Journal of Chromatography, 383 (1986) 375—386
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3341

SENSITIVE ANALYSIS OF BLOOD FOR AMODIAQUINE AND THREE METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

DWIGHT L. MOUNT, LESLIE C. PATCHEN, PHUC NGUYEN-DINH, ANN M. BARBER, IRA K. SCHWARTZ and FREDERICK C. CHURCHILL*

Division of Parasitic Diseases, Center for Infectious Diseases, Centers for Disease Control, Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA 30333 (U.S.A.)

(First received April 22nd, 1986; revised manuscript received July 17th, 1986)

SUMMARY

A high-performance liquid chromatographic method using oxidative electrochemical detection has been developed for selective and sensitive quantification of the antimalarial drug amodiaquine and three of its metabolites in the blood of dosed individuals. The method requires only one extraction step and has detection limits of 1 ng/ml for amodiaquine and its metabolites desethylamodiaquine and bisdesethylamodiaquine and 3 ng/ml for 2-hydroxydesethylamodiaquine. Minor modification of the mobile phase preserves the chromatographic separation and allows ultraviolet spectroscopic detection, which, although appreciably less sensitive, permits monitoring of levels of amodiaquine and the three metabolites in blood and urine samples if an electrochemical detector is unavailable. Levels of amodiaquine and the three metabolites were determined for two volunteers undergoing a nine-week chemoprophylactic regimen in connection with travel to a malarious area. Data are included to compare the in vitro antimalarial activities against three strains of *Plasmodium falciparum* of amodiaquine and the three metabolites considered.

INTRODUCTION

The incidence of resistance in *Plasmodium falciparum* malaria to chloroquine (Cq) continues to increase in large areas of the developing world [1]. Amodiaquine (Am), also a 4-aminoquinoline antimalarial, has been therapeutically effective for certain resistant strains and has been tested in several developing regions of the world with documented Cq-resistant strains of *P. falciparum* (refs. 2 and 3 and citations therein). Interest in and use of Am

0378-4347/86/$03.50

have increased dramatically as a result [4]. With this increased use have come recent, disquieting reports of cases of agranulocytosis associated with Am [4, 5] that will affect the use of this antimalarial for chemoprophylaxis. Continued study of Am metabolism and refinement of analytical methods are needed to permit pharmacokinetic studies useful in characterizing the therapeutic effect of Am and in investigating possible causes for the side-effects occasionally associated with the drug.

Previous work in our laboratory [2, 3] has demonstrated that the blood of persons treated with Am contains little of the parent drug but significant quantities of two metabolites, desethylamodiaquine (DEAm) and 2-hydroxydesethylamodiaquine (HDEAm). This work identified two pathways by which Am is metabolized: (1) N-deethylation and (2) hydroxylation in the 2-position. Mihaly et al. [6] published a method for high-performance liquid chromatographic (HPLC) determination of Am itself using ultraviolet (UV) spectrophotometric detection in patients treated by intravenous infusion. No mention was made in this work regarding Am metabolism. This method was later modified by Winstanley et al. [7] to permit quantification of DEAm as well as Am. The article of Mihaly et al. [6] was published in 1985, contrary to the erroneous citation in the article of Winstanley et al. [7]. Pussard and coworkers published HPLC methods, also using UV detection, which determine Am and DEAm in blood and blood fractions [8] and Am, Cq, and their monodesethyl metabolites in blood, blood fractions, and urine [9]. The metabolite HDEAm was not identified in the studies reported in refs. 6—9. DEAm has been shown to have antimalarial activity similar to that of Am [2, 8]; however, recent work has shown that HDEAm has about 1% the activity of Am for two Cq-sensitive *P. falciparum* isolates tested [3]. In the present study we set out to develop an HPLC method to permit sensitive monitoring of Am and three metabolites and to determine the relative in vitro activities of these four compounds against several strains of *P. falciparum*.

