Journal of Chromatography, 574 (1992) 305–312
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6191

Simultaneous determination of chloroquine and its three metabolites in human plasma, whole blood and urine by ion-pair high-performance liquid chromatography

P. Houzé* and A. de Reynies

Département de Biochimie–Pharmacologie–Toxicologie, C.H.R. de Versailles, 177 Route de Versailles, 78157 Le Chesnay (France)

F. J. Baud

Réanimation Toxicologique, Hôpital F. Widal, 200 Rue de Faubourg Saint Denis, 75010 Paris (France)

M. F. Benatar and M. Pays

Département de Biochimie–Pharmacologie–Toxicologie, C.H.R. de Versailles, 177 Route de Versailles, 78157 Le Chesnay (France)

(First received July 29th, 1991; revised manuscript received October 14th, 1991)

ABSTRACT

A method was developed for the separation and measurement of chloroquine and three metabolites (desethylchloroquine, bis-desethylchloroquine and 4-amino-7-chloroquinoline) in biological samples by ion-pair high-performance liquid chromatography with UV detection. The method uses 2,3-diaminonaphthalene as an internal standard and provides a limit of detection between 1 and 2 ng/ml for chloroquine and its metabolites. The assay was linear in the range 12.5–250 ng/ml and the analytical recovery and reproducibility were sufficient. The assay was applied to the analysis of biological samples from a patient undergoing chloroquine chemoprophylaxis and a patient who had ingested chloroquine in a suicide attempt.

INTRODUCTION

Despite many reported cases of poisoning [1], chloroquine (CLQ), a 4-aminoquinoline, is widely used for prophylaxis and treatment of malaria and various connective disorders [2–4]. The metabolism of chloroquine has been well studied [5] but is not yet totally established. After oral administration, chloroquine is rapidly de-ethylated in the blood to desethylchloroquine (30–40% of blood chloroquine concentration) and bisdesethylchloroquine (5–10% of blood chloroquine concentration) [5,6]. The main human metabolite is desethylchloroquine, which possesses the same antimalarial activity against chloroquine-susceptible *Plasmarium falciparum* [7] and toxicity [8]. Other metabolites can be produced during chloroquine biotranformation, such as 4-amino-7-chloroquinoline [8], carboxylic acid derivatives and other uncharacterized metabolites which were found in small amounts [7,9].

In eighteen years, several high-performance liquid chromatographic methods for the detection of chloroquine and its two main metabolites have been developed with [10,11] or without [12] an internal standard using spectrofluorimetric detection. These methods were applied to pharmacological studies in healthy volunteers after a

0378-4347/92/$05.00

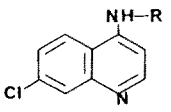
single dose [9,11,13] or in malaria patients [14,15]. To our knowledge, the toxicokinetics of chloroquine and its metabolites have not been assessed in man.

In biological fluids a method allowing the simultaneous determination of chloroquine and its three metabolites has never been reported. In this paper, we describe a selective and sensitive method to separate and determine chloroquine and its three metabolites (desethylchloroquine, M1; bisdesethylchloroquine, M2; and 4-amino-7-chloroquinoline, M3) (Fig. 1), involving organic extracts of biological fluids (blood, plasma or urine) followed by high-performance liquid chromatography. This assay is applicable to both pharmacological and toxicological research, especially to determine the toxicokinetic parameters of chloroquine and its metabolites in man after acute poisoning.

EXPERIMENTAL

Chemicals

Chloroquine sulphate and free bases of M1, M2 and M3 were generous gifts from Specia (Vitry, France). The internal standard (I.S.), 2,3-diaminonaphthalene, was purchased from Aldrich (Brussels, Belgium). Sodium 1-heptanesulphonate (ion-pair chromatographic grade) was obtained from Interchim (Besançon, France). Acetonitrile (HPLC grade) and methanol (analytical-reagent grade) were procured from Merck (Darmstadt, Germany). All other chemicals were of analytical-reagent grade and were used as received.



R = CH_3–CH–$(CH_2)_3$–$N(C_2H_5)_2$ 1

R = CH_3–CH–$(CH_2)_3$–$N^{H}_{C_2H_5}$ 2

R = CH_3–CH–$(CH_2)_3$–NH_2 3

R = H 4

Fig. 1. Structural formulae of chloroquine free base (1) and its metabolites M1 (2), M2 (3) and M3 (4).

