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Short communication

# Determination of chloroquine and desethylchloroquine in plasma and blood cells of *Plasmodium vivax* malaria cases using liquid chromatography

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## 1. Introduction

Chloroquine has been used for many decades in the prophylaxis and treatment of malaria [1]. Determination of drug concentrations in different body fluids is important for prophylaxis and treatment of malaria cases, investigation of pharmacokinetics as well as help to decide true resistance of malaria parasites to different antimalarials [2]. Many LC methods have been reported for the determination of chloroquine in different body fluids in healthy volunteers [2–4] and some extent to *Plasmodium falciparum* malaria [5,6]. Resistance of *Plasmodium vivax* to chloroquine is rather a new phenomenon and is spreading all over the world [7,8] and chloroquine concentrations in diseased state may play a vital role to confirm the status of true resistance. Edwards et al. [9] have reported pharmacokinetics of chloroquine in healthy and *P. vivax* Thai subjects following an intravenous infusion and no significant difference is found between the two groups while Rombo et al. [10] have determined whole blood concentrations of chloroquine and desethylchloroquine during and after treatment of adult patients infected with *P. vivax*, *Plasmodium ovale* and *Plasmodium malariae*. We describe a normal-phase LC method for the separation of chloroquine and its metabolite desethylchloroquine in plasma and blood cells for the determination of their concentrations in *P. vivax* malaria cases and to obtain information on the chloroquine pharmacokinetic parameters in *P. vivax* cases.

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## 2. Materials and methods

### 2.1. Chemicals and standards

HPLC grade dichloromethane and methanol were obtained from Spectrochem (Mumbai, India), while diethylether, perchloric acid and other chemicals were of analytical reagent grade and were used without further purification.

Chloroquine, desethylchloroquine and internal standard 4-(4-dimethylamino-1-methylbutylamino)-7-chloroquinoline were obtained from the Department of Clinical Pharmacology, Karolinshka Institute, Huddinge Hospital, Huddinge, Sweden. The concentration range of 0.025–1.0 μg $ml^{-1}$ were used for the calibration curves of chloroquine and desethylchloroquine while the internal standard (2.5 μg $ml^{-1}$) was used throughout the study. All solutions of drug and its metabolite were prepared in mobile phase (dichloromethane-methanol-perchloric acid (1 M); 100:9:1.2, v/v/v).

### 2.2. Instrumentation and chromatographic conditions

A Waters HPLC system (Waters Assoc. Milford, MA, USA) consisting of a 510 pump, 486 multiwavelength UV detector operated at 343 nm, or Shimadzu fluorimetric 530 detector ($E_x = 340$ nm; $E_m = 380$ nm), a Rheodyne injector and an integrator was used for the analysis. The column was a (3.9 × 300 mm; particle size 5 μm) uPorasil (Waters Assoc.) normal phase column.

The mobile phase consisting of a dichloromethane-methanol-(1 M) perchloric acid (100:9: 1.2,v/v/v) was pumped at a flow rate of 1.0 ml $min^{-1}$ at an ambient temperature. The mobile phase was filtered and degassed by ultrasonication (Dacon FS1OO, Hove, UK) before use.

### 2.3. Subjects

The subjects were 51 *P. vivax* malaria cases (confirmed through microscopic examination), age group 14–52 years, mean body weights 63.4 kg from Indian Oil Corporation, Mathura (UP), India. After ascertaining that no other antimalarials had been taken before starting the treatment, all patients were given 900 mg of chloroquine base (600 mg on day 0 and 300 mg on day 1) followed by 75 mg of primaquine base @ 15 mg $day^{-1}$.

Intravenous blood (2.0 ml) was drawn from patients on days 0, 1, 2, 3, 4 and 5. Samples on day 0 and day 1 were taken at 3 h after dosing. Samples were taken in such a way that a maximum of three samples were taken from each patient to avoid practical problems. Heparin was used as an anticoagulant. The blood was centrifuged on an IEC Centra-7 (International Equipment Company, Needham Heights, MA, USA) for 15 min at 1000 × *g* to separate plasma and blood cells. All samples were kept at 4°C until used.

