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SIMULTANEOUS DETERMINATION OF CHLOROQUINE, AMODIAQUINE AND THEIR METABOLITES IN HUMAN PLASMA, RED BLOOD CELLS, WHOLE BLOOD AND URINE BY COLUMN LIQUID CHROMATOGRAPHY

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SUMMARY

A column liquid chromatographic method for the simultaneous determination of chloroquine, amodiaquine and their monodesethyl metabolite in human plasma, red blood cells, whole blood and urine is described. The drugs and internal standard were extracted as bases with methylene dichloride and then re-extracted into an acid aqueous phase. Separation was obtained using a reversed-phase column and a mobile phase of phosphate buffer (pH 3.0)—acetonitrile (88:12). The absorbance of the drugs was monitored at 340 nm with a sensitivity limit of 10 pmol/ml. No endogenous compound interfered at this wavelength. The mean overall recovery from each biological fluid was > 75%. This method can be applied to therapeutic, pharmacokinetic and epidemiological studies. The metabolism of these two amino-4-quinolines in humans is compared.

INTRODUCTION

The widespread resistance of *Plasmodium falciparum* to chloroquine is a considerable problem for the chemoprophylaxis and treatment of malaria. The lack of cross-resistance frequently observed between chloroquine and amodiaquine suggests that the latter might be an alternative drug for prophylaxis and treatment of *P. falciparum* malaria in chloroquine-resistant areas [1].

Several methods have been developed to determine chloroquine and its monodesethyl metabolite in biological fluids using gas chromatography [2, 3] and column liquid chromatography (high-performance liquid chromatography, HPLC) [4—7], but none of these procedures has been suitable for amodiaquine. The spectrofluorimetric assay described by Trenholme et al.

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[8] was not specific enough to distinguish between amodiaquine and its metabolites. Recently, an HPLC assay of amodiaquine in human plasma was described, but the metabolites were not detected after intravenous injection [9]. The main metabolite, monodesethylamodiaquine, was later identified after oral administration of amodiaquine [10, 11].

Because of frequent self-medication with chloroquine, both amodiaquine and chloroquine are often found in the blood of malarial patients during epidemiological studies. It was therefore necessary to develop a rapid, selective and sensitive method for the simultaneous assay of chloroquine, monodesethylchloroquine, amodiaquine and monodesethylamodiaquine. The reversed-phase HPLC method described here uses UV detection and permits the simultaneous assay in plasma, red blood cells (RBCs), whole blood and urine.

EXPERIMENTAL

Chemicals

Chloroquine diphosphate (CQ) was obtained from Sigma (St. Louis, MO, U.S.A.); monodesethylchloroquine base (CQM) and the internal standard (I.S.) 6,8-dichloro-4-(1-methyl-4-diethylaminobutylamino)quinoline were generous gifts from Sterling Winthrop Research Institute (Clichy, France); amodiaquine dihydrochloride (AQ) was kindly donated by Roussel UCLAF (Romainville, France) and monodesethylamodiaquine dihydrochloride (AQM) by Parke-Davis Division of Warner-Lambert (Ann Arbor, MI, U.S.A.). The chemical structures are shown in Fig. 1. Stock solutions containing 10 μmol/ml CQ or AQ were prepared in distilled water. The CQM and AQM stock solutions were prepared in methanol. Working standards were prepared from the stock solutions. Acetonitrile and methylene dichloride (chromatographic purity; Merck, Darmstadt, F.R.G.) were used. All other chemicals were analytical grade. Screw-capped polypropylene tubes (15 ml capacity) were used for extraction.

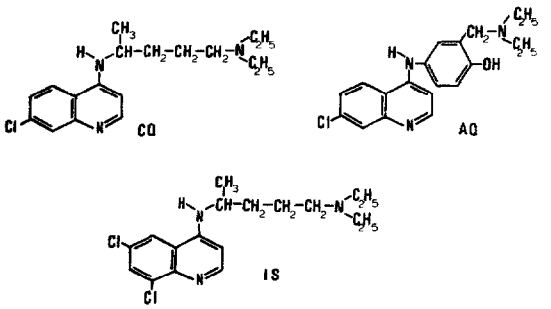


Fig. 1. The chemical structures of chloroquine (CQ), amodiaquine (AQ) and internal standard (I.S.).