Standard solutions

Stock solutions (20 mg/l) of chloroquine and M1, M2 and M3 were prepared in 10 *M* hydrochloric acid–methanol (0.1%, v/v) and methanol, respectively. The working solution in methanol was prepared by diluting the stock solutions 1:40. All solutions were stored at −20°C for up to 8 weeks. A stock solutions of I.S. (100 mg/l) was prepared in 1 *M* hydrochloric acid. A working solution (2 μg/ml) was prepared in water and kept at 4°C for up to 6 weeks.

Plasma standards (calibration standards) were prepared at concentrations of 12.5, 25.0, 50.0, 100.0, 250.0 ng/ml by adding 1 ml of drug-free human plasma to the residue obtained by evaporation of 25, 50, 100, 200 and 500 μl of working solution. A 100-μl volume of I.S. working solution (200 ng) was used for internal standardization.

Instrumentation and chromatographic conditions

The determination was carried out using a system consisting of a Model 64 HPLC pump (Knauer) equipped with a Rheodyne injector with a 50-μl sample loop (Cunow, Cergy-St. Christophe, France) and a Spectroflow 783 variable-wavelength UV detector set at 343 nm (Cunow). A 5-μm Ultrasphere IP C_{18} reversed-phase column (15 cm × 4.0 mm I.D.) (Beckman, Gagny, France) was used. Chromatograms were recorded with a Chromatopac C-R3A integrator (Shimadzu, Kyoto, Japan). The mobile phase for column equilibration and drug elution consisted of 0.02 *M* sodium 1-heptanesulphonate with 700 μl/l of diethylamine (adjusted to pH 3.4 with orthophosphoric acid)–acetonitrile (72:28). The mobile phase was used in the isocratic mode at a flow-rate of 1.5 ml/min. The column pressure ranged from 70 to 80 bar. All chromatographic separations were performed at room temperature.

Extraction procedure

Calibration graph. Plasma standards (1.0 ml) were pipetted into a 10-ml screw-capped tube containing 100 μl (200 ng) of internal standard and 500 μl of 1 *M* ammonia solution; 8.0 ml of diethyl ether were added. The tubes were shaken for 30 min and centrifuged at 4000 *g* for 10 min. The organic layer was transferred into another tube and dried (0.2–0.3 g of sodium sulphate). The ether was removed after centrifugation and evaporated to dryness by a stream of nitrogen at room temperature. The residue was dissolved in 100 μl of eluent and 50 μl were injected into the chromatograph.

Samples. In the pharmacological studies, 1 ml of plasma or whole blood treated with 500 μl of saponin or 1 ml of urine diluted 1:10 with water was mixed with the I.S. and the mixture was treated as described above. In the case of acute poisoning, samples of smaller volume were used to allow the determination of chloroquine and its metabolites.

Quantification, recovery and precision. The calibration graphs were obtained by linear regression of peak-area ratios of the drugs to the I.S. plotted against concentration. Because of the wide concentration range found for chloroquine and its metabolites in the various biological samples, different calibration graphs were drawn, and the amount of I.S. was adjusted accordingly. The extraction recovery was calculated by comparing the measured values for spiked plasma samples with those for standard aqueous solutions at two concentrations (25 and 100 ng/ml) The intra-assay precision was assessed at two concentrations (25 and 250 ng/ml) for ten determinations. The inter-assay precision was determined once at the two concentrations in ten replicates.

Biological samples

Blood samples were collected in EDTA tubes. An aliquot was centrifuged at 1500 *g* for 10 min to separate the plasma. Whole blood and plasma were stored at 4 and −20°C, respectively, until analysed. Total urine was collected and an aliquot was frozen at −20°C.

Clinical application

Whole blood and plasma aliquots were obtained from two subjects in order to determine the concentrations of chloroquine and its metabolites by HPLC. The first was a healthy white female adult taking chloroquine (100 mg/day) for chemoprophylaxis for 4 days. The second subject was a 26-year-old white female who had ingested 4 g of chloroquine in a suicide attempt and had been admitted to the intensive care unit 5 h after ingestion. Serial blood samples were obtained from admission to 57 h after chloroquine ingestion. Urine sample collection was performed daily.

