Journal of Chromatography, 414 (1987) 77–90
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3437

ANALYSIS OF ARTESUNIC ACID AND DIHYDROQINGHAOSU IN BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH REDUCTIVE ELECTROCHEMICAL DETECTION

Z.M. ZHOU

Department of Pharmacology, Institute of Chinese Materia Medica, Academy of Traditional Chinese Medicine, Beijing 100700 (China)

and

J.C. ANDERS, H. CHUNG and A.D. THEOHARIDES*

Department of Pharmacology, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC 20307-5100 (U.S.A.)

(First received July 22nd, 1986; revised manuscript received September 24th, 1986)

SUMMARY

A new high-performance liquid chromatography (HPLC) method using reductive electrochemical detection has been developed for the analysis of the antimalarial drugs artesunic acid (ARTS) and dihydroqinghaosu (DQHS) in blood. Presently, this method has been validated to 4 μg/ml for ARTS and 200 ng/ml for DQHS. Pharmacokinetic studies in the rabbit show that after intravenous administration (100 mg/kg) ARTS is metabolized rapidly to DQHS and has a $t_{1/2}$ of 1.7 min in blood. DQHS data were fit to non-linear regression models consisting of the sum of two exponential terms. For phases 1 and 2, $t_{1/2}$ values of 3.0 ± 0.4 and 29 ± 2 min were calculated, respectively. In vitro studies in which ARTS was incubated with blood from various species show that rabbit blood hydrolyzes ARTS at a much greater rate than rat or human blood. Incubation of ARTS with rabbit blood in the presence or absence of diisopropylfluorophosphate suggested that this hydrolysis reaction is catalyzed by plasma and red blood cell esterases. These results suggest that future pharmacokinetic studies in both animals and man should focus on the measurement of DQHS rather than ARTS.

INTRODUCTION

Over the last twelve years, the active antimalarial principle of the herb qinghao (*Artemesia annua* L.) has been isolated and chemically characterized by Chinese scientists [1–4]. This compound, a sesquiterpene lactone endoperoxide (Fig. 1) named qinghaosu (QHS) or artemisinin, and its synthetic derivatives have been shown to be more effective than chloroquine against the erythrocytic stages of

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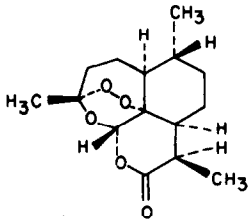

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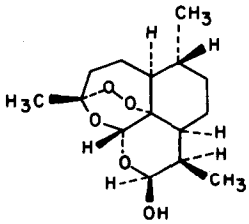
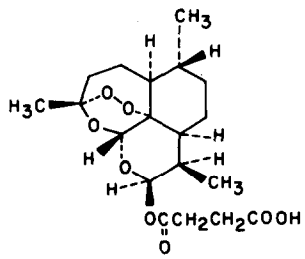


DIHYDROQINGHAOSU



ARTESUNIC ACID

Fig. 1. Structures of qinghaosu, dihydroqinghaosu and artesunic acid.

plasmodia, their parasiticidal action is faster, and they are equally effective against chloroquine-resistant strains of *Plasmodium falciparum* [5]. In recent years, over two thousand cases of vivax or falciparum malaria have been treated with QHS or its derivatives in China with excellent results [6]. Furthermore, no toxic reactions were observed in these trials [6] and it has been reported that QHS and its derivatives are superior to chloroquine with respect to chemotherapeutic index and side-effects [7].

This laboratory has recently become interested in studying the pharmacokinetics and metabolism of the water-soluble QHS ester derivative, artesunic acid (ARTS) and its metabolite, dihydroqinghaosu (DQHS) (Fig. 1). Previous studies on the pharmacokinetics of ARTS after intravenous (i.v.) administration suggested that ARTS is rapidly hydrolyzed to DQHS in rats [8]; therefore, DQHS was measured rather than ARTS. DQHS was measured by thin-layer chromatography (TLC) and staining with *p*-dimethylaminobenzaldehyde [8].

Considering the low dose of ARTS administered to humans (100 mg), the lack of sensitivity of the TLC method and the fact that little is known about the pharmacokinetics of ARTS and DQHS in other species, this laboratory sought to develop new specific and more sensitive methodologies to accurately measure both ARTS and DQHS in blood so that pharmacokinetic studies could be performed. Besides the obvious objective of developing more sensitive and specific methods, we hoped to unambiguously determine whether the rapid hydrolysis of ARTS to DQHS in vivo occurs in all species. If this is proven true, then future efforts, in animals and in clinical investigations, could focus on the measurement of DQHS for pharmacokinetic and pharmacodynamic studies. ARTS would only

be considered a "prodrug", utilized only for purposes of formulation and administration.

