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Note

Ion-paired liquid chromatographic method for the analysis of blood and plasma for the antimalarial drug halofantrine and its putative mono-debutylated metabolite

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Halofantrine hydrochloride, 1,3-dichloro-6-trifluoromethyl-9-[1-hydroxy-3-(di-*n*-butylaminopropyl)phenanthrene] (I) hydrochloride*, a phenanthrene-methanol (Fig. 1), is an antimalarial drug with demonstrated in vitro and in vivo activity against both chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum* [1], one of the most pernicious malarial species in humans. In order to study the relationship between dose, blood level, and effect, a reliable quantitative method to estimate the concentration of this drug is necessary.

Methods available to quantitate I in blood and plasma are limited. A published high-performance liquid chromatographic (HPLC) assay [2] requires extensive washing and overnight extraction of 2-ml blood samples, yet the 10 ng/ml assay detection limit is insufficient for pharmacokinetic studies in humans. The radioimmunoassay (RIA) suffers from limited sensitivity and specificity due to cross-reaction of antibodies with metabolites [3]. In a paper describing a liquid chromatographic–mass spectrometric (LC–MS) method, which did not contain clinical data, a detection limit of 1 ng/ml was reported [4].

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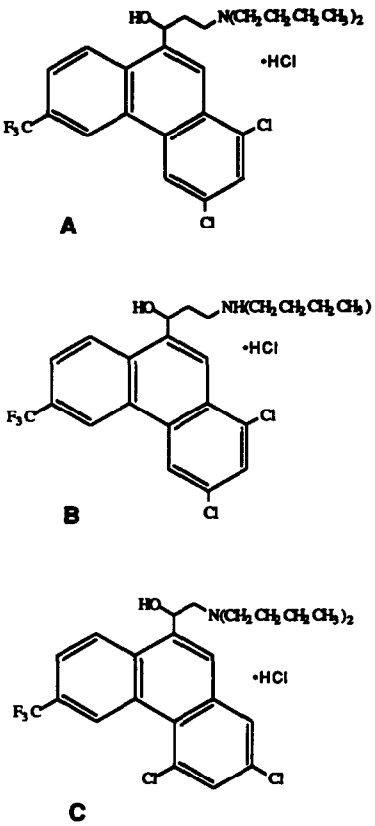

Fig. 1. Molecular structures of (A) the antimalarial drug halofantrine (hydrochloride), (B) putative metabolite 1,3-dichloro-6-trifluoromethyl-9-[1-hydroxy-3-(*n*-butylaminopropyl)-phenanthrene] (hydrochloride) and (C) internal standard, 2,4-dichloro-9-(2-dibutylamino-1-hydroxy)ethyl-6-trifluoromethylphenanthrene hydrochloride.

This paper describes the development of a simple and rapid HPLC assay for I and its metabolite, 1,3-dichloro-6-trifluoromethyl-9-[1-hydroxy-3-(*n*-butylaminopropyl)phenanthrene] (II) hydrochloride* (Fig. 1), in 0.5-ml samples of blood or plasma. Separation of the drug from endogenous components involved protein precipitation and a solid-phase cartridge elution step prior to HPLC analysis. Assay sensitivity was increased by use of fluorescence, rather than UV detection. The assay has been successfully used in several pharmacokinetic studies.

EXPERIMENTAL

The HPLC system employed a Beckman Model 110A pump (Beckman Instruments, Berkeley, CA, U.S.A.), a Perkin-Elmer Model 204 S fluorescence detector (Perkin-Elmer, Norwalk, CT, U.S.A.), a WISP 710 B autoinjector (Waters Assoc., Milford, MA, U.S.A.), a Hewlett-Packard HP 3392A integrator (Hewlett-

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Packard, Santa Clara, CA, U.S.A.), and an Altex C_8 bonded-phase column (5 μm particle size, 250 mm × 4.6 mm) (Beckman Instruments). Fluorescence detector settings were: 300 nm excitation wavelength, 375 nm emission wavelength, slits at 20 nm, sensitivity at 10, and photo multiplier gain at 4. The mobile phase consisted of acetonitrile–water (75:25), 0.2% (w/v) sodium lauryl sulfate (SDS), and 0.2% (v/v) glacial acetic acid. The flow-rate was 1.2 ml/min.