RESULTS AND DISCUSSION

Fig. 2 shows the separation and determination of chloroquine and its metabolites in human biological fluids using 2,3-diaminonaphthalene as I.S. In the chromatograms which were obtained after extraction of 1.0 ml of blank plasma, no additional peaks that could interfere with the determination of chloroquine, its metabolites and the I.S. were present (Fig. 2a). The retention times for M3, I.S., M2, M1 and chloroquine were 3.2, 4.4, 5.8, 7.2 and 8.4 min, respectively. Chloroquine, its metabolites and the I.S. were well separated. The peak shapes were symmetrical with no evidence of tailing. Optimum separation in a shorter elution time was obtained by adjusting the acetonitrile/0.02 *M* sodium 1-heptanesulphonate ratio to precisely 28:72 (Fig. 3). Chromatograms obtained on analysis of whole blood and urines samples had a similar appearance. The resolution of our method (Fig. 2b, c and d) was better than that reported for chloroquine and de-ethylated metabolites with spectrofluorimetric detection by Alvan *et al.* [11] and Brown *et al.* [12]. This HPLC method permits a rapid and good separation of chloroquine and the three identified metabolites (M1, M2 and M3).

The calibration graphs for the drug and metabolites in each biological fluid exhibited excellent linearity over the concentration range 12.5–250 ng/ml. In each biological fluid, the slopes for M3

($y = 0.011x - 0.047$; $r = 0.998$) and M2 ($y = 0.009x - 0.045$; $r = 0.998$) are greater than those for chloroquine ($y = 0.004x - 0.012$; $r = 0.999$) and M1 ($y = 0.004x - 0.007$; $r = 0.999$), but the slopes for these two compounds do not differ significantly. Hence, the same calibration graph can be used for chloroquine and desethylchloroquine (M1). The calibration graphs had the same slope regardless of whether plasma or urine was used. The corresponding graph for whole blood concentrations of chloroquine and its metabolites had slightly different slopes.

By requiring a signal-to-noise ratio of greater than 5:1, the minimum detectable concentration of chloroquine and M1 in plasma was 2 ng/ml and that of M2 and M3 was 1 ng/ml, with relative standard deviations (R.S.D.) of 18 and 25%, respectively. Pussard *et al.* [16], using HPLC with UV detection at the same wavelength (343 nm), reported a minimum detectable concentration of 5 ng/ml chloroquine and desethylchloroquine. The sensitivity of our method is the same as that of HPLC with spectrofluorimetric detection [11]. The accuracy calculated as 100 · (added concentration − measured concentration) was 2, 1, 3.5 and 5% for 50 ng/ml chloroquine, M1, M2 and M3, respectively.

The intra- and inter-assay precision data for chloroquine and metabolites in plasma, urine and whole blood are summarized in Table I. The R.S.D. values (always $< 10\%$) demonstrate that the reproducibility of the method is good.

Analytical relative recoveries of chloroquine and its metabolites in plasma and urine are summarized in Table II. The recoveries lie between 65 and 90%. These values for chloroquine and its main metabolites (M1 and M2) are in agreement with those reported in the literature. Brown *et al.*

