Paired-Ion Liquid Chromatographic Method for the Analysis of a Phenanthrenemethanol Antimalarial in Whole Blood

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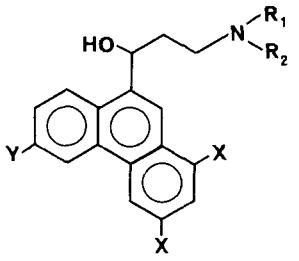
Abstract □ A sensitive and specific high-performance liquid chromatographic (HPLC) assay was developed for the determination of the candidate antimalarial (±)-(1,3-dichloro-6-trifluoromethyl-9-phenanthryl)-3-di-(*n*-butyl)aminopropanol hydrochloride in whole blood. A reversed-phase, paired-ion (lauryl sulfate) system achieved separation of the antimalarial and internal standard from interfering constitutents with a sensitivity limit of 10 ng/mL by UV detection (254 nm). Chromatographic variables (counterion concentration, pH, and column temperature) were examined to determine their effect on assay characteristics (retention, efficiency, and relative response) in clinical analysis. The antimalarial was isolated from 2.0 mL of whole blood using overnight extraction with 30% ethyl acetate in hexane followed by an acid/base partition sequence to remove major interferences. Overall recovery for the antimalarial was 84% with a CV of 5.0%, and the recovery of the internal standard was 81% (CV = 3.6%). The assay was validated by analysis of both intra- and interlaboratory samples. The assay was applied to the analysis of whole blood samples taken from a 30-year-old healthy human male who had received a single 14.1-mg/kg oral dose. The stability of the antimalarial in whole blood for up to 4 months and in sample extracts for up to 34 d at −17°C was also demonstrated.

Malaria persists as one of the most widespread and debilitating parasitic diseases in humans. Although once endemic to most temperate, semitropical, and tropical regions, it was virtually eradicated in many temperate areas.[1] Restricted use of residual pesticides and the emergence of drug-resistant malarial strains have now raised the possibility of its reintroduction into these areas without effective means for treatment or control.

A resurgence of efforts to develop new drugs capable of effecting a radical cure against resistant malarial strains has produced a number of potential antimalarials. Specifically, preclinical trials of phenanthrenemethanols have shown several of these drugs to be effective in rapid clearing of parasitemia and fever from chloroquine-resistant strains of *Plasmodium falciparum*,[2] one of the most pernicious malarial species in humans. Chloroquine-resistant strains of this species occur widely in Southeast Asia, South America, and portions of Central America.[3]

The phenanthrenemethanol antimalarial (±)-1-(1,3-dichloro-6-trifluoromethyl-9-phenanthryl)-3-di-(*n*-butyl)aminopropanol hydrochloride (**1**)[4] has recently demonstrated effectiveness in the radical cure of both chloroquine-sensitive and chloroquine-resistant *P. falciparum* without the usual phototoxicity associated with other phenanthrenemethanol candidates.[2] However, little is known concerning the metabolism, bioavailability, and pharmacokinetics of **1** in humans due to the absence of sensitive and specific analytical methodology for monitoring levels of the drug in biological fluids, particularly whole blood.



1: R_1 = R_2 = *n*-butyl, X = Cl, Y = CF_3
2: R_1 = R_2 = *n*-heptyl, X = Y = H
3: R_1 = H, R_2 = *n*-butyl, X = Cl, Y = CF_3

Both normal-[5,6] and reversed-phase[6] HPLC methodologies have been used in the separation of antimalarials. Although the general extraction and chromatographic properties of these drugs are often similar, attempts to utilize reported HPLC systems for analysis of **1** in whole blood have proven unsuccessful. Normal-phase systems[5,6] provided separation of antimalarials from high levels of endogenous interference associated with whole blood but lacked stability. During analysis of biological extracts, chromatographic characteristics of the antimalarial peaks were found to be dependent on the source of the column (manufacturer). Reversed-phase chromatography with a buffered mobile phase[6] gave a stable system but did not provide sufficient selectivity for separation of antimalarials from coextracted interferences.

A sensitive and specific method has been developed for the analysis of **1** in human blood using an analogue **2** as the internal standard. The methodology is based on reversed-phase HPLC of the antimalarials with paired-ion reagents and detection via UV absorbance at 254 nm. The chromatographic system is stable to repeated injections of whole blood extracts and provides good separation of the analyte **1** and a suspected metabolite **3**. Endogenous interference is reduced to acceptable levels by extensive purification of sample extracts prior to HPLC analysis. The method was validated by analysis of intra- and interlaboratory blood samples.

