$	AUC
HD	5	0	12.7	0.14	6.0	0.0197	39.9	17.7	1,970	136.6
	n=4		$\pm 3.3$	$\pm 0.07$	$\pm 2.3$	$\pm 0.0068$	$\pm 14.3$	$\pm 3.0$	$\pm 611$	$\pm 55.3$
HD	10	0	12.1	0.18	4.6	0.023	36.3	14.8	3,679	221.9
	n=4		$\pm 3.1$	$\pm 0.09$	$\pm 1.5$	$\pm 0.011$	$\pm 12.5$	$\pm 3.5$	$\pm 510$	$\pm 116.0$
HD	50	0	7.44	0.15	4.68	0.011	61.2	18.8	13,090	1,045.0
	n=3		$\pm 0.77$	$\pm 0.02$	$\pm 0.49$	$\pm 0.0003$	$\pm 1.9$	$\pm 1.54$	$\pm 1,205$	$\pm 130.4$
HN	5	0	14.6	0.11	6.49	0.0203	36.7	19.29	2,141	149.0
	n=5		$\pm 2.81$	$\pm 0.02$	$\pm 1.31$	$\pm 0.0046$	$\pm 11.4$	$\pm 2.49$	$\pm 308$	$\pm 11.5$
LPD	5	2.46	10.5	0.18	3.79	0.0074	104.7	18.59	2,005	196.4
	n=4	$\pm 0.93$	$\pm 2.56$	$\pm 0.01$	$\pm 0.22$	$\pm 0.0025$	$\pm 33.9$	$\pm 2.04$	$\pm 499$	$\pm 65.5$
LPN	5	1.82	9.12	0.18	4.0	0.0083	84.57	18.47	1,711	137.3
	n=3	$\pm 0.16$	$\pm 1.04$	$\pm 0.02$	$\pm 0.49$	$\pm 0.0007$	$\pm 7.35$	$\pm 2.05$	$\pm 207$	$\pm 20.2$
HPD	5	22.0	30.7	0.09	7.60	0.0047	407.14	45.48	6,296	566.9
	n=2	$\pm 0.7$	$\pm 3.55$	$\pm 0.01$	$\pm 0.70$	$\pm 0.0037$	$\pm 324$	$\pm 1.39$	$\pm 645$	$\pm 106.4$
HPN	5	22.78	17.0	0.12	6.71	0.0027	423.6	35.39	3,235	218.7
	n=2	$\pm 1.7$	$\pm 7.31$	$\pm 0.05$	$\pm 2.75$	$\pm 0.0017$	$\pm 262.5$	$\pm 5.72$	$\pm 1,196$	$\pm 10.6$

Table I. – Parameters of the pharmacokinetics of chloroquine in healthy and parasitized mice, dosed and sampled at day or at night. Group designation : HD – healthy mice, dosed at day; HN healthy mice dosed at night; LPD – low parasitemia, dosed at day; LPN – low parasitemia, dosed at night; HPD – high parasitemia, dosed at day; HPN – high parasitemia, dosed at night. n – number of mice. Units : all rate constants are given in  $\text{min}^{-1}$  and half times and  $t_{max}$  in min;  $C_{max}$  –  $\text{nmol/l}$ ; AUC –  $\mu\text{mol min/l}$ ; F/Vd – l/kg.