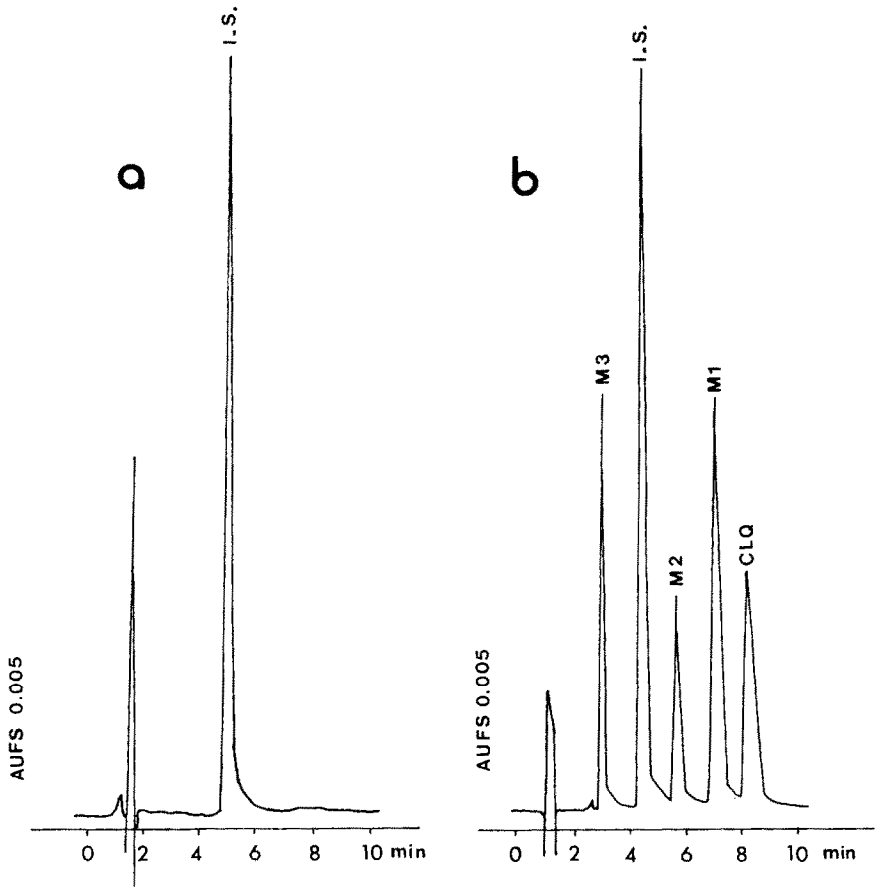


Fig. 2.

(Continued on p. 309)

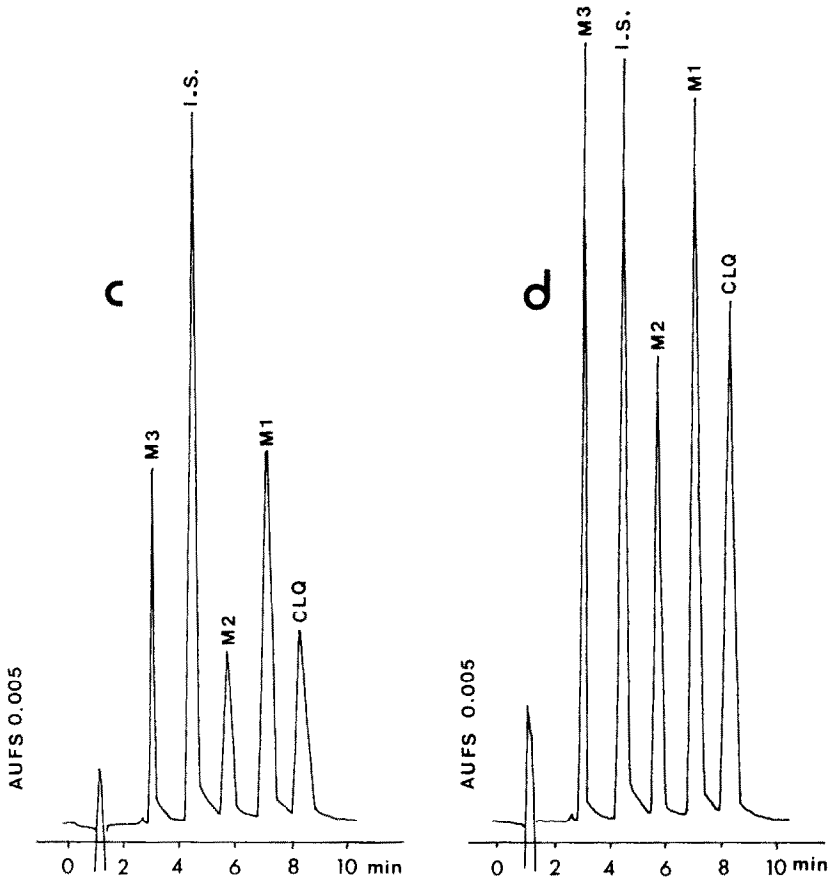


Fig. 2. HPLC profiles of (a) extracted blank plasma with internal standard, (b) spiked human plasma or (c) whole blood containing 50 ng/ml M2, and M3, 100 ng/ml CLQ and M1 and 200 ng/ml internal standard and (d) spiked human urine containing 100 ng/ml M2 and M3, 200 ng/ml CLQ and M1 and 200 ng/ml internal standard. Peaks: CLQ, chloroquine; M1, desethylchloroquine; M2, bisdesethylchloroquine; M3, 4-amino-7-chloroquinoline; I.S., internal standard.