Experimental Section

Reagents and Materials—Solvents were distilled-in-glass quality and were used as received. Acetonitrile used in the chromatography was UV grade (Burdick and Jackson, Muskegon, MI). All reagents and buffers were reagent grade and were used as received. Water was obtained from a Milli-Q water

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purification system (Millipore Corp., Bedford, MA).

The antimalarials, **1** and **2**, and the ^{14}C-labeled analogue of **1** were furnished as their hydrochloride salts (Walter Reed Army Institute of Research, Washington, DC). Stock solutions of these materials were prepared in methanol and stored at −17°C.

Trisodium phosphate:boric acid buffer (pH 10.9) was prepared from 1.64 g of boric acid and 13.68 g of trisodium phosphate dodecahydrate in 500 mL of water. Glycine buffer (pH 9.5) was prepared from 70 mL of a stock solution of 0.1 M glycine and 0.1 M NaCl and 30 mL of 0.1 M NaOH.

Reversed-phase HPLC columns, C_{18} bonded silica (250 × 4.0 mm; Partisil-10, Whatman, Clifton, NJ), were packed at 7500 psi using a modified balanced-density technique (carbon tetrachloride:2-propanol, 1:1). Carbon tetrachloride used in the slurry solvent was purified over basic alumina (Woelm Alumina B-Super I, type W200; ICN Pharmaceuticals, Cleveland, OH). A commercial reversed-phase column (250 × 4.6 mm; Partisil PXS ODS-3, Whatman) was purchased prepacked.

The column packing, C_{18} bonded silica, was prepared by refluxing 10 g of 10-μm silica (dried overnight at 150°C) with 10 mL of distilled octadecyltrichlorosilane (Petrarch Systems, Bristol, PA) in 50 mL of toluene (dried with sodium) overnight under a dry nitrogen atmosphere. Glassware used in the preparation of the packing material was oven-dried and maintained under a dry nitrogen atmosphere until used. Remaining chlorosilane bonds were hydrolyzed by washing the packing material with acetone:water (1:1). After drying the material at 100°C under reduced pressure, the resulting silanols were covered[7,8] by refluxing overnight with 10 mL of hexamethyldisilazane (Pierce Chemical Co., Rockford, IL) in 50 mL of toluene (sodium-dried) under a dry nitrogen atmosphere. The bonded packing was checked for the presence of residual silanols using methyl red indicator.[9,10] The material was acceptable only if no red or pink coloration of the surface was detected.

Instruments and Chromatographic Conditions—The chromatographic system consisted of a single pump (model M6000A; Waters Associates, Milford, MA), a fixed-wavelength UV absorbance detector (model 440; Waters) set at 254 nm, a manual injector (model U6-K; Waters) or an autosampler (model 7108; Waters), and an Omniscribe strip-chart recorder (model B-5000; Houston Instruments, Austin, TX). The HPLC column was thermostated in a water jacket at 40.5 ± 0.1°C using a temperature controller (Haake model E52; Berlin–Lechterfelde, F.R.G.).

The mobile phase consisted of acetonitrile:water containing 0.005 M lauryl sulfate. The pH of the mobile phase was adjusted to 3.5 using 10% (v/v) sulfuric acid, and the mobile phase was filtered through a 5.0-μm fluoropolymer membrane (Mitex, 47 mm; Millipore) before use. The amount of acetonitrile in the mobile phase was adjusted to give a capacity factor (k') of ~12 for **1** and was nominally 70–75% (v/v) for C_{18} bonded-silica columns. A short precolumn (50 × 2.1 mm) packed with C_{18} bonded pellicular silica (Perisorb RP-18, S/P, McGaw Park, IL; Permaphase ODS, Fisher Scientific, Pittsburgh, PA) was used to protect the analytical column.

Extraction and Purification—Frozen blood samples were thawed at room temperature for 1 h and thoroughly mixed by vortexing 30–60 s (Evapo-Mix; Buchler Instruments, Fort Lee, NJ). A 2.0-mL aliquot of each blood sample was transferred to a screw-capped culture tube (16 × 125 mm) with Teflon-lined cap. To each of the samples were added 500–800 ng/mL of internal standard buffer and 4.0 mL of 30% ethyl acetate:hexane. Samples were then extracted overnight by gentle shaking (100 oscillations/min) (shaker model 6244; New Brunswick Scientific Co., New Brunswick, NJ).