Acetonitrile (HPLC grade) was purchased from J.T. Baker (Pittsburgh, NJ, U.S.A.). SDS (99% purity) was obtained from Fluka (Hauppague, NY, U.S.A.) and was purified twice by recrystallization from methanol. I (bottle No. BK43807), II (bottle No. BK21070), and 2,4-dichloro-9-(2-dibutylamino-1-hydroxy)ethyl-6-trifluoromethylphenanthrene (III) hydrochloride (internal standard, bottle No. AX29250) (see Fig. 1) were furnished by the Walter Reed Army Institute of Research. Silanizing reagent was purchased from Aldrich (Milwaukee, WI, U.S.A.). Hydrochloric acid (36.5%) was obtained from Fisher Scientific (Santa Clara, CA, U.S.A.). All other chemicals were reagent grade. Blood and plasma for spiked samples were obtained from Irwin Memorial Blood Bank (San Francisco, CA, U.S.A.).

Stock and working solutions

Stock and working solutions were prepared. I and II were dissolved in methanol to make a stock I base solution of 0.90 mg/l and a stock II base solution of 1.4 mg/l. A 1:1 mixture of these stock solutions was used to spike biological samples. III, a geometric isomer of I, used as the internal standard, was prepared as a 50 μg/ml stock solution in methanol. The internal standard working solution was prepared by dilution of the stock solution to 100 ng/ml with acetonitrile.

Sample preparation

The assay required 0.5 ml blood or plasma for analysis. Water (0.5 ml) was added to all blood samples, which were then vortexed to lyse cells. Immediately afterwards, 1.5 ml of internal standard working solution and 1.0 ml of acetonitrile were added to blood samples to precipitate protein and to retard enzyme degradation of I and II. For plasma samples, just the 1.5 ml of internal standard working solution were added to precipitate protein. All samples were then vortexed for 20 s, blood samples were sonicated for 5 min, and all samples were centrifuged at 3000 *g* for 10 min. The supernatants were transferred to 500-mg C_8 Bond Elut columns (Analytichem International, Harbor City, CA, U.S.A.) and washed with 4 ml acetonitrile. The Bond Elut columns, which retained the drugs, were placed over silanized tubes into which the compounds of interest were eluted with 2 ml of acetonitrile containing 0.73% (v/v) hydrochloric acid. (The Bond Elut columns can be reused in this assay if they are washed with approximately 3-ml sequential rinses of eluting solution, water, acetonitrile, water, and acetonitrile. New cartridges were cleaned with approximately 3-ml sequential rinses of acetonitrile, water, methanol, water, and acetonitrile followed by the cleaning procedure described above for reuse of cartridges.) The eluted solutions were evaporated under nitrogen to dryness and the residue was redissolved in 200 μl

of mobile phase. One eigth to one third of the resulting solutions were delivered onto the HPLC column with a WISP autoinjector.

Blank blood and plasma samples (0.5 ml) were spiked with the I and II working solution to give standard curve concentrations ranging between 0.9 and 90 ng/ml for I and between 1.4 and 140 ng/ml for II. Standard curve blood samples were vortexed for 10 s, then equilibrated for 1 h at room temperature without stirring. Pooled blank blood and plasma were spiked at four concentrations of I and II, mixed on a rotator at room temperature for 1 h, and divided into 0.5-ml aliquots that were analyzed (precision and recovery samples) or kept frozen, then analyzed when appropriate (control and stability samples). Recovery was determined by comparing the I and II concentrations in spiked plasma and blood samples versus concentrations in spiked water samples at four concentrations within the linear range of the standard curve. Each recovery sample was prepared as described above, except the internal standard was not added until the nitrogen evaporation step, and the I and II spike in water was not passed through the C_8 Bond Elut cartridge. Recovery of I and II was then determined for each sample using the expression:

$$\text{recovery} = \frac{\text{peak-height ratio of compound to internal standard in plasma or blood}}{\text{peak-height ratio of compound to internal standard in water}}$$

To test drug stability, the 0.5-ml aliquots were stored at −20°C or −80°C until assayed. All other frozen samples were held at −80°C. Blind spiked plasma samples were provided by the Walter Reed Army Institute of Research to test the accuracy of the assay. All spiked blood and plasma samples were prepared for analysis as described above.