A wide variety of chromatographic techniques and approaches that could be utilized to obtain the specificity and sensitivity required were initially examined. Gas chromatographic techniques with or without derivatization were initially found to be inadequate because of the thermal lability of the compounds. Attempts to derivatize DQHS with a UV-absorbing reagent also proved unsuccessful because the reactions were not quantitative. Since our laboratory had previous experience using high-performance liquid chromatography (HPLC) with electrochemical detection (ED) [9], and because QHS in plant extracts is being measured successfully by HPLC with reductive ED [10], we decided to utilize this technique for the measurement of ARTS and DQHS in blood. In this paper, the results of validation experiments for ARTS in the low μg/ml range and for DQHS in the low μg/ml and mid ng/ml range are reported as well as the results of some in vivo and in vitro experiments obtained by utilizing these HPLC methodologies.

EXPERIMENTAL

Chemicals

QHS, DQHS and ARTS were provided by the Institute of Chinese Materia Medica, Academy of Traditional Chinese Medicine, Beijing, China. Their structures are depicted in Fig. 1. Reagent-grade ammonium acetate for HPLC was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Methyl *tert.*-butyl ether (MTBE) for extraction and acetonitrile for HPLC were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Sodium bicarbonate solution (7.5%) was obtained from Gibco Labs. (Grand Island, NY, U.S.A.) and was diluted to 3.75% for use with distilled, deionized water. Diisopropylfluorophosphate (DFP) was obtained from Sigma (St. Louis, MO, U.S.A.).

Standard solutions

All standard stock solutions of QHS (500 or 50 μg/ml) and DQHS (100, 10 or 1 μg/ml) were prepared daily in reagent-grade acetonitrile. Standard solutions of the sodium salt of ARTS were prepared daily by adding ARTS to 3.75% sodium bicarbonate solution for a final concentration of 500 μg/ml.

Extraction procedure for ARTS

Fresh heparinized (10 I.U./ml) human, rabbit (New Zealand White) or rat (Sprague–Dawley) blood (0.5 ml) was added to disposable glass extraction tubes (16×125 mm) followed by 5 μg (in 10 μl acetonitrile) of internal standard (QHS). Varying amounts of sodium bicarbonate solution and ARTS were added to samples such that the desired concentration of ARTS was obtained and that the total volume of sodium bicarbonate solution added was 0.1 ml. Samples for the standard curve contained 0, 1, 5, 10, 25 or 50 μg of ARTS and validation samples contained 2.0–40 μg ARTS. After the addition of ARTS, the sample was immediately vortexed for 10 s, then 4 ml of acetonitrile were added, and the sample

was again vortexed for 20 s. The addition of ARTS and acetonitrile was performed one sample at a time to prevent hydrolysis. The samples were centrifuged at 2400 *g* at 5°C for 5 min. The supernatant was decanted off into clean extraction tubes and evaporated to dryness under a low stream of nitrogen at room temperature. The samples were dissolved in 0.5 ml mobile phase for analysis by HPLC–ED.

Extraction procedure for DQHS

To disposable extraction tubes (16×125 mm), varying amounts of DQHS in acetonitrile were added and acetonitrile was evaporated off with a Buchler vortex evaporator. Heparinized (10 I.U./ml) rat, rabbit or human blood was added to the tubes (0.5 ml for the μg range or 1.0 ml for the ng range), followed by 5 μg or 700 ng of internal standard (QHS) for the μg or ng ranges, respectively (in 10 μl acetonitrile). Samples for standard curves contained 0, 1.0, 2.5, 5, 10, 25 and 50 μg or 0, 100, 200, 500, 700 and 1000 ng of DQHS for the μg and ng ranges, respectively. After addition of blood and internal standard to the tubes containing DQHS, the samples were vortexed for 10 s each, 4.0 ml MTBE were added and the samples were vortexed for 30 s. (The addition of MTBE and subsequent vortexing is performed one sample at a time.) The samples were then centrifuged at 2400 *g* for 5 min, the upper MTBE layer was transferred to clean extraction tubes and evaporated to dryness with a Buchler vortex evaporator at room temperature. Samples were dissolved in 0.5 ml mobile phase for HPLC–ED analysis.