### 2.4. Extraction procedure

The extraction of chloroquine and desethylchloroquine from plasma and blood cells was performed as described by Alvan et al. [3]. Briefly to 0.5 ml of sample, 80 μl of internal standard, 1 ml of 1 M NaOH and 6 ml of diethylether were added and shaken for 15 min. The extracted sample was centrifuged at 1000 × *g* for 10 min and the ether layer was separated and evaporated by a stream of nitrogen. The residue was redissolved in mobile phase for HPLC analysis.

### 2.5. Recovery and reproducibility

The recovery was determined at concentrations of 0.025, 0.10, 0.50 and 1.0 μg $ml^{-1}$ of chloroquine and desethylchloroquine in plasma and blood cells by comparing peak-height ratios of spiked standard with the ratio obtained by direct injection of pure standards. Within-day and day-to-day reproducibility of the method were determined by repeated assay of different concentrations of chloroquine and desethylchloroquine.

### 2.6. Stability of chloroquine and desethylchloroquine

The stability of chloroquine and desethylchloroquine were determined in the samples

stored over a period of 3 months at 4°C and their concentrations were determined at regular intervals.

### 2.7. Pharmacokinetics

The terminal half-life ($T_{1/2}$), volume of distribution ($V_d$) and area under the curve (AUC) were calculated according to the methods reported earlier [11].

## 3. Results and discussion

HPLC separation of chloroquine and desethylchloroquine in plasma and blood cells was accomplished by using a modification in the mobile phase composition of a normal phase HPLC method used for the determination of sulfalene [12]. The composition of mobile phase dichloromethane-methanol-perchloric acid (1 M) was 100:9:1.2 (v/v/v) for the separation of chloroquine, desethylchloroquine and internal standard instead of 96:9:1.0 as reported for sulfalene. The mobile phase was pumped at 1.0 ml $min^{-1}$ on uPorasil normal phase column. This modification in the mobile phase composition proved to give base-line separation. Fig. 1 showed the chromatographic behaviour of a blank plasma extract and a plasma extract taken on day 3 from a *P. vivax* infected patient treated with 900 mg chloroquine. Some endogenous compounds peaks from plasma and blood cells appeared in the chromatogram but they appeared before the peaks of chloroquine, desethylchloroquine and internal standard thus did not interfere in the quantification. The chromatographic behaviour of the extract from plasma or blood cells was similar. Other common antimalarials like, quinine, sulfadoxine, primaquine did not interfere in the determination of chloroquine and its metabolite desethylchloroquine by this method [12].

During the study, a large number of calibration curves were obtained for the concentration range of 0.025–1 μg $ml^{-1}$ for chloroquine and desethylchloroquine. The results obtained showed linear relationships over the concentration ranges used: the regression equation obtained for chloroquine was $y = 3.2x \pm 0.04$ ($r = 0.99$, $n = 25$) and that for desethylchloroquine was $Y = 9x \pm 0.05$ ($r = 0.99$, $n = 25$).

No degradation was detected for chloroquine and desethylchloroquine storage in plasma or blood cells at 4°C for over 3 months. The limit of detection for chloroquine and desethylchloroquine with UV and fluorimetric detection were 10 and 5 ng, respectively with a signal to noise ratio of 3:1 using the prescribed method while the limit of quantification was 20 ng $ml^{-1}$. There was no difference in the values of limit of detection and limit of quantification between plasma and blood cells matrix. Within-day and day-to-day coefficients of variations (C.V) in plasma for chloroquine averaged 2.3 and 2.76%, respectively (Table 1) while for desethylchloroquine were 2.83 and 3.36%, respectively. Mean extraction recoveries of chloroquine in plasma and blood cells were 91.05 and 90.5%, respectively while for desethylchloroquine were 90.6 and 89.7%, respectively (Table 2).