Samples were centrifuged (model CL, International Clinical Centrifuge; International Equipment Co., Needham Heights, MA) for 5 min, and the organic layer was transferred to a clean screw-capped culture tube. The aqueous layer was rinsed with an additional 2.0 mL of 30% ethyl acetate:hexane, and the organic phase was combined with the initial extract. The sample extracts were evaporated to dryness under a nitrogen stream, and the residue was partitioned between 4.0 mL of hexane and 4.0 mL of 0.1 M citric acid solution (90% methanol:water). The mixture was vortex-mixed for 15 min and centrifuged to complete the separation of layers, and the hexane layer was removed and discarded. The purification step was repeated with an additional 4.0 mL of hexane, which was also discarded. The aqueous:methanol layer was reduced to ~0.5 mL under a nitrogen stream (primarily to remove the methanol), and 4.0 mL of trisodium phosphate:boric acid buffer was added. The buffer was partitioned with 4 mL of 30% ethyl acetate:hexane (vortexing–centrifugation), and the organic layer was transferred to a 1-dram vial with a Teflon-lined cap and completely evaporated under a nitrogen stream. The resulting sample residue was reconstituted in 200 μL of an acetonitrile:methanol solution (1:1) via ultrasonication (5 min) for HPLC injection.

Quantitation—Concentrations of **1** were estimated from peak height ratios (**1**/**2**) using a calibration curve of peak height ratio versus amount of analyte/mL of whole blood. Calibration curves were prepared by analyzing replicate samples of outdated whole blood spiked with **1** at five concentration levels over the range of 50–1500 ng/mL. The curve was weighted towards the low end by analyzing quadruplicate samples at the two lowest concentration levels (50 and 100 ng/mL) and triplicate samples at the remaining levels (500, 1000, and 1500 ng/mL). The correlation coefficient (r) and regression line were determined by the linear least-squares method. Results were reported as the hydrochloride salt.

Results and Discussion

Extraction and Clean-up Procedures—The antimalarial agent mefloquine has been reported to concentrate in the erythrocytes as well as bind to plasma protein.[6] As the distribution of **1** in blood has not yet been established, analytical methodology was developed for hemolyzed blood.

The analyte **1** is highly lipophilic and thus easily extracted from basic aqueous solutions into relatively nonpolar solvents. The nature of whole blood, however, placed special requirements on the extraction procedure. In particular, pH adjustment (sodium hydroxide or ammonium hydroxide) of the sample prior to extraction led to clotting of the sample and a tendency to form emulsions. Diluting the blood with an equal volume of 0.1 M glycine buffer (pH 8–10) prevented clotting and reduced the tendency for emulsification. Optimum recovery was obtained with buffers at pH 9–9.5 (seven replicates, CV = 3%). Because of the significant buffering capacity of whole blood, the final pH of the blood–buffer mixture was 8.0–8.5. Gentle shaking of the samples (~100 ocillations/min) further reduced emulsion formation. Under these conditions, the partition equilibrium was attained slowly; the extraction was carried out over a period of at least 4 h. An overnight extraction proved convenient.

The UV absorbance band of **1** is broad, with a large extinction coefficient at 254 nm. Thus, good detection sensitivity (4 ng = 10% FS at 0.005 AUFS) can be obtained using a filter photometer with a low-pressure mercury source. The amount of UV-active material extracted from whole blood was excessive and could not be resolved from the analyte and internal standard. It was, therefore, necessary to incorporate an extract purification procedure into the analytical method to reduce the background to an acceptable level.

The purification procedure consisted of a simple acid/base

partition scheme designed to efficiently remove endogenous acidic and neutral components. The extraction solvent (30% ethyl acetate:hexane) was specifically chosen to minimize the degree of coextraction for endogenous blood components. Each step of the procedure was optimized for maximum recovery of **1** using ^{14}C-labeled drug. The overall recovery of **1** from whole blood using the combined extraction/purification procedure was 84% (17 samples, CV = 5%) over the range of 50–1000 ng/mL, as determined by HPLC.