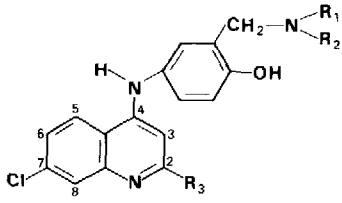
EXPERIMENTAL*

Standards

Amodiaquine base, desethylamodiaquine dihydrochloride, bisdesethylamodiaquine (bDEAm) dihydrochloride hemihydrate, and the isobutyl analogue of desethylamodiaquine dihydrochloride (the internal standard) were supplied by Parke-Davis Division of Warner-Lambert (Ann Arbor, MI, U.S.A.). A quantity of 2-hydroxydesethylamodiaquine, purified as described previously [3], was used as a standard. Structures of the free bases corresponding to each of these compounds are illustrated in Fig. 1. The standards were dissolved in 0.001 *M* aqueous hydrochloric acid and diluted in this same solvent to provide fortifying chromatographic standards of appropriate concentrations.

---

*Use of trade names and commercial sources is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.



Amodiaquine $R_1 = R_2 = CH_2CH_3$; $R_3 = H$.
Desethylamodiaquine $R_1 = CH_2CH_3$; $R_2 = R_3 = H$.
Metabolite II $R_1 = CH_2CH_3$; $R_2 = H$; $R_3 = OH$.
Tentatively identified as
2-hydroxydesethylamodiaquine

bis-Desethylamodiaquine $R_1 = R_2 = R_3 = H$.

Internal standard $R_1 = CH_2-CH(CH_3)-CH_3$; $R_2 = R_3 = H$.

Fig. 1. Structures of amodiaquine, three of its metabolites, and the internal standard compound.

Reagents and solvents

Methanol and ethyl acetate were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Ethanesulfonic acid was obtained from Eastman Kodak (Rochester, NY, U.S.A.). 1-Pentanesulfonic acid, sodium salt, was from Aldrich (Milwaukee, WI, U.S.A.). Diethylamine (Aldrich) was distilled before use. Disodium ethylenediaminetetraacetate (EDTA) was from Fisher Scientific (Fair Lawn, NJ, U.S.A.). All other chemicals used were of reagent grade or better.

Apparatus

The liquid chromatographic system consisted of a Spectra-Physics Model SP8700 ternary-gradient solvent delivery system, a Spectra-Physics Model SP8500 dynamic mixer, a Rheodyne Model 7125 loop injector, an Alltech Absorbosphere C_{18} reversed-phase column (5 μm particle diameter; 15 cm × 2.1 mm I.D.), a Bioanalytical Systems Model LC-4B amperometric detector with a glassy carbon electrode set at 0.80 V (versus the Ag/AgCl electrode, 3.0 *M* in NaCl) and a Spectra-Physics Model SP 4270 digital recorder—integrator. An Altex Ultrasphere ODS reversed-phase column (5 μm particle diameter; 15 cm × 2.0 mm I.D.) was used in later phases of the work.

The eluent contained 30 parts methanol (solvent A), 10 parts of an aqueous solution 0.025 *M* in sodium pentanesulfonate, 0.01 *M* in diethylamine, and 0.0002 *M* EDTA (solvent B), and 60 parts of an aqueous solution 0.025 *M* in ethanesulfonic acid, 0.01 *M* in diethylamine, and 0.0002 *M* in EDTA (solvent C). The three solvents were mixed by the ternary system to yield the indicated proportions. The flow-rate of the mobile phase was 0.95 ml/min. Chromatography was performed at ambient temperature. The pump pressure was ca. 34 MPa. A column heater with thermostat (Bio-Rad, Glattbrugg, Switzerland) was procured near the end of the study and used to provide a column tempera-

ture of 40°C. The increase in temperature permitted separation to occur at a column pressure of about 21 MPa. A mobile phase composition of 28% solvent A, 10% solvent B, and 62% solvent C at a flow-rate of 0.8 ml/min gave the same separation and analysis time at the higher temperature and lower column pressure as was achieved at room temperature and higher pressure.

Dosing of volunteers

A male volunteer (volunteer I) weighed 86 kg and received a single 400-mg dose (as base) of Camoquine (amodiaquine dihydrochloride dihydrate, Parke-Davis Division of Warner-Lambert). A single venipuncture blood collection was made 4 h after dosing. Sufficient blood (20 ml) was drawn to provide samples for studies of storage stability of analytes in whole blood.

Volunteer I and a second volunteer weighing 89 kg underwent a nominal Am chemoprophylaxis regimen, 5 mg/kg per week (400 mg as base per week) in connection with travel to Kenya, a country endemic for malaria. (Since volunteer I had taken the above-mentioned single dose of Am, 2.5 months had passed.) Venipuncture samples (5 ml) were taken at intervals to establish the concentration profile over time for Am and detectable, identifiable metabolites.