Bergqvist and Churchill [2] have given a review on the HPLC methods for the determination of chloroquine in different body fluids and later on an overview on this subject has also been compiled by Karbwang and Na-Bangchang in 1993 [11]. The approach for the separation of chloroquine and its metabolite on normal phase chromatography was to use diethylamine or amomnia as a modifier in the mobile phase and it gave very good separation. However, strict experimental conditions for the reproducibility are needed. Moreover column back pressure increases due to water contents and the resolution of internal standard and desethylchloroquine became poor [3]. Contrary to this mobile phases containing ion-pair agents like perchlorate or haptanesulphonic acid were used on reversed-phase chromatography. However, it is clearly indicated that the efficacy of reversed-phase column decreases if the pH of the mobile phase was above 3–5 [13] besides the separation was not as good as on normal phase column. Recently Karim et al. [5] have used heptanesulphonic acid as ion-pairing agent together with diethylamine as a modifier base to improve the separation on reversed-phase column. It is to point out that the use of diethy-

lamine in the mobile phase on reversed-phase chromatography may reduce the column life besides efficacy and reproducibility factors as mentioned above.

The chromatographic system described here is sensitive and specific for the separation of chloroquine and its metabolite desethylchloroquine in plasma and blood cells. The separation followed normal phase phenomenon with ion-pair mode. Moreover, one column may last for more than 6 months without affecting separation. It may be noted that the internal standard has a long retention time than chloroquine and desethylchloroquine which allowed for a more accurate measurements as stated earlier [5].

Mean chloroquine and desethylchloroquine concentrations in plasma and blood cells of *P. vivax* malaria patients during and after treatment with 900 mg chloroquine base are given in Table 3. The chloroquine plasma concentrations in all samples up to 5 days were found higher than the required chloroquine plasma concentration of 16 μg $l^{-1}$ to clear *P. vivax* parasites [1,14]. Similar