RESULTS

The assay procedures described above were used to obtain excellent results in the analysis of plasma and blood samples for I and II. Standard statistical measurements of linearity, precision, and accuracy were within acceptable limits. Also tested were recovery both of the drug and of its putative metabolite from blood and plasma. In addition, stability of both compounds in blood and plasma was measured at −20°C and −80°C.

Fig. 2 illustrates typical chromatograms for blank plasma, plasma spiked with I, its metabolite, and the internal standard, and plasma collected 144 h after a subject received a 500-mg oral dose of I (given as the hydrochloride). The separation was performed under isocratic conditions. The peaks of interest and the internal standard are baseline-separated. The retention times were 10.2, 16.9, and 22.0 min for II, I, and the internal standard, respectively. Flushing the column with acetonitrile–water (50:50) at the end of each analysis day restored column resolution and increased column life.

When standard curve samples in the general concentration range 0–100 ng/ml were analyzed by the linear regression method, the blood (plasma) concentra-

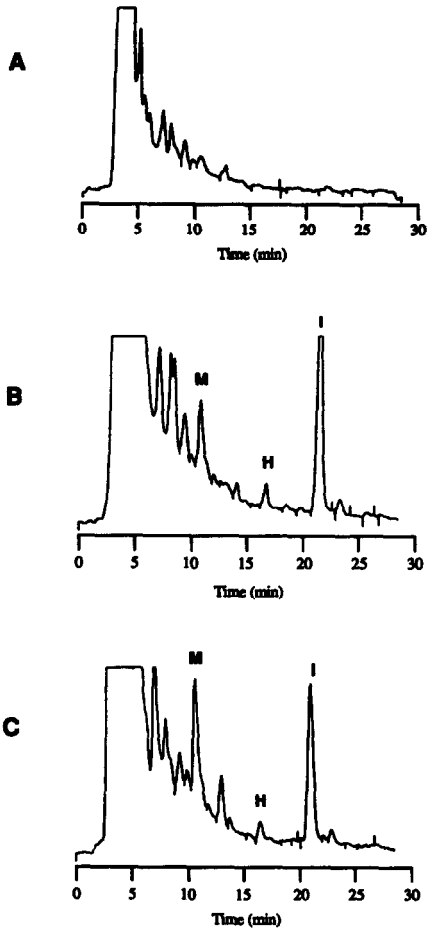

Fig. 2. Chromatograms of (A) blank plasma, (B) plasma spiked to 2.70 ng/ml with the halofantrine base (H), 4.20 ng/ml with the metabolite base (M), and with the internal standard (I) and (C) plasma sample collected 144 h after a 500-mg oral dose of halofantrine hydrochloride (concentrations are 8.22 ng/ml halofantrine base and 32.6 ng/ml metabolite base).

tions of the drug and its metabolite versus the respective peak-height ratios relative to the internal standard gave correlation coefficients (r^2) in the range 0.9983–0.9991. Precision of the method over the working range was determined by the analysis of replicate ($n=6$) spiked samples. The coefficients of variation (C.V.) for concentrations ranging from 4.21 to 90.6 ng/ml of I and II in either plasma or blood were below 10% in every case. The accuracy of the method for plasma concentration measurements was determined by the analysis for I in blind spiked plasma samples obtained from the Walter Reed Army Institute of Research. Spiked levels and calculated levels are presented in Table I. The limits of detection are 0.90 ng/ml for I and 1.40 ng/ml for II in plasma and blood (three times the signal to noise ratio). Thus, the method is accurate and precise for concentration measurements in the 0–100 ng/ml range.