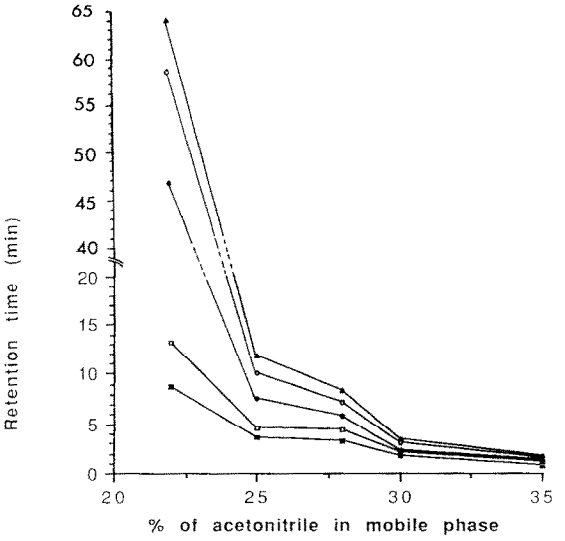


Fig. 3. Relationship between the retention time of (■) M3, (□) I.S., (●) M2, (○) M1 and (▲) chloroquine and the percentage of acetonitrile in mobile phase.

[12] reported a recovery of 85% of chloroquine and its de-ethylated metabolites without adding an internal standard. Pussard *et al.* [16] reported analytical recoveries greater than 75% with internal standardization.

To reduce plasma contamination by blood cells containing high chloroquine and metabolite concentrations, plasma is separated from whole blood by centrifugation at 1500 *g* for 10 min as reported by Bergqvist and Domeij-Nyberg [10].

The assay is shown to be selective, without interference from endogenous material (Fig. 2a) or other animalarial drugs. Under our conditions, the retention times for amodiaquine and quinine

TABLE I

REPRODUCTIBILITY OF THE HPLC METHOD

CLQ = chloroquine; M1 = desethylchloroquine; M2 = bis-desethylchloroquine; M3 = 4-amino-7-chloroquinoline; *n* = number of determinations.

Concentration (ng/ml)	Sample	R.S.D. (%)			
		CLQ	M1	M2	M3
Intra-assay (n = 10)					
25	Plasma	4.2	3.9	4.2	4.8
	Whole blood	5.1	4.0	3.5	3.5
	Urine	3.5	3.8	4.3	4.1
250	Plasma	5.6	4.5	5.0	5.9
	Whole blood	7.1	5.2	4.3	5.5
	Urine	4.2	5.0	5.1	3.8
Inter-assay (n = 10)					
25	Plasma	4.6	6.1	5.2	5.5
	Whole blood	4.2	3.5	4.2	5.0
	Urine	3.8	5.6	5.0	4.7
250	Plasma	5.0	4.0	4.2	5.3
	Whole blood	5.2	4.8	3.5	3.8
	Urine	4.5	4.6	4.0	5.1

are 10.5 and 12 min, respectively. Halofantrine and mefloquine are not detected at 343 nm.

The HPLC procedure described above was used for the assay of human whole blood and plasma samples obtained from a patient taking chloroquine for chemoprophylaxis. Whole blood chloroquine, M1, M2 and M3 concentrations were 1300, 440, 150 and 270 ng/ml, respectively. M1 and M2 accounted for 34 and 11.5% of the whole blood chloroquine concentrations. These values are in agreement with those reported in the literature [5,7]. In our study, M3 represented 21% of the whole blood chloroquine concentration. Previous studies [6,10,17] have shown that 4-amino-7-chloroquinoline represents a small amount of unchanged drug. However, Ette *et al.* [9] reported that this metabolite can amount to twice that of chloroquine in healthy volunteers who had taken chloroquine chronically.

In plasma samples, only chloroquine, M1 and M2 were found, at concentrations of 330, 200 and 8 ng/ml, respectively (66 and 3.5% of plasma chloroquine concentrations).