Determination of Am, DEAm, bDEAm, and HDEAm in blood

To 15-ml, screw-cap centrifuge tubes, each containing 300 μl of 1.5 ng/μl internal standard solution, were added 1-ml quantities of sample blood using 1-ml graduated serologic pipets. Each pipet was rinsed twice with 0.5 ml of water. Aqueous 50% K_2HPO_4 (1 ml) and ethyl acetate (6 ml) were added to each sample. The centrifuge tubes were capped and shaken on a reciprocal shaker at 240 cps for 30 min. After centrifugation to separate layers, each organic layer was transferred by Pasteur pipet to a clean tube, the solvent was evaporated on a 60°C water bath by a gentle stream of nitrogen, and 200 μl of mobile phase were added to reconstitute the sample. Each sample was sonicated for 10–15 min to break up the large globules of lipid which were present. Each reconstituted extract was filtered through a silanized glass wool plug in a disposable pipet tip (Item No. RT-20, Rainin Instruments, Emeryville, CA, U.S.A.) using low-speed centrifugation of the extract-filled tip in a 2-ml vial. The centrifugation forced the extract through the glass wool into the bottom of the vial and removed much of the lipid phase. Small amounts of emulsified lipid occasionally were observed in the filtrate but did not interfere with separation and quantification and had no apparent adverse effect on the column when injected. A 10-μl quantity of each sample extract was injected on the HPLC column. The samples were quantified by peak-area comparisons with curves generated from standards prepared by fortifying 1-ml quantities of control blood with each of the four analytes and carried through the above procedure.

Storage-stability study

Blood samples, withdrawn from volunteer I 4 h after he received a 400-mg (as base) dose of Camoquine, were analyzed in duplicate. A portion of the blood was stored in a refrigerator at 5°C and analyzed at weeks 1, 2, 4, 8, and 16.

In vitro sensitivity testing

The in vitro tests were conducted essentially as previously described [2, 3, 10]. An isolate from Southeast Asia (Indochina 1-CDC) known to be Cq-resistant as well as a Cq-susceptible isolate, NF-54 [11], and a Cq-susceptible cloned line of an isolate from Honduras (clone B_3 of isolate Honduras 1-CDC) were tested. Appropriate amounts of the parasites were collected from continous culture, diluted in uninfected human type O-positive erythrocytes, and resuspended in culture medium [RPMI-1640 supplemented with 30 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (HEPES) buffer and 10% type O-positive human serum] to yield a parasitemia of 0.2–0.7% at an erythrocyte suspension of 2%. Aliquots of 100 μl of this culture material were distributed into micro-titer plate wells, either control wells or wells predosed with the various compounds to be tested. From stock solutions of Am hydrochloride, DEAm hydrochloride, HDEAm hydrochloride or bDEAm hydrochloride in 0.001 *M* aqueous hydrochloric acid, serial dilutions had been made to give a total of ten different final concentrations from 30 to 0.001 μM Am, DEAm, HDEAm or bDEAm in buffered medium. Additional control wells had been predosed with the corresponding serial dilutions of hydrochloric acid alone. The plates were incubated for 48 h at 37°C, the cultures were harvested, Giemsa-stained thin smears were made, and the number of parasites per 10^4 erythrocytes were counted. The end-point for inhibition was the concentration of the compound that prevented any increase in parasite count during the incubation period.

RESULTS AND DISCUSSION

Characterization of the method

Ethyl acetate was chosen as extraction solvent because of its effectiveness in extracting all four analytes, including HDEAm. Only one extraction is required, which simplifies sample preparation but leaves the concentrated extract with suspended lipid matter after reconstitution in the mobile phase. The expedient of filtering each concentrated extract through silanized glass wool in a disposable pipet tip by centrifugation in a small vial substantially clarifies the extracts. Injection of a 10-μl aliquot from the filtrate in the bottom of the vial onto the HPLC column permits quantification of the four analytes.