A structurally analogous internal standard **2** was chosen based on its recovery using the extraction/purification procedure already optimized for **1**. The recovery of **2** from whole blood (125 ng/mL) using the complete method was 81% (17 replicates, CV = 4%). The UV response (254 nm) for **2** was nearly identical to **1**.

Chromatography—The difficulties associated with the reversed-phase HPLC of ionic or ionizable compounds are well established.[11–13] Since the analyte and internal standard both contain a tertiary aliphatic amine ($pK_a > 8$), they proved to be no exception. Buffered mobile phase systems (e.g., phosphate, sulfate, acetate) at pH ≤ 4.5 provided improved chromatography for the antimalarials through control of the ionic equilibria. However, in all cases, the retention of the resultant species coincided with retention of several particularly large interferences derived from the whole blood matrix. Attempts to modify the isolation/purification procedure were not successful in removing these interferences. Therefore, attention was directed toward application of paired-ion chromatographic (PIC) techniques as a means of selectively adjusting the analyte and internal standard retention relative to endogenous interferences.

Evaluation of PIC reagents with progressively increasing aliphatic character (butane-, pentane-, hexane-, heptane-, octane-, and tetradecanesulfonic acids and lauryl sulfate) produced, as expected, a corresponding increase in the retention of the antimalarials, while the retention of most endogenous constituents in whole blood remained essentially unchanged. Lauryl sulfate gave the most satisfactory results (Fig. 1a) with the analyte and internal standard (denoted by asterisks) eluting in a relatively "clean" area. Figure 1a is representative of the blank chromatograms obtained from both outdated whole blood and freshly drawn blood using the aforementioned procedures.

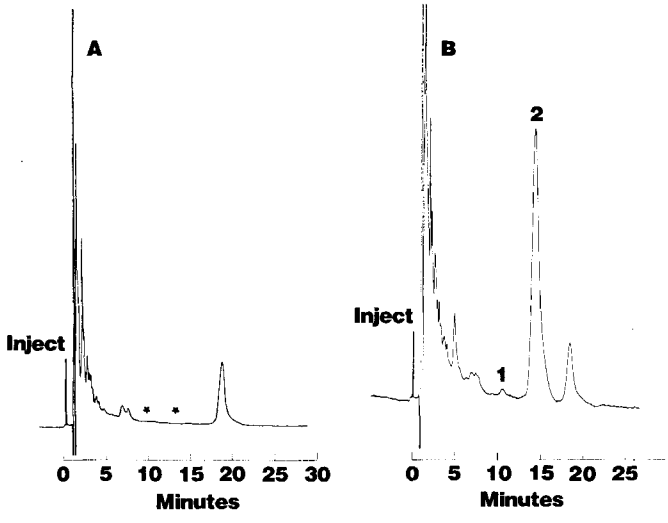

Figure 1—*Chromatograms of outdated whole blood extract. Key: (A) unspiked sample (asterisks denote retention times of 1 and 2, respectively); (B) spiked sample (11 ng/mL in whole blood). Conditions: column, C_{18} bonded silica (250 × 4.0 mm); mobile phase, 70% acetonitrile:0.005 M lauryl sulfate (pH 3.5); flow rate, 2.0 mL/min; detector, 254 nm at 0.01 AUFS (A) or 0.005 AUFS (B); column temperature, 40°C; sample volume, 20 μL.*

The paired-ion chromatography procedure was optimized for various parameters, including the effects of mobile phase components, counterion concentration, pH, and temperature. Acetonitrile was quickly shown to be the mobile phase component of choice since complete resolution of **1** and **2** was obtained with this solvent, while no separation of the two compounds was observed using methanol. Ultraviolet-grade acetonitrile must be used since other grades produced erratic baselines. After adjusting the percentage of organic modifier in the mobile phase to maintain a convenient retention for analyte and internal standard, the specific effects of other separation parameters were then evaluated.

The effect of the counterion concentration in the chromatography of the antimalarials was evaluated for mobile phase concentrations of 0.0005–0.001 M lauryl sulfate while maintaining pH, column temperature, and mobile phase composition content constant. The upper concentration limit of 0.01 M was dictated by the solubility of lauryl sulfate in the mobile phase. An increase in antimalarial retention was noted for an increase in the lauryl sulfate concentration. An initial decrease in compound retention was observed for lauryl sulfate concentrations between 0.0005 and 0.001 M; this observation remains unexplained. From 0.001 to 0.01 M lauryl sulfate concentration, retention times increased by ~45% for **1** and **2**. Since neither a constant nor maximum retention was reached at any of the concentrations studied, 0.005 M was chosen as the operating concentration for lauryl sulfate, a value well below the limit of solubility for the PIC reagent.