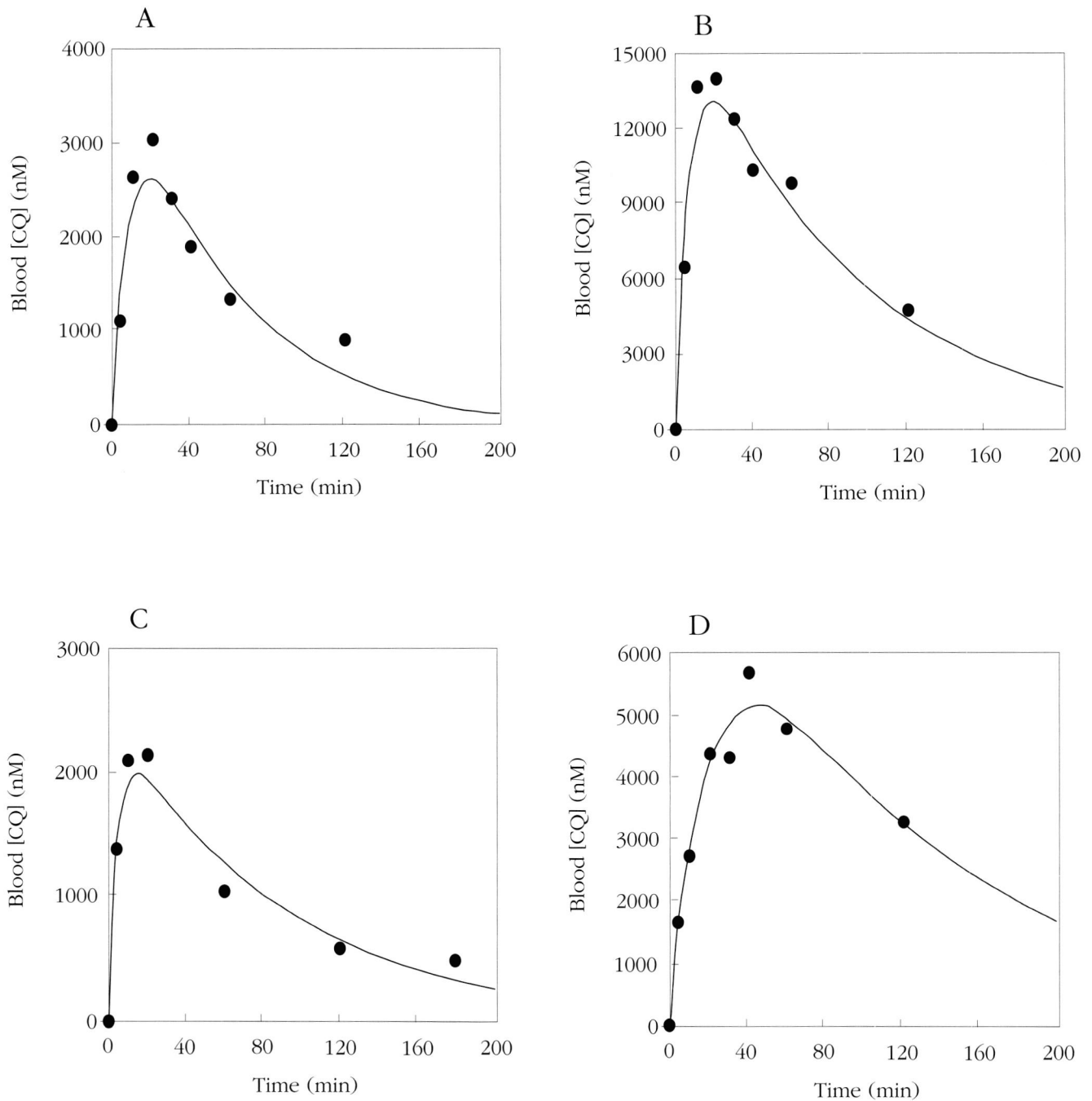


Fig. 1. – Time-dependence of whole blood CQ concentration in individual mice and the derived pharmacokinetic curve - representative examples.

Healthy or infected mice were injected sc with CQ, and blood was sampled at various time intervals and its C content was determined as described in Materials and Methods. The time-dependence was analyzed by non-linear least square analysis and the best fitting parameters were used to generate the continuous curve in each case. All examples are from day-treated animals. A : healthy mouse, 5 mg/kg CQ; B : healthy mouse, 50 mg/kg CQ; C : slightly parasitized mouse, 5 mg/kg CQ; D : heavily infected mouse, 5 mg/kg CQ.