Recoveries (mean ± S.D.) of I and II averaged 84.2 ± 3.8% and 94.0 ± 6.7%, re-

TABLE I

ACCURACY OF 1,3-DICHLORO-6-TRIFLUOROMETHYL-9-[1-HYDROXY-3-(DI-*n*-BUTYLAMINOPROPYL)PHENANTHRENE] CONCENTRATION MEASUREMENTS IN BLIND PLASMA SAMPLES

Number of samples	Spiked level (ng/ml)	Measured level (mean ± S.D.) (ng/ml)	Bias (%)
1	0	0	0
6	2.60	2.58 ± 0.18	− 0.77
5	17.4	19.2 ± 0.88	10.3
6	39.0	39.3 ± 1.89	0.77

spectively, for plasma samples and 93.9 ± 7.2% and 92.0 ± 7.3%, respectively, for blood samples in analyses at four concentrations within the linear range of the standard curve.

Stability of I and II was tested in blood at four concentrations by analysis of samples held at −80°C or at −20°C for up to two months. No appreciable degradation of I or II in blood was seen in samples stored two months at −80°C. However, at least 20% of I and II were degraded in samples stored less than one month at −20°C.

DISCUSSION

The adsorption to glassware by I, its metabolite, and the internal standard would adversely affect the reliability of any assay. These compounds all possess phenanthrenemethanol structures (Fig. 1), which have highly conjugated aromatic rings, and contain relatively long alkyl side-chains. Such non-polar structures presumably contribute to adsorption to glassware, which is especially noticeable when extremely low concentration measurements are necessary. In addition, I and II do not elute from the moderately non-polar C_8 Bond Elut or from an HPLC reversed-phase column when an acetonitrile–water mobile phase is used, but do elute when acid is added to the mobile phase. To take advantage of this property, the sample clean-up procedure includes protein precipitation by acetonitrile, removal of other undesirable endogenous materials from the sample with an acetonitrile wash, and subsequent elution from the C_8 column with an acetonitrile–hydrochloric acid mixture. Lower method detection limits were attained by restricting the absorption to glassware with use of silanized tubes and by the sample clean-up procedure before injection onto the HPLC column.

I, the metabolite, and the internal standard all possess a highly conjugated structure and are highly fluorescent. We chose to use fluorescence detection rather than UV detection, which resulted in a gain in selectivity, a reduction in background noise, and a reduction in the number of interference peaks. In this investigation, unlike the previous report in the literature [2], fluorescence detection was shown to increase sensitivity four-fold over that of UV detection at 254 nm.