The effect of counterion concentration on chromatographic efficiency was also determined. From low to high counterion concentration, an exponential decay in efficiency (HETP) was noted. This trend is consistent with results reported for cationic PIC systems in general.[14] Since the chosen operating concentration (0.005 M) fell on the relatively flat area of the curve, small changes in the counterion concentration due to batch-to-batch variation did not measurably influence the analytical separation. In addition, the measured error in UV response introduced by a variation in the counterion concentration of ±0.001 M was only ±1%.

The effect of mobile phase pH on the chromatography of the antimalarials was determined for the pH range of 2.5–5.0 while maintaining the organic mobile phase modifier concentration, counterion concentration, and column temperature constant. Retention values for the two compounds increased (k' = ~6.7–8.7 for **1**; k' = ~9.2–12.4 for **2**), but the overall separation of the two compounds was not affected. Also, the pH of the mobile phase did not appear to exert an appreciable influence on chromatographic efficiency over the observed pH range, in keeping with the assumption that the antimalarials are completely ionized throughout this range. Mobile phase was adjusted to pH 3.5 for all subsequent studies.

Changing column temperature over a 40°C range (from ambient) led to a decrease in retention, an observation consistent with effects reported by others.[15,16] Retention of both **1** and **2** showed nearly linear decrease with increasing temperature. The chromatographic efficiency was relatively unaffected by temperature variation and may indicate less contribution from mass transport (solvent viscosity and diffusion) parameters as compared with ionic equilibria. The major effect of column temperature changes was manifested in baseline drift. A change in column temperature of only a few degrees over a 4-h period was found to produce a drift on the order of 0.005 AU/h. At the high sensitivity (0.005 AUFS) required in this analysis, such drift effectively rendered the system unusable. Simple shielding of the column from laboratory drafts and air movements proved insufficient, while precise temperature control

(±0.1°C) allowed rapid equilibration and use of the system within 15 min after introducing the mobile phase. All assays were subsequently conducted with a column temperature of 40°C.

Analysis of Biological Samples—The intra-assay reproducibility was determined using replicate samples (n = 7) of outdated whole blood fortified at a level of 150 ng/mL of **1**. Analysis of the samples gave a CV of 1.1%, with an average error of 4.1% ($\bar{x}$ = 156 ± 1.7 ng/mL). Duplicate analyses of samples fortified at concentrations of 225, 375, 825, and 1325 ng/mL of **1** resulted in average errors of 2, 4, 5, and 6%, respectively.

Results from a single analysis of interlaboratory blinds in fresh dog blood are shown in Table I. Samples were prepared, immediately frozen, and remained frozen (−17°C) until analyzed. The average error of analysis was 3%, with a maximum error of 12% at the lowest concentration (121 ng/mL).

A second set of interlaboratory blinds was analyzed using fresh human blood as the matrix. As shown by the results in Table II, no significant difference between the two matrices was noted either in the chromatography or the analytical results. The average error for single analysis of each sample in the second set was 3%, with a maximum error of 6% at 388 ng/mL.

The stability of **1** in outdated whole blood was determined by analysis of interlaboratory blinds stored at −17°C for a 4-month period. The results for single analyses of each sample (Table III) did not reflect a significant loss of **1** over the range of 136–1500 ng/mL. The average error for the analysis was 8%, with a maximum of 17% at 375 ng/mL.

The stability of the antimalarial in whole blood extracts was determined by reanalyzing a set of interlaboratory blinds after having stored the sample extracts in sealed vials at −17°C for 34 d. Results of the second analysis (day 34) were calculated using a new standard curve and compared with the results of the initial analysis (day 0). The data in Table IV show relatively uniform decrease in antimalarial concentration over the specified period.

Table I—Analysis of Interlaboratory Blinds Fortified with 1 in Dog Blood

Sample Conc., ng/mL	Conc. Found, ng/mL[a]	Error, %
121	107	−12
170	165	−3
194	193	−1
267	254	−5
291	305	5
413	405	−2
583	591	1
995	901	−9
1093	1016	−7
1165	1052	−10

[a] Standard curve (peak height ratio versus concentration of 1): slope, 0.00121; intercept, +0.00253; r, 0.994.