Chromatographic equipment

The method was devised using a Waters liquid chromatograph equipped with a Waters Intelligent Sample Processor 710B (WISP®), an M6000 high-pressure pump, a Model 440 absorbance detector with 254- or 340-nm filters and an Omniscribe® recorder. Chromatography was carried out on a (15 × 0.39 cm I.D.) C_{18} Novapack column (Waters Assoc.) with a particle size of 5 μm.

Solvent system

The mobile phase for column equilibration and drug elution consisted of 45 m*M* potassium dihydrogen phosphate buffer (adjusted to pH 3.0 with orthophosphoric acid) containing 12% acetonitrile. Column elution was done at room temperature using a flow-rate of 0.6 ml/min and a pressure ranging between 66 and 72 bar.

Sample collection

One healthy human volunteer weighing 70 kg was given 2 mg/kg chloroquine base and 5 mg/kg amodiaquine base in a single oral dose. Blood samples were collected into EDTA tubes at times 0, 6 and 24 h after drug administration. An aliquot of whole blood was saved. Another was centrifuged at 1500 *g* for 15 min, and the upper two thirds of the plasma and the lower two thirds of RBCs were separated. These biological samples were immediately frozen and stored at −20°C. Total urine during the 6 h following drug administration was collected and an aliquot was frozen at −20°C. Drug-free plasma, RBCs, whole blood and urine were frozen for standard curve calibration.

Extraction procedure

Samples were thawed quickly just before extraction and mixed on a vortex for 10 s. RBCs and whole blood were haemolysed in distilled water and urine was diluted in distilled water (1:10). A 30-μl aliquot of 10 n*M* I.S. solution and a 250-μl aliquot of dipotassium hydrogen phosphate (pH 9.5) were added to 1-ml samples of plasma, RBCs or whole blood lysates of 500 μl of diluted urine in the extraction tube. Methylene dichloride (7 ml) was added to the mixture and the tubes were shaken in a reciprocal shaker (100 oscillations per min) at room temperature for 15 min. The aqueous phase was discarded after centrifugation. A 1-ml aliquot of 1 *M* sodium hydroxide was added. After shaking for 10 min (as above) and centrifuging, the aqueous phase was removed by aspiration. Then, 250 μl of 0.1 *M* hydrochloric acid were added. After shaking for 15 min and centrifuging, 100—200 μl of the aqueous phase were injected into the chromatograph.

Calibration, recovery and precision

Evaluation of the assay was carried out using six standard calibration points in the concentration range 30—1000 nmol/l CQ, CQM, AQM and AQ in the four biological media. The calibration curves were obtained by linear regression of the peak-height ratios of each drug or metabolite/I.S. versus the concentration of each drug. Because of the wide concentration range found for these drugs in the various biological samples, different calibration curves were drawn and the amount of I.S. was adjusted accordingly. Recovery was calculated by comparing the measured values of spiked samples of each medium with those of standard aqueous solutions at two concentrations (100 and 600 nmol/l). Intra-assay precision was determined at two concentrations (50 and 600 nmol/l) for ten analyses. Inter-assay precision was determined once at the same two concentrations in five replicates.

RESULTS

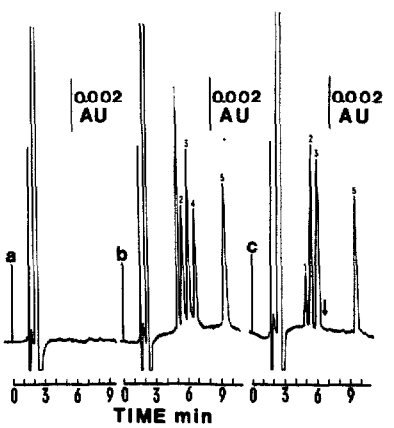
Figs. 2b, 3b, 4b and 5b illustrate typical chromatograms obtained in the assay of spiked plasma, RBCs, whole blood and urine. Optimal separation was obtained by adjusting the pH of the mobile phase to exactly 3.0 and the acetonitrile—buffer ratio to 12:88, precisely. The retention times for CQM, AQM, CQ, AQ and I.S. were 4.5, 5.0, 6.0, 7.0 and 10.0 min, respectively.