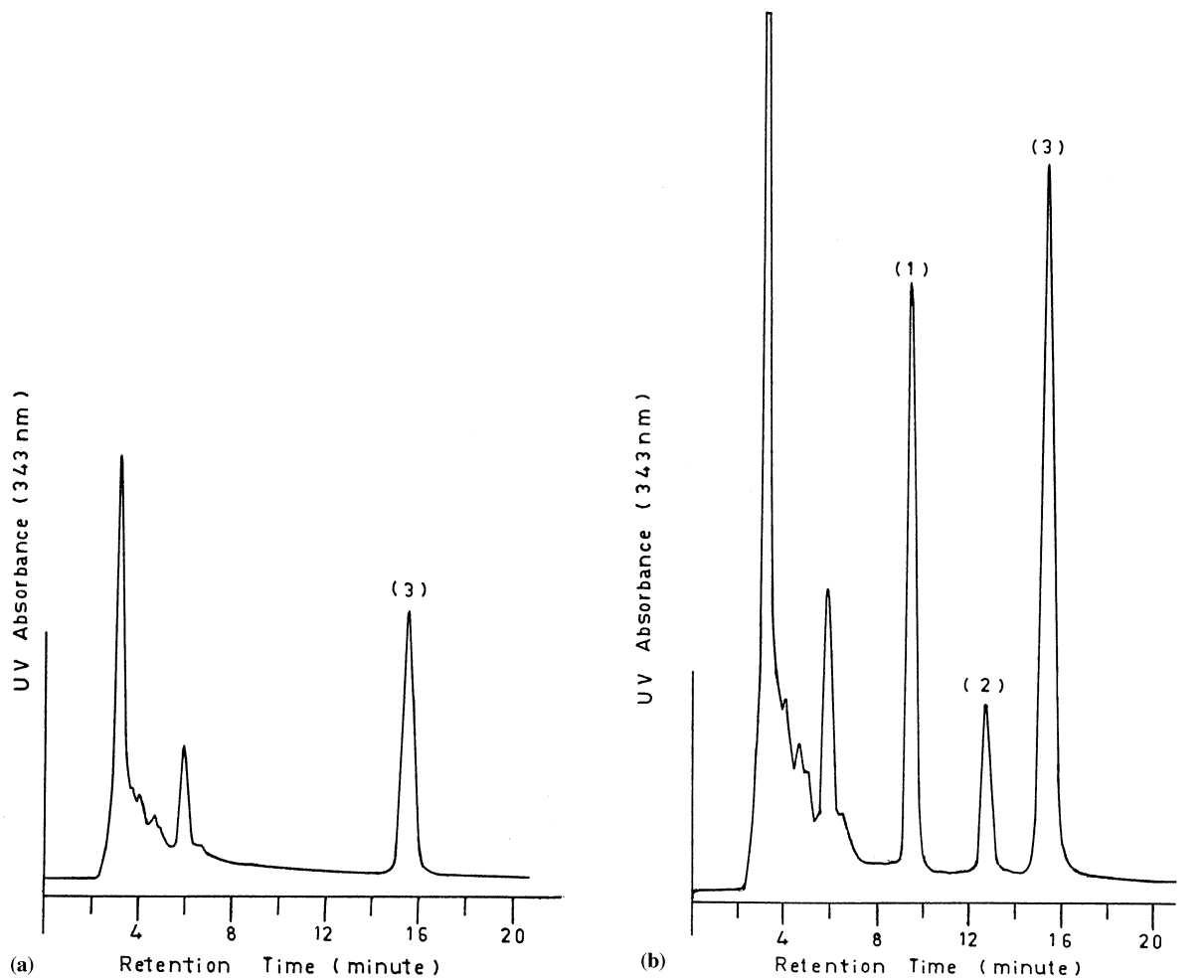


Fig. 1. (a) Chromatogram of a blank plasma extract containing internal standard (IS:4-(4-dimethylamino-1-methylbutylamino)-7-chloroquine); peak 3 obtained from a *P. vivax* patient before drug administration. (b) Chromatogram of a plasma extract taken on day 3 after treatment with 900 mg chloroquine to a *P. vivax* patient. Peak 1, Chloroquine (0.44 μg $ml^{-1}$); peak 2, desethylchloroquine; peak 3, Internal standard (IS)

Table 1
Precision of the HPLC method for chloroquine and desethylchloroquine in plasma (spiked samples)

| | Concentration (μg ml $^{-1}$) | $n$ | Coefficient of variation (C.V.)% | |
|---|---|---|---|---|
| | | | Chloroquine | Desethylchloroquine |
| Within-day | 0.025 | 5 | 2.7 | 3.0 |
| | 0.10 | 5 | 2.4 | 2.8 |
| | 1.0 | 5 | 1.8 | 2.7 |
| Mean ± SD | | | 2.3 ± 0.45 | 2.83 ± 0.15 |
| Day-to-day | 0.025 | 5 | 2.4 | 3.2 |
| | 0.10 | 5 | 3.1 | 4.0 |
| | 1.0 | 5 | 2.8 | 2.9 |
| Mean ± SD | | | 2.76 ± 0.35 | 3.36 ± 0.56 |

observations were recorded earlier by Rombo et al. [10] in the patients infected with *P. vivax*, *P. ovale* and *P. malariae*. It may be noted that the mean parasite clearance time was 57 h and no recrudescence was recorded during and after 20 days of treatment. The blood cells chloroquine concentrations were higher than plasma in all samples. Chloroquine to desethylchloroquine ratio in plasma was higher in the starting of treatment and decreased with time while in blood cells, this ratio varied between 2 and 3 times during the entire study period. This could indicate that chloroquine binding is saturated in blood cells and more chloroquine would became available for metabolism as suggested by Frisk Holmberg et al. [15]. Large interindividual difference of chloroquine was found in plasma as well as in blood cells were in agreements with the earlier reports [5,6].