The method was also applied to the assay of samples obtained from a subject who had ingested chloroquine in a suicide attempt. The concentrations of chloroquine and its metabolites were determined in whole blood and plasma collected regularly during the time course of poisoning. In all samples, we found only chloroquine and desethylchloroquine (M1). Fig. 4a shows the time course of whole blood chloroquine concentrations, which can be described by a biexponential curve. The mean ratio of whole blood M1 to chloroquine concentrations was 3.9 ± 1.3%, ranging from 1.3 to 5.4% (n = 8) (Fig. 4b). In six plasma samples the mean ratio of desethylchloroquine to chloroquine concentrations ranged from 7.8 to 12.6% (mean ± S.D., 9.5 ± 1.9%). This result seems to indicate that the distribution of chloroquine and its main metabolites in red blood cells is different. In contrast, Verdier *et al.* [18] reported a similar distribution of chloro-

TABLE II

RECOVERY OF THE ANALYTICAL METHOD

CLQ = chloroquine; M1 = desethylchloroquine; M2 = bisdesethylchloroquine; M3 = 4-amino-7-chloroquinoline; *n* = number of determinations.

Concentration (ng/ml)	Sample	Recovery (mean ± S.D., *n* = 5) (%)			
		CLQ	M1	M2	M3
25	Plasma	78 ± 1.5	80 ± 2.0	65 ± 3.1	83 ± 2.0
	Urine	80 ± 3.0	85 ± 2.5	68 ± 2.5	80 ± 2.2
100	Plasma	85 ± 4.0	81 ± 1.3	82 ± 2.5	90 ± 3.5
	Urine	88 ± 1.0	79 ± 0.5	89 ± 3.2	88 ± 4.5

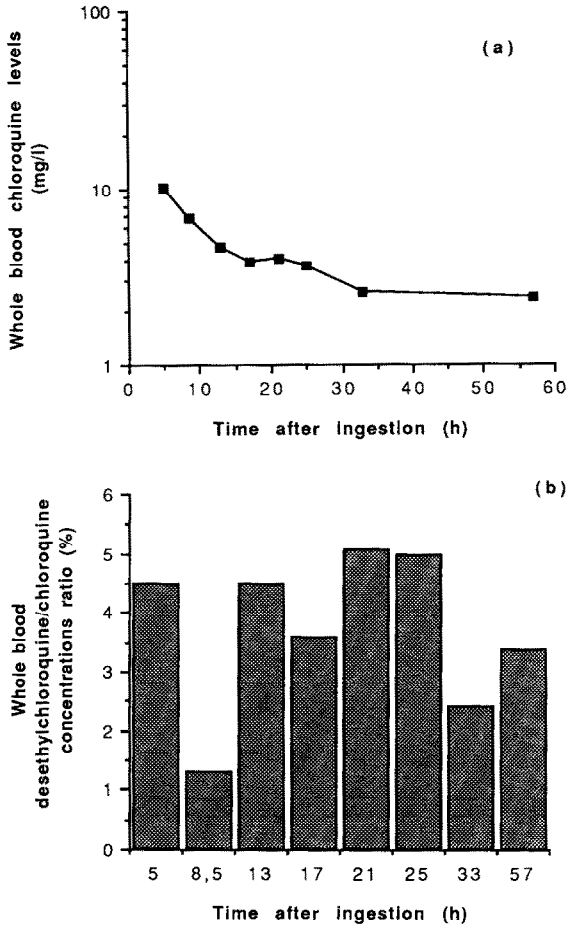


Fig. 4. Time courses of (a) whole blood chloroquine concentration and (b) whole blood M1/chloroquine concentration ratio during acute poisoning.

TABLE III

URINARY EXCRETION OF CHLOROQUINE AND ITS METABOLITES DURING ACUTE POISONING

CLQ = chloroquine; M1 = desethylchloroquine; M2 = bis-desethylchloroquine; M3 = 4-amino-7-chloroquinoline.

Time after ingestion (h)	Urine output (ml)	CLQ (μg/ml)	M1 (μg/ml)	M2 (μg/ml)	M3 (μg/ml)
9	400	334.4	14.5	7.4	0.9
33	1600	110.4	9.6	5.9	–[b]
57	–[a]	847.4	99.4	24.7	1.8

[a] Not determined.
[b] Not detected.

quine and desethylchloroquine in red blood cells in healthly volunteers and malaria patients.