Extraction recoveries of drugs and metabolites are shown in Table I. All values were > 70% at two concentrations in each biological fluid. The recovery of I.S. averaged 91 ± 2.1% (n = 10) for a concentration of 300 nmol/l.

The within-day and day-to-day coefficients of variation for each drug (50 and 600 nmol/l) in the four media are described in Table II. These values (always < 10%) demonstrate that the reproducibility of the method is good.

The standard curves for drugs and metabolites in each biological fluid exhibited excellent linearity over the concentration range 30—1000 nmol/l. The coefficient of correlation was consistently at least 0.997 ($P < 0.001$). An example of a calibration curve in plasma is shown in Fig. 6. In each biological fluid, the slope of CQM is greater than that of CQ, but the slopes of AQ and AQM do not differ significantly. Thus, the same calibration curve can be used for AQ and AQM.

Figs. 2a, 3a, 4a and 5a show plasma, RBCs, whole blood and urine blank extract detected at 340 nm. There is no peak interference with drug or I.S. detection at this wavelength, but endogenous components extracted at pH 9.5 produced chromatographic interference at 254 and 229 nm. However, the molar extinction coefficient for these drugs was slightly less at 340 than at 254 nm, but the peak detection for AQM was more selective, therefore, there was



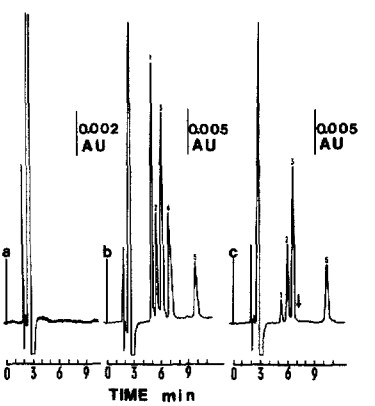
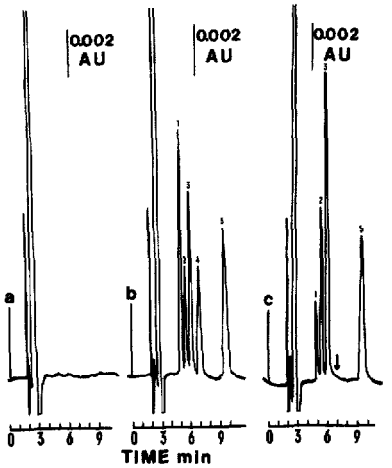


Fig. 2. Chromatograms obtained from human plasma samples. (a) Blank plasma; (b) control plasma containing 100 pmol/ml AQ, AQM, CQ, CQM and 300 pmol/ml I.S.; (c) subject's plasma 6 h after a single oral dose of amodiaquine (5 mg/kg) and chloroquine (2 mg/kg). Peaks: 1 = CQM; 2 = AQM; 3 = CQ; 4 = AQ; 5 = I.S.

Fig. 3. Chromatograms obtained from human red blood cell samples. (a) Blank; (b) control red blood cells containing 300 pmol/ml AQ, AQM, CQ, CQM and 250 pmol/ml I.S.; (c) subject's red blood cells 6 h after a single oral dose of amodiaquine (5 mg/kg) and chloroquine (2 mg/kg). Peaks: 1 = CQM; 2 = AQM; 3 = CQ; 4 = AQ; 5 = I.S.