HPLC with reductive ED

The chromatographic system consisted of a Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), a Valco injector (Model C6U, Valco Instruments, Houston, TX, U.S.A.) with a 100-μl sample loop, an LC-4B amperometric electronic controller with an LC-19 transducer package [Bioanalytical Systems (BAS), West Lafayette, IN, U.S.A.], an SP4270 integrator (Spectra-Physics, San Jose, CA, U.S.A.), 250×4.6 mm I.D. or 250×2.0 mm I.D. 5-μm Spherisorb phenyl steel columns (Phase Sep, Thomson Instrument, Newark, DE, U.S.A.) and an MPLC Spheri 5 cyano 30×4.6 mm I.D. guard cartridge (Brownlee Labs., Santa Clara, CA, U.S.A.). All HPLC analyses reported in this paper were performed at ambient temperature. The mobile phase was aqueous 0.1 *M* ammonium acetate, pH 7.1–acetonitrile (82:18) and the flow-rate 1.5 ml/min for the 4.6 mm I.D. column and 0.7 ml/min for the 2.0 mm I.D. column. Samples were reconstituted in 0.5 ml mobile phase and 20% of each sample was injected after deoxygenation (see below). The electrochemical detector was operated in the reductive mode at an applied potential of −0.8 V using an Hg/Au thin-layer cell (Model TL-6A, BAS) and a Ag/AgCl reference electrode (Model RE-1, BAS), at a sensitivity of 32–128 nA f. s. The electrochemical detector signal was filtered at a maximum cut-off frequency of 0.02 Hz with Model 102A frequency filter (Spectrum Scientific, Newark, DE, U.S.A.).

Reductive ED

Since electrochemical detection in the reductive mode requires rigorous deoxygenation procedures, the liquid chromatography system was configured as rec-

ommended by BAS with the following modifications: 3.2 mm O.D. and 1.6 mm O.D. stainless-steel tubing is used throughout the apparatus; since the special steel connector recommended by BAS to connect the column outlet to the cell clogged frequently, regular 0.2 mm (I.D.) tubing was used with combination nut and ferrule PTFE finger-tight fittings. The mobile phase was kept in a 5-l reflux apparatus, the condenser was cooled with a refrigerated circulating water-bath containing ethylene glycol–water (50:50). After preparation and initial deoxygenation, the mobile phase was continuously purged with argon at a flow-rate of 10 ml/min, which was regulated with a needle valve. Mobile phase was then pumped through the system and recycled back into the mobile flask. (Mobile phase can be used for approximately one month, however a gradual increase in retention is observed due to slow preferential acetonitrile evaporation.) Sample deoxygenation before injection was accomplished by purging the sample with distilled, deionized water saturated argon for approximately 1 min. Loading the sample into the injector loop was performed by immersing the injector inlet line into the sample. Positive pressure forced the sample into the loop and the injection was then made.

Quantitation

Quantitation of DQHS or ARTS was achieved by using peak-height ratios of either compound to internal standard (QHS). For each analysis, a standard curve was generated by adding known and varying amounts of DQHS or ARTS and a constant amount of the internal standard to either human, rat or rabbit blood. Spiked samples were treated as unknowns to evaluate the precision and accuracy of the methods. The recovery (%) was determined by comparison of the peak heights of DQHS or ARTS for each unknown sample to an external standard curve of μg DQHS or ARTS on-column versus peak height.

In vivo infusion and sampling methodologies

New Zealand white male rabbits weighing 2–3 kg were used to determine the feasibility and utility of the developed methodologies. Animals were housed in standard cages and given food (Rabbit Chow, Ralston Purina, St. Louis, MO, U.S.A.) and water ad libitum. Animals were placed in a Nalgene® restraint cage for the 2-h duration of the experiment. Drug was administered as a bolus injection (less than 30 s) i.v. in the marginal ear vein. Samples for ARTS and DQHS analysis were removed from the medial ear artery of the opposite ear according to the method of Paulsen and Valentine [11]. The sodium salt of ARTS was prepared by dissolving ARTS in 3.5% sodium bicarbonate (Gibco Labs.) at a concentration of 200 mg/ml. This solution was administered to the animal as a bolus injection such that the dose was 100 mg/kg. Heparinized blood samples were frozen immediately after sampling with a dry ice–acetone bath and kept at $-70\,^{\circ}C$ for the duration of the experiment. Samples were then thawed at room temperature, extracted and analyzed immediately as described above. Blood concentration–time data were analyzed using model equations with an iterative nonlinear curve fitting computer program with a non-weighted least-squares crite-