Fig. 2

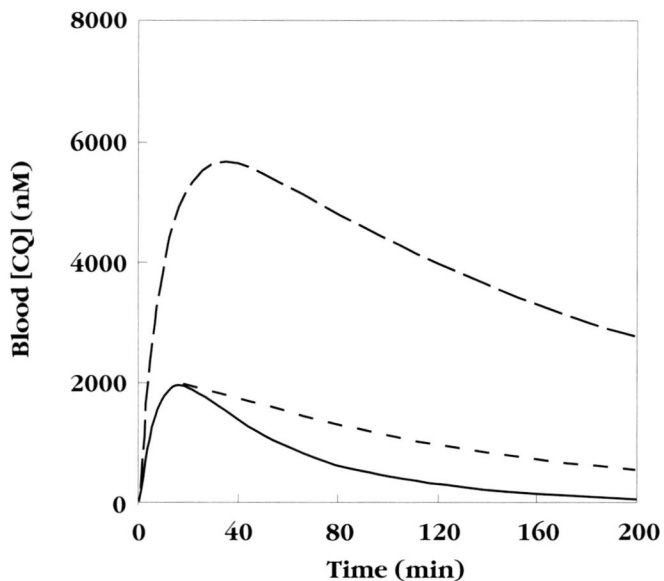


Fig. 3

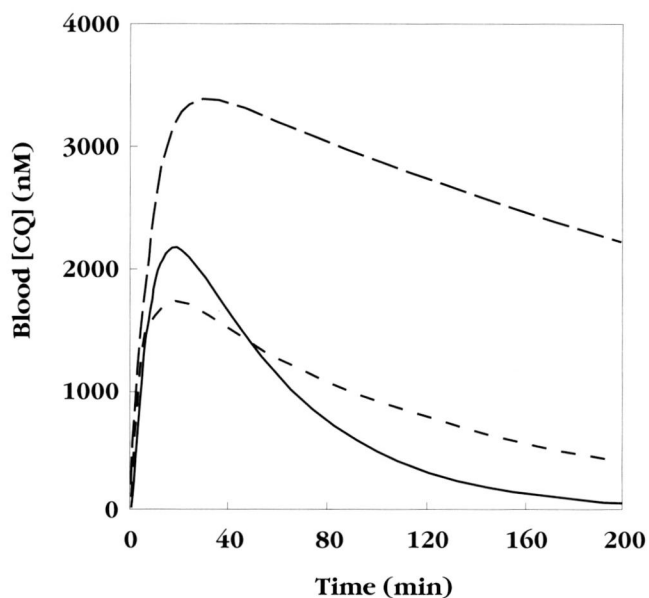


Fig. 4

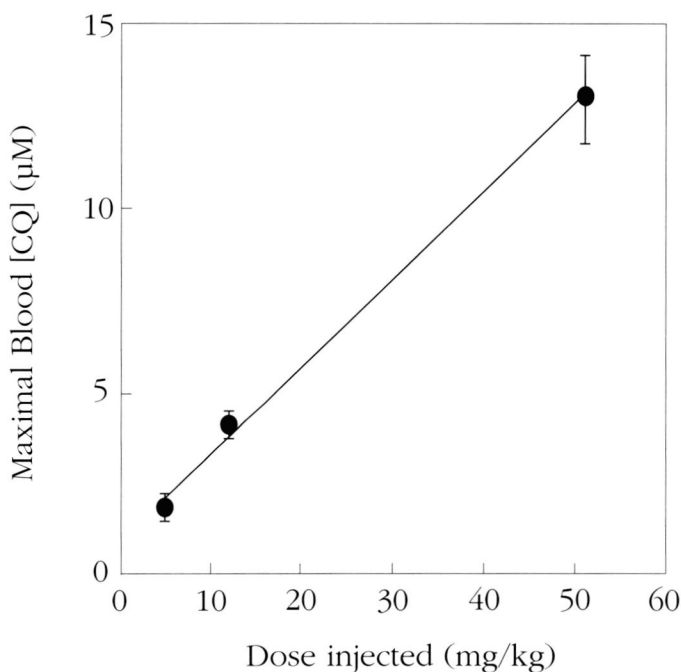


Fig. 2. – Pharmacokinetics of CQ in day treated healthy and infected mice. Healthy ( ——— ), slightly parasitized ( - - - ) and heavily infected ( — — — ) mice were given 5 mg/kg CQ sc at noon, and blood samples were collected at different time intervals and analysed for CQ content. The pharmacokinetic data for each mouse were retrieved, averaged, and used to reconstruct the time-dependent changes in blood [CQ]. Fig. 3. – Pharmacokinetics of CQ in night treated healthy and infected mice. Details are given in the legend to Fig. 2, except that drug was injected at virtual midnight. Fig. 4. – Effect of chloroquine dose on the maximal blood drug concentration. Mice, 4 in each group, were injected sc with 5, 10 and 50 mg/kg chloroquine diphosphate. Blood was sampled and the chloroquine concentration was determined as described in Materials and Methods. The maximal drug concentration was calculated from the derived pharmacokinetic data, and the mean  $\pm$  SD were plotted against dose. The line was obtained by linear regression ( $r=0.999$ ).

structurally related to amodiaquine, in the rat, the rabbit and the healthy human. Terminal  $t_{1/2}$  were 14.5 hr, 22 hr and 5 days, respectively (Pussard *et al.*, 1988; Verdier *et al.*, 1989), indicating a correlation between the size of the animal and this parameter. Since the precise determination of the final  $t_{1/2}$  value depends on the duration of the study, and because in man CQ decay was best fitted according to a 3 exponential model, it is likely that in the present study in which only data up to 120 min were used for the pharmacokinetic analysis,  $K_e$  does not represent terminal elimination but only tissues distribution and/or metabolism. Mono-desethylchloroquine (the main metabolite of CQ) concentrations were not taken into account in the present study.

The aim of the present experiments was to study the rapid pharmacokinetic phases of CQ in the mouse, namely, the absorption and the tissue distribution of the drug, since these are relevant to the rationale of chronotherapy. The concentration of the drug was determined in whole blood rather than in plasma, although it is known that normal erythrocytes are capable of concentrative accumulation of CQ. This does not pose a problem in healthy and slightly parasitized animals, since in the first case there is a straight correlation between plasma and whole blood concentration (Frisk-Holmberg *et al.