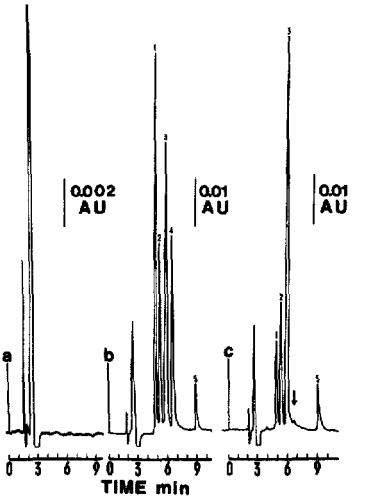


Fig. 4. Chromatograms obtained from human whole blood samples. (a) Blank; (b) control whole blood containing 100 pmol/ml AQ, AQM, CQ and CQM and 300 pmol/ml I.S.; (c) subject's whole blood 6 h after a single oral dose of amodiaquine (5 mg/kg) and chloroquine (2 mg/kg). Peaks: 1 = CQM; 2 = AQM; 3 = CQ; 4 = AQ; 5 = I.S.

Fig. 5. Chromatograms obtained from human urine samples. (a) Blank; (b) control urine containing 800 pmol/ml AQ, AQM, CQ, CQM and 450 pmol/ml I.S.; (c) subject's urine 6 h after a single oral dose of amodiaquine (5 mg/kg) and chloroquine (2 mg/kg). Peaks: 1 = CQM; 2 = AQM; 3 = CQ; 4 = AQ; 5 = I.S.

TABLE I

RECOVERY OF THE ANALYTICAL METHOD (n = 5)

Concentration (nmol/l)	Sample	Recovery (mean ± S.D.) (%)			
		CQM	AQM	CQ	AQ
100	Plasma	95 ± 4.1	83 ± 1.0	97 ± 4.5	83 ± 3.4
	Red blood cells	87 ± 1.8	75 ± 1.1	89 ± 4.1	76 ± 2.0
	Whole blood	92 ± 3.2	82 ± 3.3	91 ± 2.2	80 ± 3.1
	Urine	96 ± 2.5	89 ± 3.1	98 ± 2.5	91 ± 4.6
600	Plasma	92 ± 2.0	81 ± 1.8	93 ± 1.4	85 ± 1.6
	Red blood cells	88 ± 3.5	78 ± 3.0	88 ± 3.2	73 ± 2.5
	Whole blood	91 ± 4.0	79 ± 2.1	90 ± 4.0	79 ± 3.9
	Urine	97 ± 4.1	82 ± 2.6	98 ± 3.0	86 ± 4.2

no appreciable loss in analytical sensitivity. The minimum detectable concentration for simultaneous determination of the four compounds was ca. 10 pmol/ml (i.e. 3.6 ng/ml for AQ, 3.3 ng/ml for AQM, 3.2 ng/ml for CQ and 2.9 ng/ml for CQM) by requiring a signal-to-noise ratio of greater than 5:1.

Figs. 2c, 3c, 4c and 5c show chromatograms of extracted samples from a 40-year-old healthy subject, 6 h after absorption of a single oral dose of amodiaquine (5 mg/kg) and chloroquine (2 mg/kg). The concentrations of CQM, AQM and CQ were 125, 634 and 665 nmol/l in whole blood, 30, 150 and 100 nmol/l in plasma and 122, 969 and 915 in RBCs, respectively. After

TABLE II

REPRODUCIBILITY OF THE HPLC METHOD

Concentration (nmol/l)	Sample	Coefficient of variation (%)			
		CQM	AQM	CQ	AQ
Within-day (n = 10)					
50	Plasma	2.2	6.4	3.6	2.8
	Red blood cells	4.3	9.1	4.6	7.9
	Whole blood	4.3	8.2	4.0	7.7
	Urine	1.7	4.4	1.6	3.1
600	Plasma	1.3	3.2	0.5	1.6
	Red blood cells	3.0	5.7	3.6	3.5
	Whole blood	1.9	4.9	2.1	2.0
	Urine	1.1	2.0	0.8	1.2
Day-to-day (n = 5)					
50	Plasma	2.8	7.5	5.1	6.3
	Red blood cells	3.4	9.1	6.9	8.3
	Whole blood	3.2	8.3	6.1	7.7
	Urine	3.2	4.1	3.4	4.5
600	Plasma	2.4	4.1	2.7	3.7
	Red blood cells	2.3	6.5	3.3	4.2
	Whole blood	2.2	5.9	3.1	3.9
	Urine	1.9	3.9	1.8	2.5