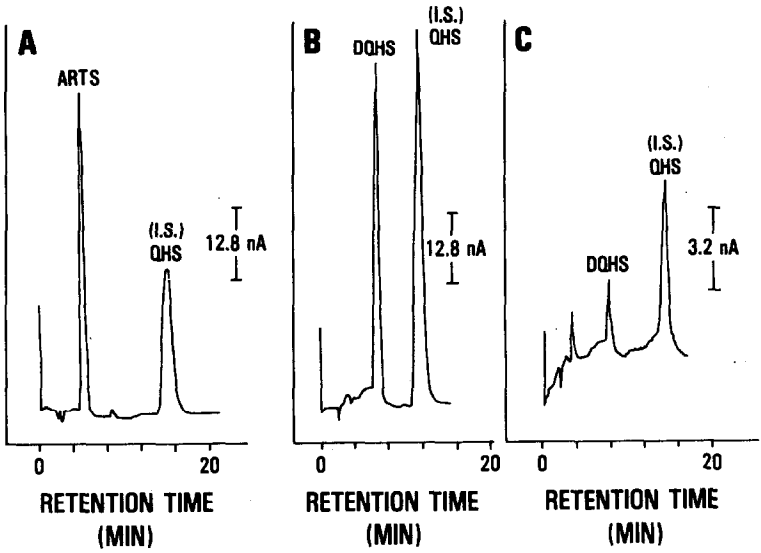


Fig. 2. Chromatograms of human blood spiked with (A) ARTS (10 μg per 0.5 ml), (B) DQHS (2.5 μg per 0.5 ml) and (C) DQHS (200 ng/ml). Chart speed; 0.25 cm/min. Analysis performed with 250×4.6 mm I.D. column.

rion of fit [12]. Three experiments were performed in the same animal with approximately two weeks between experiments.

In vitro studies on the hydrolysis of ARTS in blood

Species differences in the hydrolysis of ARTS were examined by incubating heparinized (10 I.U./ml) rat, rabbit or human blood with ARTS (100 μg/ml) in a Dubnoff incubator at 37°C. ARTS was added after a 5-min preincubation and 0.5-ml samples were removed at various time points and extracted and assayed as described above.

Experiments to determine whether the hydrolysis of ARTS to DQHS in blood is catalyzed by endogenous esterases were conducted as described above except that whole blood was preincubated for 30 min in the presence or absence of $5 \cdot 10^{-4}$ *M* DFP. Addition of DFP to blood was accomplished by dissolving it in saline–ethanol (95:5) at $5 \cdot 10^{-2}$ *M* and adding this solution directly to the blood (10 μl/ml of blood). Equal volumes of saline–ethanol (95:5) were also added to control samples.

RESULTS*

Separation

Fig. 2 illustrates typical chromatograms obtained with human blood samples spiked with either ARTS or DQHS with the 4.6 mm I.D. HPLC column. ARTS elutes at 5.0 min, DQHS at 7.3 min and the internal standard, QHS, at 13.7 min.

*All confidence intervals reported in this paper represent standard deviation of the mean.

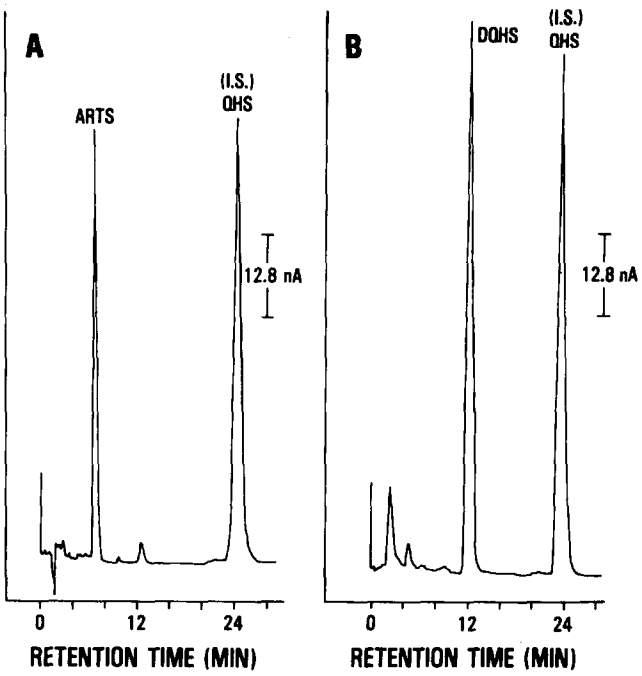


Fig. 3. Chromatograms of rabbit blood spiked with (A) ARTS (5.0 μg per 0.5 ml) and (B) DQHS (5.0 μg per 0.5 ml). Analysis performed with 250×2.0 mm I.D. column.

Fig. 3 shows typical chromatograms obtained from rabbit blood samples spiked with either ARTS or DQHS and chromatographed on the 2.0 mm I.D. column. ARTS elutes at 6.0 min, DQHS at 11.0 min and internal standard, QHS, at 22.0 min. Excellent resolution is obtained in both chromatographic systems. These chromatograms are also representative of those obtained from unknown samples. Analysis of blank blood extracts showed that endogenous blood contaminants do not interfere with the quantitation of these compounds. It is also readily apparent from the chromatograms, especially Fig. 2C (30 ng DQHS on-column), that the ultimate sensitivity of the system has not yet been approached (see Fig. 2C).