*, 1984). In the second case, although infected cells accumulate much more drug, the light parasitemia is not expected to change significantly the concentration of CQ in the blood. Indeed, neither  $F/V_d$ , nor AUC or  $C_{max}$  were significantly different in healthy and slightly parasitized animals (Table D). In a study on humans (including children) slightly infected (1-2 % parasitemia) with *P. falciparum*, no differences in the  $K_e$  and  $K_a$  between healthy and sick subjects was observed (Adelusi *et al.*, 1982). A much higher erythrocyte to plasma distribution of CQ was determined at the beginning of the treatment (25 mg/kg over 3 days), which then decreased in parallel to the drop in parasitemia, indicating the larger drug uptake into infected cells.

Injection of increasing doses of CQ into healthy mice, resulted in a linear increase of peak blood concentrations (Fig. 4) and AUC, but not in a significant parallel increase in  $V_d$ , suggesting that the blood compartment may have become saturated, or that metabolization of the drug was enhanced. Mice dosed at night can be included in this generalization, suggesting that handling of CQ by the mouse is not affected by the circadian rhythm.

CQ absorption into the blood compartment from sc injection was very rapid,  $t_{1/2}$  ranging from 4 to 6.5 min, compared to about 20 min in malaria patients

(White *et al.*, 1987), with no effect of time of dosing or parasitemia. The apparent half-time of [CQ] decline ( $t_{1/2e}$ ) in healthy mice is around 40 min, similar to that observed in falciparum malaria patients using a double exponential decay model (White *et al.*, 1987), or >700 min in healthy humans using a triple exponential decay model (Frisk-Holmberg *et al.*, 1984). Our values may be an overestimate because the decline of blood [CQ] was analyzed according to a single exponential decay, rather than to a double or a triple exponential decay as done when the total time of sampling was prolonged. The time to peak concentration ( $t_{max}$ ) in healthy mice was around 18 min, compared to 15 min found after intramuscular injection in humans (Aderounmu 1986). Since  $t_{max}$  is expected to increase hyperbolically with increasing  $t_{1/2e}$  (when  $K_a$  is kept constant), an overestimated  $t_{1/2e}$  in the present study may explain the larger than expected values (based on body size differences) observed in mice.