The lower traces in Fig. 2 depict chromatograms resulting from the analysis of whole blood for Am and the three indicated metabolites, by HPLC with electrochemical detection (ED). Statistical evaluation of the results of whole blood analysis by this method for DEAm and HDEAm showed that precision was excellent for both analytes (Table I). Response was also found to be linear for Am and bDEAm to concentration levels well above those found in the blood of volunteers. The amounts of Am and bDEAm were small in such samples relative to the quantities of DEAm and HDEAm found. The linear least-squares relationship for Am in this experiment was $y = 0.002085x - 0.00500$. The corresponding equation for bDEAm was $y = 0.001067x - 0.00452$. Both linear functions gave r^2 values of better than 0.99. The detection limit is 1 ng/ml for Am, DEAm, and bDEAm and 3 ng/ml for HDEAm, defining minimum detectable peak height as that corresponding to three times

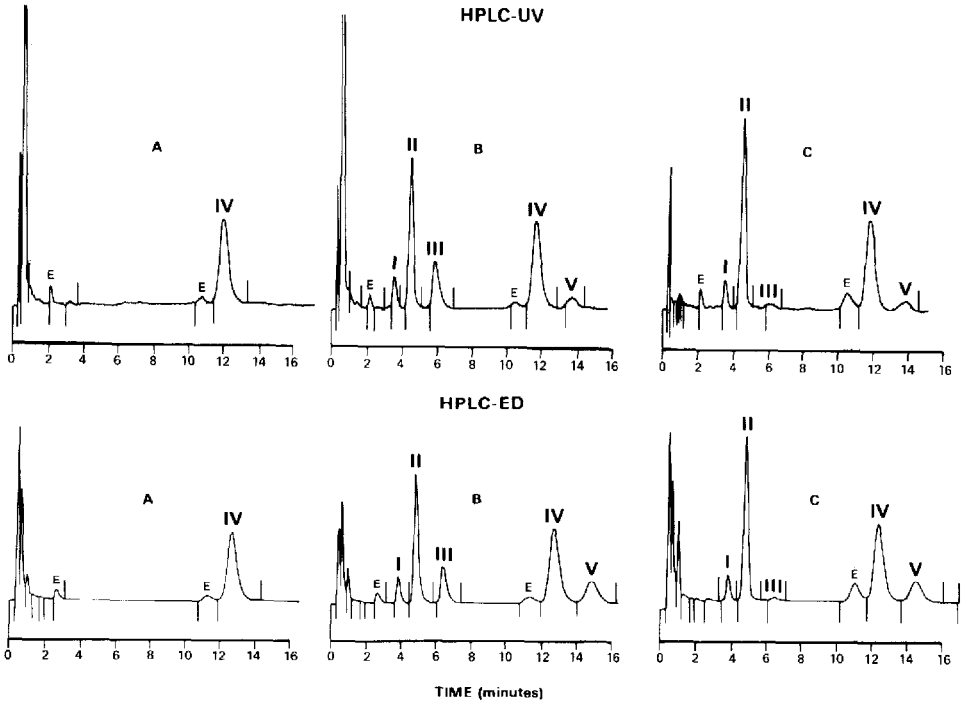


Fig. 2. Chromatograms resulting from application of the method to: (A) a 1.0-ml blood blank; (B) a 1.0-ml blood standard containing 162.0 ng of Am, 393.4 ng of DEAm, 248.9 ng of HDEAm, and 150.0 ng of bDEAm; (C) a 1.0-ml blood sample taken 4 h after volunteer I was given Am in the fifth week of the prophylaxis regimen. HPLC—ED was used to generate the three lower traces and HPLC—UV at 340 nm produced the three upper traces. Injection volume was 10 μl for ED-quantified determinations, 20 μl for UV-quantified determinations. Peaks: I = bDEAm; II = DEAm; III = Am; IV = internal standard; V = HDEAm; E = endogenous impurities.

peak-to-peak baseline noise. Extraction efficiences were 68, 80, 52, 69, and 84%, respectively, for Am, DEAm, bDEAm, HDEAm, and the internal standard.

Samples were stored in the refrigerator at 5°C and analyzed after 1, 2, 4, 8, and 16 weeks to determine whether any loss of DEAm or HDEAm was apparent during this period. No detectable decrease in the levels of these two analytes was seen.