Linearity

The graphical relationships between the peak-height ratio of ARTS or DQHS to QHS versus amount of ARTS or DQHS added to blood were always linear. Mean coefficients of determination (r^2) and y-intercepts were: 0.988 ± 0.015 and -0.3 ± 0.9 for ARTS ($n=10$); 0.995 ± 0.005 and 0.3 ± 1.1 for DQHS in the μg range ($n=5$); and 0.992 ± 0.006 and 35 ± 13 for DQHS in the ng range ($n=4$).

*Precision and accuracy**

Precision and accuracy of the methods for ARTS and DQHS were determined by the analysis of replicate spiked samples. Table I shows that for analysis of

*Results were obtained from samples which were spiked individually rather than as a pool because of lability of compounds. Precision and accuracy data would improve if blood was spiked once. Tables I–III show all validation experiments that were performed and is not a selected representation.

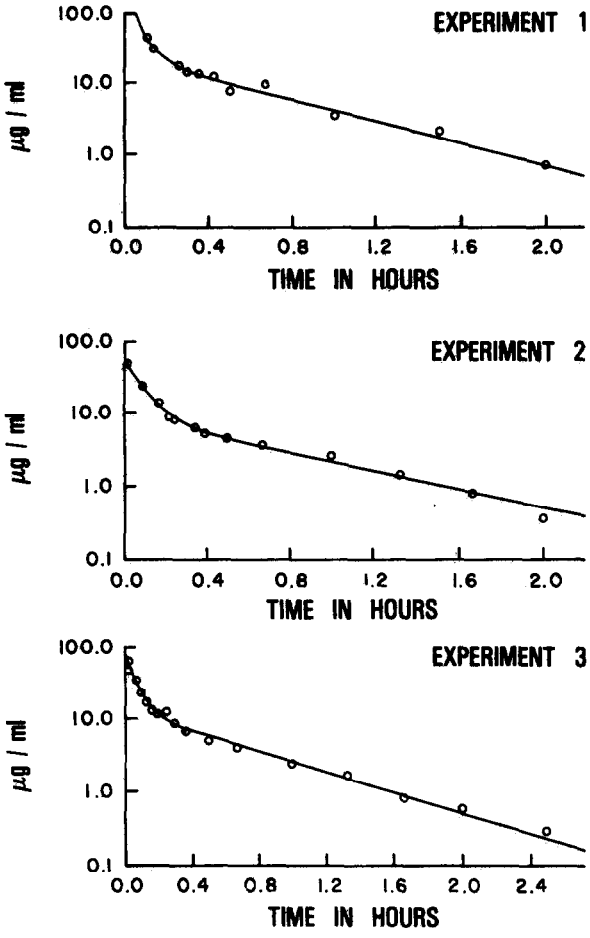


Fig. 4. Whole blood concentrations of DQHS versus time after intravenous administration of ARTS (100 mg/kg). Solid line represents the curve of fitted regression equation.

ARTS in human blood in the low μg range a mean coefficient of variation of 13% and a mean error in accuracy of 9% were determined for four different experiments on four different days. The results from similar experiments with DQHS in the low μg range are presented in Table II. Mean coefficient of variation was 7% overall with an overall mean error in accuracy of 8%. Validation results for analysis of DQHS in the ng range are shown in Table III for 200 and 500 ng/ml samples. Overall mean coefficient of variation and accuracy error were 5.2 and 4.5%, respectively, for four different experiments and five samples at each DQHS level and experiment.

Recovery

Extraction efficiencies for both ARTS and DQHS were determined by running appropriate external standard curves during the same time period as analysis of validation samples, calculating the amount of ARTS or DQHS actually put on-

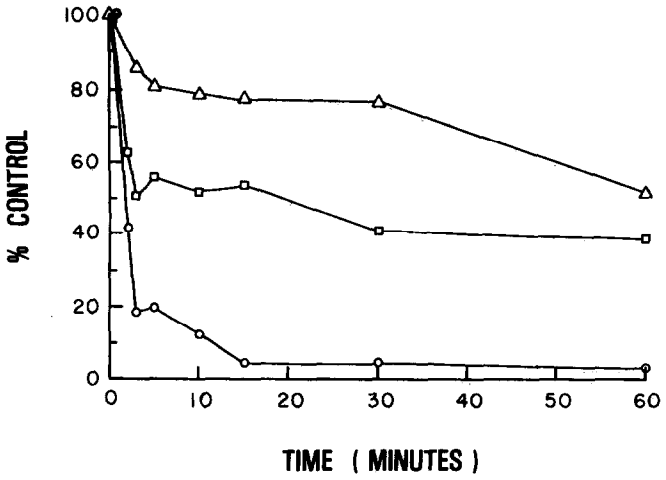


Fig. 5. Time course of disappearance of ARTS (50 μg/ml) during incubation with rabbit (○), rat (△) or human (□) blood.

column from the external standard curve and then dividing this amount by the amount of DQHS or ARTS that should have been present if the extraction efficiency was 100%. Mean recovery of ARTS was 49.7 ± 8.3% ($n=15$). Mean recovery for DQHS was 99 ± 9% ($n=11$) and 72 ± 7% ($n=8$) for μg and ng ranges, respectively.