Slight infection of 1.5-3 % parasitemia, has already a perceptible effect on the kinetics of blood [CQ] (Figs. 2 and 3), mostly due to a substantial decrease in  $K_e$  (increase in  $t_{1/2e}$ ) and a small but insignificant increase in  $K_a$  (decrease in  $t_{1/2a}$ ). These observations suggest that the handling of CQ by the slightly infected mouse has been altered for yet unknown reasons. Whereas this does not result in an alteration in  $t_{max}$  compared to healthy mice, the antimalarial plasma [CQ] may prevail for longer times.

Whatever may be the real situation, the kinetics of blood [CQ] in mice is extremely rapid, and therefore suitable for testing the notion of chronotherapy. It has been previously shown that a single injection of 5 mg/kg CQ into *P. chabaudi*-infected mice eliminates specifically and selectively the mid-term trophozoites (Cambie *et al.*, 1991). This would mean that the drug concentration is maintained for a sufficient length of time to exert their antimalarial effect, but shorter than the time needed for the previous stage of young trophozoites to mature to the susceptible stage, i.e., 3 hrs.

Heavy infection markedly alter the pharmacokinetics of CQ :  $F/V_d$ , AUC,  $t_{1/2e}$  and  $t_{max}$  are considerably and significantly ( $p < 0.05$ ) increased. These results are reminiscent of those obtained in rhesus monkeys heavily infected (20-25 % parasitemia) with *P. knowlesi* (Prasad *et al.*, 1985). Both AUC and  $t_{1/2e}$  are larger in day-treated mice (Table D). Knowing the cell cycle of *P. chabaudi*, at the time of injection around noon, most of the parasites are at the mid-trophozoite stage, while when the drug has been given at night, most of the parasites were at the late schizont or the ring stage. Both of these stages have a lower capacity for drug accumulation compared to the trophozoite

stage. The excess CQ observed in heavily infected animals (compared to uninfected animals) is probably due to the presence of infected cells as is evident from the following calculations. Assume that the hematocrit in healthy and heavily infected mice is more or less constant at 50 %. In healthy animals  $C_{\max} = C_p * f_p + C_u * f_u$  whereas in infected animals  $C_{\max} = C_p * f_p + C_u * f_u + C_i * f_i$ , where C's are concentrations and f's are the fractional volume of the whole blood occupied by plasma (p), uninfected cells (u) and infected cells (i). Taking the  $C_{\max}$  values for healthy and heavily infected animals from Table I, we obtain that in day-treated and night-treated animals infected cells accumulate 20 and 4.5 times more CQ than uninfected cells, respectively. The doubling of  $t_{\max}$  is essentially due to the increase in  $t_{1/2e}$  since  $t_{1/2a}$  is not much different from control mice. The high  $t_{1/2e}$  is due to the heavy load of parasites that increases the capacity of the blood compartment to accumulate drug, and a rate of drug efflux that is smaller than the rate of influx. One must conclude that CQ is dynamically retained in infected cells, and that plasma concentration of the drug is likely considerably lower than that found in healthy controls or in slightly infected animals, thus accounting for the low Ke values observed in heavy infection.

The present study establishes a baseline for the pharmacokinetics of CQ in the mouse malaria model. In conjunction with our previous investigations, it shows that when testing drugs in murine models, one has to know the stage-dependence of drug action, the precise pharmacokinetics of the drug at different stages of parasite development, and the effect of parasitemia on the disposition of the drug. Using the mouse malaria model for the *in vivo* testing of drugs, requires full knowledge of these parameters. The modifications of kinetic parameters (increase in  $t_{\max}$ , AUC and  $t_{1/2e}$ ), observed in infected mice, are similar to those observed in malaria patients. Thus, the mice malaria model seems to be quite appropriate to study CQ kinetics in order to investigate the possible implications, in humans, of malaria chronotherapy. If CQ is correctly used, i.e., combining the stage-dependence and the kinetic dependence of drug action, it should enable the improvement of therapeutic treatment of malaria, and the reduction or at least the delay of emergence, development and spread of *P. falciparum* drug-resistance.

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