Separation of the four analytes from each other and from two endogenous compounds, one of which elutes near the internal standard peak and the other near the bDEAm and DEAm peaks, was facilitated by the fine-tuning permitted by variation in the ratio of ethanesulfonate and pentanesulfonate concentrations. (See ref. 12 for a discussion of the use of mixed ion-pair reagents.) The use of ethanesulfonate yielded conditions in which HDEAm eluted much later than DEAm. This is in contrast to results obtained with a previous mobile phase containing heptanesulfonate in which HDEAm eluted before DEAm [3]. Thus, use of the ethanesulfonate results in the early elution of DEAm, the metabolite of greatest interest due to its activity, and the pentanesulfonate is added in sufficient amount to retard the elution of DEAm and the internal

TABLE I

STANDARD CURVE DATA FOR DEAm AND HDEAm IN 1-ml SAMPLES OF WHOLE BLOOD

Concentration added (ng/ml)	Metabolite/internal standard peak-area ratio	Concentration found* (ng/ml)
DEAm		
0.00	0.0000	3.72
39.4	0.0785	41.0
78.8	0.170	84.5
157.4	0.295**	143.9
	0.327	159.1
	0.314	152.9
	0.313	152.4
393.4	0.795	381.4
786.8	1.662	793.2
HDEAm		
0.00	0.0000	0.20
62.2	0.246	58.3
124.4	0.505***	119.4
	0.558	131.9
	0.491	116.1
	0.540	127.7
248.9	1.085	256.3
497.8	2.095	494.7

*Calculated from the least-squares straight line, $y = mx + b$ (for DEAm, $m = 2.105 \cdot 10^{-3}$, $b = -7.843 \cdot 10^{-3}$, $r^2 = 0.9994$, $n = 6$; for HDEAm, $m = 4.236 \cdot 10^{-3}$, $b = -8.428 \cdot 10^{-4}$, $r^2 = 0.9995$, $n = 5$).

**R.S.D. (inter-standard, $n = 4$) = 4.14%, R.S.D. (intra-standard, four injections) = 2.8%.

***R.S.D. (inter-standard, $n = 4$) = 5.89%, R.S.D. (intra-standard, four injections) = 3.0%.

standard relative to the two endogenous potential interfering compounds and permit separation (Fig. 2). The two different columns used provided comparable results except that, in our hands, the Altex column provided slightly better column efficiency and less tailing of peaks.

The standard DEAm was seen to have small amounts of two impurities, both of which eluted before the DEAm itself. The first-eluted impurity emerges before bDEAm in the chromatogram, while the second-eluted one emerges between the bDEAm and DEAm. A peak with retention time coincident with the second-eluted DEAm impurity was seen in some samples. When the peak separation of bDEAm was less than baseline from this following peak, peak-height rather peak-area comparisons were used for quantification of bDEAm. Area-percent estimates of the purity of the DEAm standard by both HPLC–UV and HPLC–ED gave values of about 95%. Because of the assumptions involved in this approach, a purity factor was not applied in calculating the DEAm results of Tables II and III. The concentration values for DEAm in these tables, however, are likely to be about 5% high.

The chromatographic method may be used with UV spectrophotometric detection at 340 nm to quantify Am and its metabolites as shown in the upper traces of Fig. 2. A wavelength of 254 nm or less may not be used because of

TABLE II

COMPARISON OF VALUES FOR DESETHYLAMODIAQUINE IN THE BLOOD OF A VOLUNTEER DETERMINED BY HPLC—ED AND BY HPLC—UV

Sample designation*		DEAm concentration (ng/ml)	
Week	Hour	HPLC—ED	HPLC—UV**
1	8	174.6	170.1
	168	63.8	51.1
2	8	322.4	314.1
4	168	162.4	144.2
5	4	577.1	572.0
	8	540.0	551.7
	168	139.0	130.6
8	8	444.9	430.8
	168	199.5	223.8
9	8	464.4	481.2
	168	213.6	205.4

*The volunteer received Camoquine, 400 mg as base per week for nine consecutive weeks.
**Observing wavelength was 340 nm.

interference from endogenous compounds. The sensitivity of UV spectrophotometric detection at 340 nm is substantially less than that for ED. Detection limits for Am, DEAm, and bDEAm are 15, 10, and 20 ng/ml, respectively, using a 20-μl injection volume and a criterion that the height of the minimum detectable peak be three times peak-to-peak baseline noise. The molar absorptivity of HDEAm is low at 340 nm [3] so that there is response discrimination against this analyte. The limit by UV detection is 100 ng/ml. These limits may be improved by injection of greater volumes of reconstituted extract from the 200-μl volume of extract. The only change in the procedure to permit UV detection at 340 nm is that no EDTA is included in the mobile phase. The EDTA, added to eliminate baseline drift for ED, itself causes a drift in detector response when UV detection is used. Table II compares results for DEAm by the two means of quantitation for a set of samples from a volunteer.