Stability studies

The stability of both ARTS and DQHS in frozen blood stored at −20°C for one, three or ten days or one and ten days, respectively, was studied. Experimental design and data are shown in Table IV. ARTS was not stable in frozen blood for any duration of storage when compared to either the daily control group or the experiment control group. DQHS was stable when stored for one day when compared to both control groups, but not stable after ten days of storage (significantly different than both control groups).

TABLE I

PRECISION AND ACCURACY DATA FOR ANALYSIS OF ARTS IN BLOOD

Data represent a compilation of four separate experiments. A standard curve of 2–100 μg of ARTS per ml blood bracketed spiked unknowns.

Amount added (μg/ml)	Amount measured (mean ± S.D.) (μg/ml)	Coefficient of variation (%)	Accuracy* (%)	*n*
4.0	5.0 ± 1.0	20	20	15
10.0	10.1 ± 0.9	9	1	10
40.0	38.6 ± 2.0	9	4	15
80.0	90.4 ± 12	13	12	10

*Accuracy is expressed as the percentage difference of the mean of the amount measured versus amount of drug added.

TABLE II

PRECISION AND ACCURACY DATA FOR THE ANALYSIS OF DQHS IN BLOOD IN THE μg RANGE

Data represent a compilation of six separate experiments. A standard curve of 2–100 μg of DQHS per ml blood bracketed spiked unknowns.

Amount added (μg/ml)	Amount measured (mean ± S.D.) (μg/ml)	Coefficient of variation (%)	Accuracy* (%)	n
5.0	5.5 ± 0.5	10	9	10
10.0	9.7 ± 0.9	9	1	10
50.0	53.3 ± 2.0	4	7	15
100.0	84.2 ± 4.3	5	16	10

*See footnote of Table I.

In vivo experiments

Extremely rapid disappearance of ARTS in blood was observed after i.v. administration in the rabbit. Attempts to obtain an adequate number of blood samples for pharmacokinetic analyses were unsuccessful in experiments 1 and 2. In experiment 3, blood levels of 90, 83, 75, 22, 13 and 6 μg/ml were measured at 1.0, 1.5, 2.0, 4.5, 6.0 and 8.0 min post-dosing, respectively. Linear regression analysis of these data showed that ARTS has a $t_{1/2}$ of 1.7 min in the rabbit ($r^2 = 0.996$; change in log ARTS concentration with respect to time). Fig. 4 shows the blood levels of DQHS measured after i.v. administration of 100 mg/kg of the sodium salt of ARTS to a rabbit and a plot of the computerized non-linear regression best fit of the data. These data were obtained from the same three experiments from which the ARTS data described above were collected. Blood levels ranged from approximately 66 μg/ml at 1.0 min post-dosing and decreased to 293 ng/ml at 150 min post-dosing (experiment 3). Excellent fits of the data ($r^2 = 0.993 \pm 0.003$) were obtained with a two-compartment open model consisting of the sum of two exponential terms [12]. Of the total area under the curve (11.1 ± 0.8 μg h/ml), 95% was characterized during the sampling period and distribution and elimination half-lives of 3.0 ± 0.4 min (α) and 29 ± 2 min (β) were calculated for phase

TABLE III

PRECISION AND ACCURACY DATA FOR THE ANALYSIS OF DQHS IN BLOOD IN THE ng RANGE

Data represent a compilation of four separate experiments. A standard curve of 100–1000 ng of DQHS per ml blood bracketed spiked unknowns.

Amount added (ng/ml)	Amount measured (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Accuracy* (%)	n
200	186 ± 10	5.4	7	20
500	488 ± 25	5.1	2	20

*See footnote of Table I.

TABLE IV

STABILITY OF DQHS AND ARTS IN FROZEN HUMAN BLOOD: EFFECTS OF DURATION OF STORAGE

Samples were spiked and treated as described in Experimental with the following exceptions: after spiking, samples for storage were quickly frozen in a dry ice–acetone bath and then stored at −20°C until analysis. Samples were thawed at room temperature and then extracted and analyzed immediately along with control samples. Target [DQHS] = 500 ng/ml (n = 5); target [ARTS] = 10 μg/0.5 ml (n = 5), N.D. = not determined.