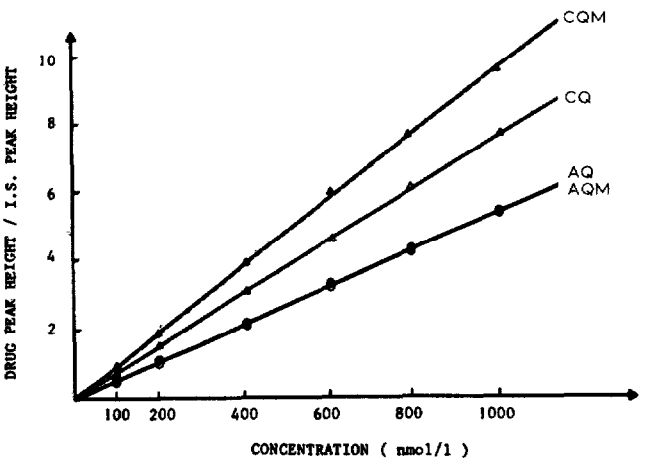


Fig. 6. Plasma calibration curves of (▲) monodesethylchloroquine, CQM (y = 0.0095x — 0.079, r = 0.9997), (△) chloroquine, CQ (y = 0.0074x — 0.09, r = 0.9997), (●) monodesethylamodiaquine, AQM (y = 0.00525x — 0.03, r = 0.9999) and (○) amodiaquine, AQ (y = 0.0053x — 0.05, r = 0.9994).

24 h, these values were: 59, 366 and 227 nmol/l in whole blood; no CQM detected; 73 and 37 nmol/l in plasma; 47, 468 and 342 nmol/l in RBCs. No amodiaquine peak was detected at 6 and 24 h after administration. In urine, the corresponding values were 6056, 14 900 and 31 800 nmol/l at 0.1 a.u.f.s.

An amodiaquine peak of 900 nmol/l was detected in the same sample at 0.01 a.u.f.s.

DISCUSSION

It can be seen in Figs. 2, 3, 4 and 5 that the separation method of the two drugs and their respective main metabolites could be used to separate any bisdesethyl metabolites present. They were not found in the samples in this study. The bisdesethyl metabolites are more polar and have shorter retention times (t_R) (bisdesethylamodiaquine: t_R = 3.0 min; bisdesethylchloroquine: t_R = 2.5 min).

The simultaneous determination of CQ and AQ is carried out by extraction at pH 9.5. In chloroquine assays, drug extraction is carried out at a minimum pH of 11 [4, 5, 7] but because the amodiaquine molecule is more acidic, it cannot be extracted from whole blood at this pH value.

The detection is more sensitive at 254 and 229 nm, but endogenous compounds also extracted at this pH interfere at these wavelengths. The Novapack column with 5-μm particles gives a result at 340 nm that is two to three times as sensitive as that obtained with a 10-μm Bondapak.

As described by Barrow [12], the metabolic pathways of these amino-4-quinolines are different. Chloroquine is weakly and slowly metabolized after oral administration [4, 5]. In whole blood, 85% unchanged CQ and 15% CQM is found 6 h after oral administration. But at 6 and 24 h after oral administration of AQ, only AQM is detected in the blood. The AQ half-life calculated by Mihaly et al. [9] was 4 and 10 h (in two subjects) after intravenous infusion. This shows that AQ is rapidly metabolized during a hepatic first pass after oral administration. Therefore, the metabolite AQM is the active form of the drug in prophylaxis and treatment.

In addition, AQM uptake by RBCs (RBC/plasma concentration) is less than CQ uptake [11].

During epidemiological studies, we have observed that self-medication with CQ for general ailments is frequent in tropical areas. Furthermore, in acute malaria, intravenous administration of quinine is the most effective therapy. Therefore, CQ, CQM and quinine blood levels could interfere with AQM assay. CQ, AQ, CQM and AQM were separated using the method described. Quinine retention time is greater than that of the I.S. This assay is suitable for pharmacokinetic studies in animals and man, and is also suitable for routine monitoring in epidemiological studies and for surveillance of *P. falciparum* drug resistance.

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