Pharmacokinetic parameters of chloroquine in plasma and blood cells of *P. vivax* patients after treatment with 900 mg chloroquine are given in Table 4. Terminal half-life of chloroquine in blood cells (168.40) was more as compared to plasma (136.40). Large variations in the terminal half-life are reported for chloroquine in healthy and malaria cases [9]. Adelusi et al. [16] have found chloroquine half-life $T_{1/2}$ in plasma and red blood cells as 131.5 and 139 h, respectively in *P. falciparum* cases. Volume of distribution ($V_d$) and area under the curve (AUC) in plasma were 49.55 I kg$^{-1}$ and 35.74 μg h$^{-1}$ ml$^{-1}$ while in blood cells were 31.16 I kg$^{-1}$ and 70.18 μg h$^{-1}$ ml$^{-1}$, respectively. Large variations in the volume of distribution and area under the curves for plasma chloroquine are reported earlier due to dose dependence and inter-individual difference [15]. Limited chloroquine pharmacokinetic studies are

Table 2
Extraction recovery of the HPLC method for chloroquine a desethylchloroquine in plasma and blood cells[a]

| Concentration μg ml$^{-1}$ | Recovery (%) (mean ± SD $n = 4$) | | | |
|---|---|---|---|---|
| | Chloroquine | | Desethylchloroquine | |
| | Plasma | Blood cells | Plasma | Blood cells |
| 0.025 | 88.3 ± 3.7 | 87.2 ± 2.4 | 89.6 ± 2.7 | 87.2 ± 2.7 |
| 0.100 | 90.1 ± 3.2 | 89.5 ± 3.1 | 91.3 ± 3.0 | 89.7 ± 2.1 |
| 0.50 | 92.5 ± 4.1 | 90.5 ± 2.1 | 90.1 ± 2.9 | 89.1 ± 1.8 |
| 1.0 | 93.3 ± 2.2 | 91.0 ± 1.8 | 92.7 ± 1.9 | 90.3 ± 1.5 |
| Mean ± SD | 91.05 ± 2.28 | 89.55 ± 1.68 | 90.92 ± 1.38 | 89.07 ± 1.34 |

[a] $n$, Number of observations.

carried out in blood cells. Edward et al. [9] have found the area under the curve in blood cells of Thai *P. vivax* subjects as 151637 μg $h^{-1}ml^{-1}$ and compared it with healthy cases and stated that the difference of AUC in both groups was insignificant. Our chloroquine pharmacokinetic data along with chloroquine concentrations in plasma and blood cells of *P. vivax* cases may work as a reference for future studies.

Table 3
Plasma and blood cells concentrations[a] of chloroquine[b] and desethychloroquine in *P. vivax* cases

| Day | Chloroquine | | Desethylchloroquine | |
|---|---|---|---|---|
| | Plasma | Blood cell | Plasma | Blood cell |
| D0 | 0.39 ± 0.15[c] (0.18–1.50)[d] | 0.42 ± 0.28 (0.13–1.70) | 0.05 ± 0.16 (0.02–0.62) | 0.19 ± 0.23 (0.02–0.85) |
| D1 | 0.49 ± 0.19 (0.20–1.25) | 0.97 ± 0.27 (0.47–1.71) | 0.18 ± 0.07 (0.10–0.33) | 0.43 ± 0.26 (0.09–0.90) |
| D2 | 0.35 ± 0.13 (0.20–0.60) | 0.70 ± 0.46 (0.28–1.56) | 0.19 ± 0.19 (0.06–0.80) | 0.33 ± 0.15 (0.15–0.60) |
| D3 | 0.24 ± 0.10 (0.08–0.41) | 0.51 ± 0.09 (0.25–0.65) | 0.17 ± 0.25 (0.02–0.90) | 0.24 ± 0.18 (0.04–0.70) |
| D4 | 0.17 ± 0.05 (0.10–0.39) | 0.41 ± 0.10 (0.25–0.68) | 0.08 ± 0.06 (0.01–0.22) | 0.14 ± 0.07 (0.04–0.30) |
| D5 | 0.15 ± 0.03 (0.08–0.26) | 0.37 ± 0.13 (0.19–0.65) | 0.07 ± 0.06 (0.01–0.22) | 0.19 ± 0.12 (0.07–0.44) |

[a] Concentration (μg $ml^{-1}$).
[b] CQ treatment 600 D0, 300 D1.
[c] Mean ± SD
[d] Figures in parentheses are the ranges.