Volunteer study

The whole blood concentrations of Am and three metabolites found at selected times in two volunteers receiving nominal 5 mg/kg regimens of Am as malaria chemoprophylaxis while visiting Kenya are shown (Table III). Each volunteer weighed between 85 and 90 kg and received 400 mg of Camoquine as base per week. As seen in the data for volunteer I, the peak whole blood concentrations of DEAm increase during the first two weeks, but by the fifth week appear to have leveled off, peaking after each dose in the range 400—600 ng/ml. The other two metabolites, bDEAm and HDEAm, also increase in concentration over the first several weeks and have established a steady-state concentration–time profile by the fifth week. Fig. 3 illustrates the concentration–time relationship for Am and the three metabolites for volunteer I during and after weeks 8 and 9, the final weeks for chemoprophylaxis.

TABLE III

LEVELS OF AMODIAQUINE AND THREE METABOLITES IN THE BLOOD OF TWO VOLUNTEERS UNDERGOING CHEMOPROPHYLAXIS

Values are for volunteer I; values in parentheses are for volunteer II. N.D. = not determined.

Dosing week	Time after dosing (h)	Concentration (ng/ml)			
		Am	DEAm	HDEAm	bDEAm
1	4	61.4	174.9	107.5	27.2
	8	15.2	174.6	78.2	28.9
	24	1.6	116.7	53.6	22.6
	72	N.D.	76.7	29.8	N.D.
	168	<1.0	63.8	16.9	28.9
2	4	27.7	310.9	152.2	43.1
	8	18.7	322.4	144.6	50.4
	24	4.9	188.6	120.4	33.4
5	0	<1.0	162.4	74.2	48.5
	4	45.0	577.1	291.8	95.2
	8	24.0	540.0	243.2	103.6
	24	5.2	335.5	92.7	96.0
	72	<1.0	240.7	84.1	59.2
	168	<1.0	139.0	59.8	44.7
8	0	<1.0 (<1.0)	168.5 (72.9)	111.0 (36.0)	59.4 (11.2)
	4	43.2 (10.4)	420.6 (253.8)	189.4 (179.2)	112.6 (47.0)
	8	20.5 (5.5)	444.9 (276.1)	211.0 (143.8)	119.6 (51.6)
	24	5.1 (<1.0)	311.5 (191.7)	136.7 (91.0)	86.7 (32.5)
	72	<1.0 (<1.0)	260.5 (118.7)	103.5 (75.9)	80.3 (13.8)
	168	<1.0 (<1.0)	199.5 (125.1)	83.4 (84.4)	70.7(<1.0)
9	4	15.8 (8.9)	363.5 (335.6)	149.6 (181.2)	118.9 (70.8)
	8	11.5 (<1.0)	464.4 (282.2)	223.5 (179.9)	121.6 (90.8)
	24	9.1 (<1.0)	361.7 (270.0)	194.0 (94.8)	89.2 (35.0)
	72	<1.0 (<1.0)	278.7 (164.5)	120.3 (54.1)	67.4 (N.D.)
	168	<1.0 (<1.0)	213.6 (97.3)	84.7 (35.1)	71.9 (24.9)
	336	<1.0 (<1.0)	116.7 (57.3)	67.4 (30.5)	36.7 (19.0)
	504	<1.0 (<1.0)	99.7 (N.D.)	44.6 (N.D.)	42.1 (N.D.)
	672	<1.0 (<1.0)	88.9 (29.0)	28.8 (17.6)	41.9 (N.D.)
	1008	<1.0	62.9	23.5	28.8

Antimalarial activity of Am and three metabolites

Table IV provides data on the relative activity of Am and three metabolites when tested by an in vitro, 48-h test against two Cq-sensitive strains and one Cq-resistant strain of *P. falciparum*. Am was found to be equally effective in vitro against the two Cq-sensitive strains and the Cq-resistant strain. DEAm was seen to exhibit from 1 to 1/3 the activity of Am against the three strains. HDEAm was 1/30 to 1/100 as effective as Am, although in these tests bDEAm was about 1/3 as effective as Am for Cq-sensitive strains and 1/10 as effective for the resistant strain.