Duration of storage[a] (days)	Concentration measured (mean ± S.D.)	Coefficient of variation (%)	Percentage of experiment control	Percentage of daily control
DQHS (ng/ml)				
Experiment control	477 ± 9	2	–	–
Daily control	500 ± 61	12	105[b]	–
1	456 ± 69	15	96[b]	91[d]
Daily control	451 ± 30	7	95[b]	–
10	289 ± 42	15	61[c]	64[e]
ARTS (μg/0.5 ml)				
Experiment control	12.6 ± 1.1	8.7	–	–
Daily control	N.D.	–	–	–
1	9.3 ± 0.8	8.6	74[c]	N.D.
Daily control	10.1 ± 1.8	17.8	80[b]	–
3	7.9 ± 0.7	8.9	63[c]	78[e]
Daily control	10.8 ± 0.8	7.4	86[c]	–
10	7.0 ± 0.9	12.9	56[c]	65[e]

[a]Since new solutions of DQHS, ARTS and internal standard must be prepared daily, these experiments were designed to account for any differences in solutions preparation. Samples in experiment control and in day 1 and 10 groups were spiked with the same solutions of DQHS or ARTS. Experiment control group was analyzed immediately without freezing. Daily control group represents samples which were spiked with same solutions used to prepare standard curve for that day. Daily control groups were analyzed immediately after spiking without freezing and during the same time period as corresponding spiked controls.
[b]Not significantly different than experimental control group ($p \leqslant 0.05$).
[c]Significantly different than experimental control group ($p \leqslant 0.05$).
[d]Not significantly different than daily control group ($p \leqslant 0.05$).
[e]Significantly different than daily control group ($p \leqslant 0.05$).

1 and for phase 2, respectively. As shown in experiment 3 of Fig. 4, frequent sampling at very early times after ARTS administration failed to show an increase and peak in DQHS levels as would be expected for metabolite formation. This observation is unusual, but can be explained by the extremely rapid hydrolysis of ARTS to DQHS in rabbit blood.

In vitro experiments

Since the in vivo experiments demonstrated that ARTS is metabolized very rapidly to DQHS in the rabbit, some in vitro experiments were performed to determine whether the reaction occurs in blood and is enzymatic in nature. Experiments with blood from other species were also performed to determine if

the rapid hydrolysis is a species-specific phenomenon. Incubation of rabbit blood with ARTS results in a rapid disappearance of ARTS (see Fig. 5) and corresponding increase in DQHS (not shown), suggesting that ARTS is enzymatically hydrolyzed to DQHS in blood. Preincubation of rabbit blood with DFP before addition of ARTS inhibited the hydrolysis 100% over the entire 30-min time course of the experiment, whereas control incubations showed a profile of hydrolysis very similar to that shown in Fig. 5. These studies strongly suggest that the hydrolysis is catalyzed by esterases in the blood, since DFP is a potent irreversible inhibitor of acetyl- and butyrylcholinesterases [13]. Fig. 5 also shows the time course of disappearance of ARTS in rat and human blood. Both rat and human blood metabolize ARTS to DQHS, however, the rates and extents of the reaction are much lower than in rabbit blood.

DISCUSSION

In this study, methodologies have been developed utilizing HPLC with reductive ED to determine blood levels of ARTS and DQHS. Different extraction procedures for the two compounds had to be utilized, because of the acidic versus neutral functional groups on the two molecules and because of the lability of ARTS. The extraction procedure utilized for ARTS (acetonitrile precipitation) could also be used for measurement of DQHS; however, much lower recovery of DQHS is obtained with this method than with MTBE extraction.

The methods were validated in the low μg range for both compounds and ng range for DQHS. Although it is felt that higher sensitivities are attainable with this method, we chose to validate the assays in these ranges so that pharmacokinetic studies could be started, and at the same time, studies to increase the sensitivity of the method could be pursued. It is likely that a ten- to twenty-fold increase in sensitivity could be achieved if the problem of baseline drift at high gain can be overcome. Lewis et al. [14] have shown that the signal-to-noise ratio of the baseline is a function of the amount of dissolved oxygen in the mobile phase. Although rigorous deoxygenation procedures have been incorporated into these methodologies, the use of elevated mobile phase temperature (40–50°C) has not yet been attempted. Furthermore, other investigators have also reported that small fluctuations in temperature have significant effects on baseline stability [15]. We are currently investigating whether this problem can be resolved by taking measures to control these parameters with greater precision. During the development of these methodologies, the potential of using a zinc scrubber column before the analytical column to remove any remaining dissolved oxygen from the mobile phase as reported by MacCrehan and May [16] was extensively investigated. This approach improved baseline stability and signal-to-noise ratio significantly, and initially seemed very promising. However, a major drawback to utilizing this procedure is that zinc ions resulting from reaction with oxygen from the scrubber column slowly contaminated the analytical column and dramatically decreased the retention of the compounds of interest. This decrease in capacity factor (k') occurred slowly over two-week time periods and could be partially reversed by washing the column with 0.05 M oxalic acid. As suggested

by MacCrehan and May [16], incorporation of an ion-exchange column after the scrubber column could correct this difficulty. This approach was not attempted since it was felt that control of these additional parameters would further complicate the system.