Table 4
Pharmacokinetic data of chloroquine in *P. vivax* infected patients

| Sl. No. | Parameters | Plasma | Blood cells |
|---|---|---|---|
| 1. | Terminal half-life ($T_{1/2'}$) | 136.40 | 168.40 |
| 2. | Volume of distribution ($V_d$, I $kg^{-1}$) | 49.55 | 31.16 |
| 3. | Area under curve (AUC, μg $h^{-1}$ $ml^{-1}$) | 35.74 | 70.18 |

## 4. Conclusion

The chromatographic system described here is sensitive and specific for the determination of chloroquine and its metabolite desethylchloroquine in plasma and blood cells. The separation followed normal phase phenomenon with ion-pair mode. The limit of quantification was 20 mg $ml^{-1}$. Within-day and day-to-day coefficients of variation for chloroquine averaged 2.3 and 2.76%, respectively. Mean extraction recoveries of chloroquine from plasma and blood cells were 91.05 and 90.5%, respectively. The plasma and blood cells concentrations within 3 h of dosing reached above therapeutic chloroquine concentration to clear *P. vivax* parasites. The blood cells concentrations were higher than plasma in all samples. Terminal half-life for chloroquine in plasma and blood cells were 136.4 and 168.4 h, respectively.

## References

[1] L.J. Bruce-chwatt, Chemotherapy of Malaria, 2nd edition: WHO Monograph Series No 27, 1986.
[2] Y. Bergqvist, F.C. Churchill, J. Chromatogr. 434 (1988) 1–20.
[3] G. Alvan, L. Ekman, B. Lindstrom, J. Chromatogr. 229 (1982) 241–247.
[4] B. Oosterhuis, B.M. Van Den, J.C.F.M. Wetsteyn, C.J. VanBoxtel, Pharm. Week. Sci. Ed. 3 (1981) 263–267.
[5] E.A Karim, K.E. Ibrahim, M.A. Hassabalrasoul, B.O. Saeed, R.A. Bayoumi, J. Pharm. Biomed. Anal. 10 (1992) 219–223.
[6] U. Hellgren, C.M. Kihamia, L.F. Mahikwano, A. Bjorkman, O. Eriksson, L. Rombo, Bull. WHO 67 (1989) 197–202.
[7] V.K. Dua, P.K. Kar, V.P. Sharma, Trop. Med. Int. Health 1 (1996) 816–819.
[8] J.K. Baird, H. Baski, M.J. Purnomo Bangs, B. Subianto, C. Patchen, S.L. Haffman, Am. J. Trop. Med. Hyg. 44 (1991) 547–552.
[9] G. Edwards, S. Looareesuwan, A.J. Davies, Y. Wattanagoon, R.E. Phillips, D.A. Warrell, Br. J. Clin. Pharmac. 25 (1988) 477–485.
[10] L. Rombo, A. Bjorkman, E. Sego, O. Eriksson, Trans. R. Soc. Trop. Med. Hyg. 80 (1986) 763–766.

[11] J. Karbwang, K. Na-Bangchang, in: K.J. Karbwang, W.H. Wernsdorfer (Eds.), Clinical Pharmacology of Antimalarials, Mahidal University, Thailand Publication, Bangkok, 1993, pp. 37–53.
[12] V.K. Dua, R. Sarin, V.P. Sharma, J. Chromatogr. 563 (1991) 330–340.
[13] Y. Bergqvist, M. Frisk-Holmberg, J. Chromatogr. 221 (1980) 119–127.
[14] J. Brohult, L. Rombo, V. Sirleaf, E. Bengtsson, Anal. Trop. Med. Parasitol 73 (1979) 401–405.
[15] M. Frisk-Holmberg, Y. Bergqvist, E. Termond, B. Domeij-Nyberg, Eur. J. Clin. Pharmacol. 26 (1984) 521–530.
[16] S.A. Adelusi, A.H. Dawodu, L.A. Salako, Br. J. Clin. Pharmac. 14 (1982) 483–487.