The data of Tables III and IV may be used to estimate the contributions to antimalarial activity of Am and the three metabolites, using the integrated

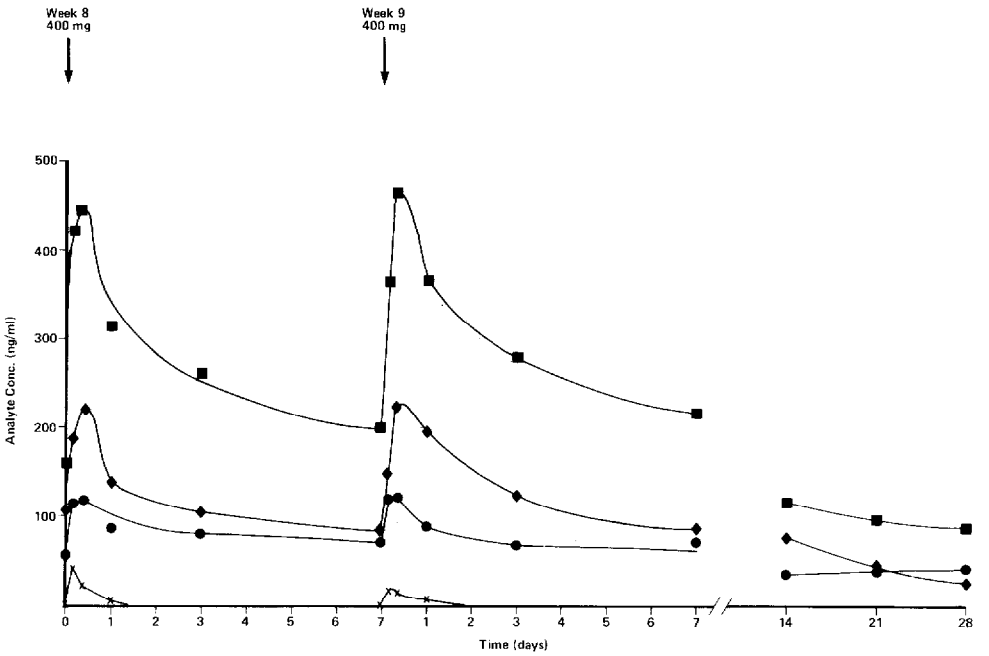


Fig. 3. Concentration versus time plots for blood levels of Am and its metabolites for volunteer I during weeks 8—12 of a nine-week chemoprophylactic regimen of Camoquine. (×) Am; (▪) DEAm; (♦) HDEAm; (●) bDEAm.

blood levels for Am and each of its metabolites through weeks 8 and 9 of the chemoprophylactic regimen (Fig. 3) and the estimates of the relative in vitro activities of the four compounds for the Indochina 1 strain of *P. falciparum* (Table IV). If the admittedly tenuous assumption is made that in vivo activity parallels in vitro activity, individual contributions to activity by each compound may be calculated by multiplying the integral (over the two-week period) of each compound's molar concentration by the estimated in vitro activity (Table IV). Contributions of each compound may then be divided by the summed products of all four compounds (total activity) for each volunteer and the Indochina 1 isolate to make a statement regarding the contributions of each compound to total activity. Such an evaluation attributes 88, 9, 2, and

TABLE IV

COMPARISON OF ACTIVITIES OF AMODIAQUINE AND THREE METABOLITES AGAINST SEVERAL *P. FALCIPARUM* STRAINS USING A 48-h IN VITRO TEST

P. falciparum strain designation	Inhibitory concentration* (μM)				
	Cq	Am	DEAm	HDEAm	bDEAm
NF 54	0.08	0.1, 0.1	0.3, 0.1	3, 3	0.3, 0.3
Indochina 1	0.6	0.1, 0.1	0.3, 0.3	10, 10	1.0, 1.0
HB 3	0.08	0.1, 0.1	0.3, 0.1	3, 3	0.3, 0.3

*Where two numbers are given under a compound heading, they represent results of separately run in vitro tests.