Chloroquine and metabolite excretion was determined daily in the urine samples (Table III). In all samples we found the main chloroquine metabolites (M1 and M2), but M3 was not always excreted as reported previously [17]. Three days after poisoning, M1 and M2 represented 11.7 and 2.8%, respectively, of the excreted chloroquine. The present results on M1 differ from those of previous pharmacological studies which reported that M1 and M2 accounted for 25 and 3%, respectively, of unchanged excreted drug [17,19].

In two urine samples, 4-amino-7-chloroquinoline was detected in small amounts (0.2–0.3% of the chloroquine excreted). These values agree with previous studies [17,19].

In conclusion, the HPLC assay described showed good reproducibility, sensitivity, accuracy and selectivity. It has the advantage of being a relatively convenient and simple procedure and can be used for pharmacokinetic studies, therapeutic monitoring, diagnosis and prognosis of chloroquine poisoning.

ACKNOWLEDGEMENT

We thank Dr. Leonardo Basco for helpful criticism of the manuscript.

REFERENCES

1 P. Kintz, S. Ritter-Lohner, J. M. Lamant, A. Tracqui, P. Mangin, A. A. J. Lugnier and A. J. Chaumont, *Human Toxicol.*, 7 (1988) 140.
2 A. M. Bradley-Moore, B. M. Greenwood, A. K. Bradley, B. R. Kirkwood and H. M. Gilles, *Ann. Trop. Med. Parasitol.*, 79 (1985) 575.
3 M. Ostensen, N. D. Brown, P. K. Chiang and J. Aarbakke, *Eur. J. Clin. Pharmacol..*, 28 (1985) 357.
4 C. O. Khoromana, C. C. Campbell, J. J. Wirima and D. L. Heymann, *Am. J. Trop. Med. Hyg.*, 35 (1986) 465.
5 E. W. McChesney and P. E. Carson, *J. Pharmacol. Exp. Ther.*, 151 (1966) 482.
6 L. L. Gustafsson, O. Walker and G. Alvan, *Br. J. Clin. Pharmacol.*, 15 (1983) 471.
7 A. F. Aderounmu, *Am. Trop. Med. Parasitol.*, 78 (1984) 581.
8 E. W. McChesney and C. D. Fitch, in W. Peters and W. H. G. Richards (Editors), *Antimalarial Drugs II. Current Antimalarial and New Drug Developments*, Springer, New York, 1984, p. 3.

9 E. I. Ette, E. E. Essien, W. O. Thomas and E. A. Brown-Awala, *J. Clin. Pharmacol.*, 29 (1989) 457.
10 Y. Bergqvist and B. Domeij-Nyberg, *J. Chromatogr.*, 272 (1983) 137.
11 G. Alvan, L. Ekman and B. Lindström, *J. Chromatogr.*, 229 (1982) 241.
12 N. D. Brown, B. T. Poon and J. D. Chulay, *J. Chromatogr.*, 229 (1982) 248.
13 L. A. Salako, A. F. Aderounmu and O. Walker, *Bull. WHO*, 65 (1987) 47.
14 W. Olaapo, A. H. Dawodu, A. A. Adeyokunnu, L. A. Salako and G. Alvan, *Br. J. Clin. Pharmacol.*, 16 (1983) 701.
15 G. Edwards, S. Looareeensuwan, A. J. Davies, Y. Wattanagoon, R. E. Phillips and D. A. Warrell, *Br. J. Clin. Pharmacol.*, 25 (1988) 477.
16 E. Pussard, F. Verdier and M. C. Blayo, *J. Chromatogr.*, 374 (1986) 111.
17 A. Viala, A. Durand, J. P. Cano and J. Jouglard, *J. Eur. Toxicol.*, 72 (1972) 189.
18 F. Verdier, F. Clavier, P. Deloron and M. C. Blayo, *Pathol. Biol.*, 32 (1984) 359.
19 M. Frisk-Holmberg, T. Bergqvist, E. Termand and B. Domeij-Nyberg, *Eur. J. Clin. Pharmacol.*, 26 (1984) 521.