QHS was chosen as internal standard for the methods because we felt that much better accuracy and precision could be achieved than with a structurally dissimilar molecule. The only drawback to using QHS as an internal standard is the possibility that DQHS might be oxidized in vivo to QHS. However, this possibility is unlikely considering the other areas of the molecule prone to oxidative attack. We also examined whether QHS was formed in vivo by administering ARTS intravenously into a rat, drawing blood 30 min after infusion and assaying for QHS. No measurable QHS was found. Furthermore, samples from the rabbit pharmacokinetic studies reported above which were not spiked with QHS showed no traces of QHS in the chromatograms.

Our in vivo and in vitro studies confirm the observations of the Chinese investigators [8] that ARTS is rapidly hydrolyzed to DQHS, and also demonstrate that the reaction occurs in whole blood. Therefore, we feel that further studies on increasing the sensitivity of the methods should focus on DQHS and not on ARTS. In vivo semiautomated microdilution assays with DQHS and ARTS have demonstrated a time dependent activity of ARTS against cultures of *P. falciparum* [17]. While both drugs exhibit an equimolar activity in long-term assays (24 h), DQHS is five-fold more potent than ARTS using short-term assays [17]. This efficacy data also suggests that measurement of DQHS is much more relevant from the pharmacodynamic perspective. The results reported in this paper in conjunction with the antimalarial data suggest that the antimalarial activity of ARTS results from rapid hydrolysis of ARTS to DQHS in the cultured blood cells.

In summary, these results do show that this technique is a workable and sensitive means of detecting and quantitating these compounds. The methods developed thus far are adequate to accomplish our initial objective which was to begin animal pharmacokinetic studies and unambiguously determine whether measurement of DQHS after i.v. infusion of ARTS represents a valid and rational approach to studying the absorption, disposition, metabolism and excretion of ARTS. Further studies to improve the sensitivity of the methods are in progress.

ACKNOWLEDGEMENTS

The authors greatly appreciate the excellent technical assistance of Mr. Mitchell Kim and Mr. Hector Velazquez and also would like to thank Dr. William F. Ridder for the help provided in the in vivo experiments. Z.H.Z. received financial support from the UNDP/World/WHO Special Programme for Research and Training in Tropical Diseases.

REFERENCES

1 4th Meeting of the Scientific Working Group on the Chemotherapy of Malaria, Beijing, People's Republic of China, WHO Report TDR/CHEMAL-SWG(4)/QHS/81.

2 Qinghaosu Research Group, Scientia Sinica, 23 (1980) 380.
3 J. Liu, M. Ni, J. Fan, Y. Tu, Z. Wu, Y. Qu and W. Chou, Acta Chim. Sinica, 37 (1979) 129.
4 China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalarials, J. Trad. Chin. Med., 2 (1982) 3.
5 China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalarials, J. Trad. Chin. Med., 2 (1982) 17.
6 China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalarials, J. Trad. Chin. Med., 2 (1982) 45.
7 China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalarials, J. Trad. Chin. Med., 2 (1982) 31.
8 China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalarials, J. Trad. Chin. Med., 2 (1982) 25.
9 J.C. Anders, A.D. Theoharides, L.M. Thomas, M.H. Smyth and H. Chung, J. Chromatogr., 311 (1984) 117.
10 N. Acton, D.L Klayman and I.J. Rollman, Planta Medica, 5 (1985) 445.
11 R. Paulsen and J.L. Valentine, Lab. Animal, 13 (1984) 34.
12 R.E. Desjardins, C.L. Pamplin, J. von Bredow, K.G. Barry and C.J. Canfield, Pharmacol. Ther., 26 (1979) 372.
13 P. Taylor, in L.S. Goodman and A. Gilman (Editors), The Pharmacologial Basis of Therapeutics, Macmillan, New York, 7th ed., 1985, pp. 110–115.
14 J. Lewis, J. Zodda, E. Deutsch and W. Heineman, Anal. Chem., 55 (1983) 708.
15 L. Elrod, Jr., Curr. Sep., 7 (1986) 31.
16 W.A. MacCrehan and W.E. May, Anal. Chem., 56 (1984) 625.
17 W. Milhous, 1986, unpublished results.