1% of the total activity to DEAm, bDEAm, Am, and HDEAm, respectively, for volunteer I and the Indochina 1 strain. The corresponding values for volunteer II are 93, 5, 1, and 1%, respectively. These examples indicate that, while DEAm is of central importance in considering the antimalarial effect of Am chemoprophylactic and curative regimens, the contributions of Am itself and other metabolites in the above cases, although small, are non-negligible. The in vitro test comparisons take place under conditions quite different from those present in vivo, and questions of variable distribution of the metabolites in vivo are pertinent. However, results of the calculations outlined above provide some perspective as to the likely contributions to antimalarial activity of the four analytes discussed.

Considerations in selecting an assay method for Am and its metabolites in blood

The present work and earlier reports [2, 3, 8, 9] serve to place the nature of the antimalarial effect of Am regimens in perspective. The present work and refs. 2 and 3 consider the whole blood concentrations and in vitro activities against a number of *P. falciparum* strains of metabolites resulting from both major primary metabolic pathways of Am. These three studies demonstrate that DEAm contributes the preponderance of antimalarial activity and is the most important entity for quantification in individuals who have received Am.

One may choose from among the several methods of analysis identified in the Introduction and those described in the present work, depending on the nature of the Am study planned. The method outlined in the present work using ED permits the most sensitive and complete assay to date of Am and known metabolites (DEAm, bDEAm and HDEAm). Demonstration of the relative inactivity of HDEAm makes assay of this metabolite of less practical interest. Our work (see above) suggests that bDEAm contributes a small but non-negligible activity, so that its determination is of some interest. The methods of Winstanley et al. [7] and Pussard et al. [9] permit determination of Am and DEAm and thus suffice for many purposes. Indeed, when interpreted in light of the current understanding of Am metabolism, use of the hydrolysis—fluorescence method of Trenholme et al. [13] should prove effective in estimating the level of active metabolite if DEAm is substituted for Am as standard.

The present work suggests the option of applying HPLC—ED to investigate the metabolism of important Am analogues in body fluids. Such analogues, which include amopyroquine, cycloquine, and pyronaridine, also contain moieties which can be expected to undergo facile two-electron oxidation to quinonimines to permit sensitive quantification by HPLC—ED.

REFERENCES

1 W.H. Wernsdorfer, W.H.O. Chron., 37 (1983) 11—13.
2 F.C. Churchill, L.C. Patchen, C.C. Campbell, I.K. Schwartz, P. Nguyen-Dinh and C.M. Dickinson, Life Sci., 36 (1985) 53—62.
3 F.C. Churchill, D.L. Mount, L.C. Patchen and A. Björkman, J. Chromatogr., 377 (1986) 307—318.
4 C.S.R. Hatton, C. Bunch, T.E.A. Peto, G. Pasvol, S.J. Russell, C.R.J. Singer, G. Edwards and P. Winstanley, Lancet, i (1986) 411—414.

5 Anonymous, Centers for Disease Control Morbidity and Mortality Weekly Report, 35 (1986) 165—166.
6 G.W. Mihaly, D.D. Nicholl, G. Edwards, S.A. Ward, M. L'E. Orme, D.A. Warrell and A.M. Breckenridge, J. Chromatogr., 337 (1985) 166–171.
7 P.A. Winstanley, C.L. Rodick, G. Edwards, M. L'E. Orme and A.M. Breckenridge, Br. J. Clin. Pharmacol., 21 (1986) 94P.
8 E. Pussard, F. Verdier, M.-C. Blayo and J.-J. Pocidalo, C.R. Seances, Acad. Sci. III (Paris), 301 (1985) 383—385.
9 E. Pussard, F. Verdier, and M.-C. Blayo, J. Chromatogr., 374 (1986) 111—118.
10 P. Nguyen-Dinh and W. Trager, Am. J. Trop. Med. Hyg., 29 (1980) 339—342.
11 J.D. Chulay, I. Schneider, T.M. Cosgriff, S.L. Hoffman, W.R. Ballou, I.A. Quakyi, R. Carter, J.H. Trosper and W.T. Hockmeyer, Am. J. Trop. Med. Hyg., 35 (1986) 66—68.
12 B.A. Bidlingmeyer, J. Chromatogr. Sci., 18 (1980) 525—539.
13 G. Trenholme, R. Williams, E. Patterson, H. Frischer, P. Carson and K. Rieckmann, Bull. W.H.O., 51 (1974) 431—434.