To demonstrate the suitability of this assay for pharmacokinetic studies, sam-

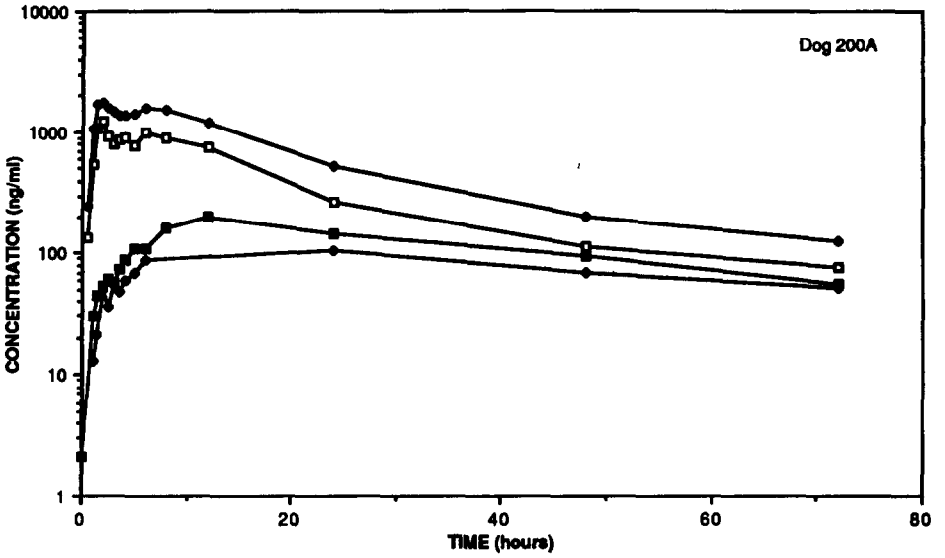

Fig. 3. Concentration-time profiles of the halofantrine and metabolite bases in plasma and blood. Data for a dog given 25 mg/kg of oral halofantrine hydrochloride. (□) Halofantrine in blood; (■) metabolite (II) in blood; (◇) halofantrine in plasma; (♦) metabolite (II) in plasma.

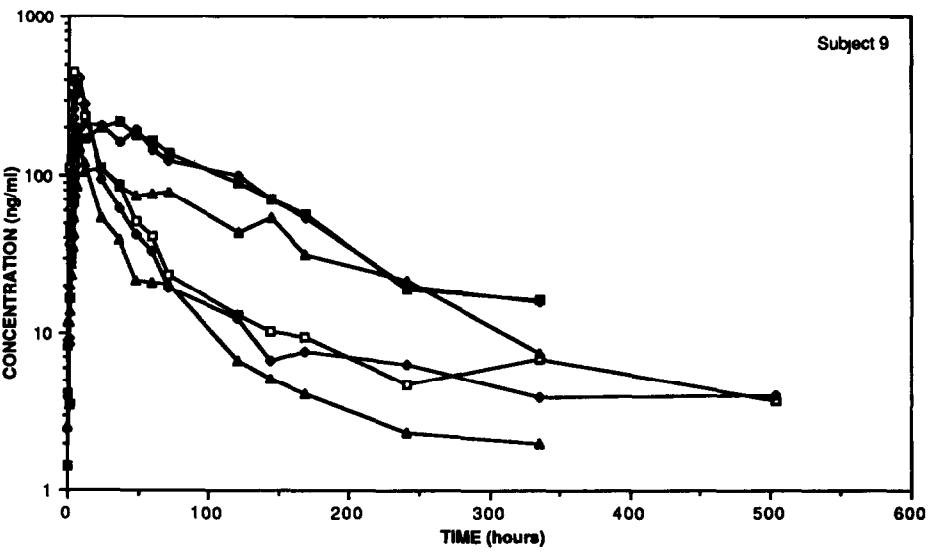

Fig. 4. Concentration-time profiles for halofantrine and metabolite bases in plasma for one human volunteer given a 500-mg oral dose of halofantrine hydrochloride. Treatment A (tablet): (□) halofantrine; (■) metabolite (II). Treatment B (capsule): (◇) halofantrine; (♦) metabolite (II). Treatment C (suspension): (△) halofantrine; (▲) metabolite (II).

ples obtained from patients given I in clinical trials and from animals given doses of the drug were analyzed for I and its putative metabolite. Fig. 3 shows the concentration of I and its metabolite in blood and in plasma versus time for samples taken from a dog that was given a capsule of I (25 mg/kg as hydrochloride). The analytical method is also demonstrated in Fig. 4 for the plasma concentrations

of I and its metabolite versus time for three oral formulations of I (500 mg as hydrochloride) given to one healthy volunteer. The data of I and II levels in blood and plasma from Figs. 3 and 4 indicates relatively rapid absorption, extensive distribution, and prolonged elimination phases. It is interesting that the level of the metabolite is much higher than the level of I in plasma in humans but not in dogs. This is probably caused by species differences. The metabolite was found to have an approximately equivalent antimalarial activity against *P. berghei* in mice [1].

In conclusion, adequate sample purification, precautions against adsorption of drugs onto glass, and use of a suitable detector permit improved measurements of I and its metabolite in biological samples. This method is sufficiently sensitive, accurate, and precise that reliable measurements of I and its metabolite can be obtained at low concentrations in plasma and blood without resorting to an LC–MS method [4]. The HPLC method described here has significant advantages over other techniques for measuring the presence of I and its putative metabolite in blood and plasma. The method is currently used for pharmacokinetic studies in human subjects.

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