Table II—Analysis of Interlaboratory Blinds Fortified with 1 in Human Blood

Sample Conc., ng/mL	Conc. Found, ng/mL[a]	Error, %
61	60	−2
97	95	−2
134	137	2
158	152	−4
219	217	−1
291	280	−4
388	366	−6
558	554	−1
704	696	−1
850	828	−3
1505	1427	−5
1700	1632	−4

[a] Standard curve (peak height ratio versus concentration of 1); slope, 0.00130; intercept, +0.00973; r, 0.9999.

Table III—Analysis of 1 in Outdated Whole Blood Stored at −17°C for 4 Months

Sample Conc., ng/mL	Conc. Found, ng/mL[a]	Error, %
136	127	−7
136	149	10
250	232	−7
250	214	−14
375	327	−13
375	311	−17
975	977	1
975	966	−1
1500	1488	−1
1500	1400	−7

[a] Standard curve (peak height ratio versus concentration of 1); slope, 0.00106; intercept, +0.0014; r, 0.9995.

Table IV—Stability of 1 in Whole Blood Extracts Stored at −17°C for 34 d

Sample Conc., ng/mL	Conc. Found, ng/mL		CV (Day 0–Day 34)
	Day 0[a]	Day 34[b]	
121	107	102	3%
170	165	154	5%
194	193	183	4%
267	254	239	4%
291	305	286	5%
413	405	381	4%
583	591	561	4%
995	901	840	5%
1093	1016	952	5%
1165	1052	988	4%

[a] Standard curve (peak height ratio versus concentration of 1); slope, 0.00121; intercept, 0.0025; r, 0.9994. [b] Standard curve (peak height ratio versus concentration of 1); slope, 0.00127; intercept, 0.0164; r, 0.9999.

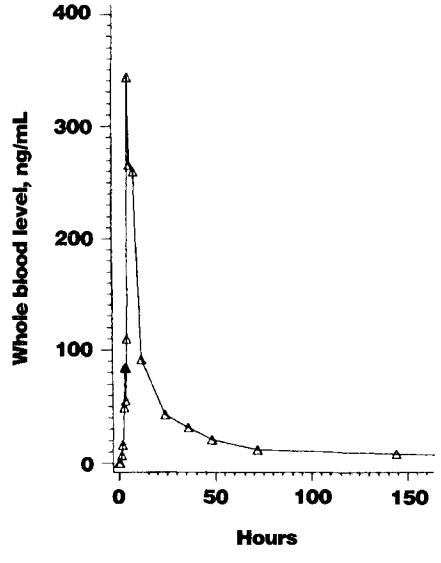

Figure 2—*Whole blood levels (human) of 1 over a 1-week period after administration of a single oral dose (1000 mg).*

The instrumental limit of detection (5 times the baseline noise level) was found to be ~2 ng (injected amount) for **1** using a fixed-wavelength UV absorbance detector at 254 nm. Since the antimalarial possesses a λ_{max} of 258 nm (ϵ 51,800) near this wavelength, the detector provided near optimum sensitivity.

The lower limit for quantitation of **1** in whole blood extracts was ~10 ng/mL (Fig. 1b) based on injection of 10% of the sample extract at 80% recovery of the drug. However, interlaboratory evaluation was performed only to a limit of 50 ng/mL.

Whole blood levels of parent drug **1** were determined for a healthy (malaria-free) 30-year-old male receiving a single oral dose of 1000 mg of **1** (14.1 mg/kg). Samples of venous blood (~6 mL) were collected over a period of 16 d (21 samples). The samples were frozen immediately following collection in glass screw-capped culture tubes with Teflon-lined caps. The samples remained frozen (−17°C) until analyzed (~1 month of storage).

The results (blood level versus time) are shown in Fig. 2 for a single analysis of each sample. The first evidence of the antimalarial was detected at 1.5 h following administration, with a maximum blood level being achieved at 5 h. The maximum blood level was quite low compared with that reported[5] for another antimalarial, mefloquine, which reached levels of ~700 ng/mL with a 500-mg oral dose. The blood concentration of **1** also was found to drop to undetectable levels far more rapidly (6–13 d for **1** versus >57 d for mefloquine) with a terminal-phase half-life for **1** estimated at 40 h using NONLIN.[17] Representative chromatograms are shown in Fig. 3.

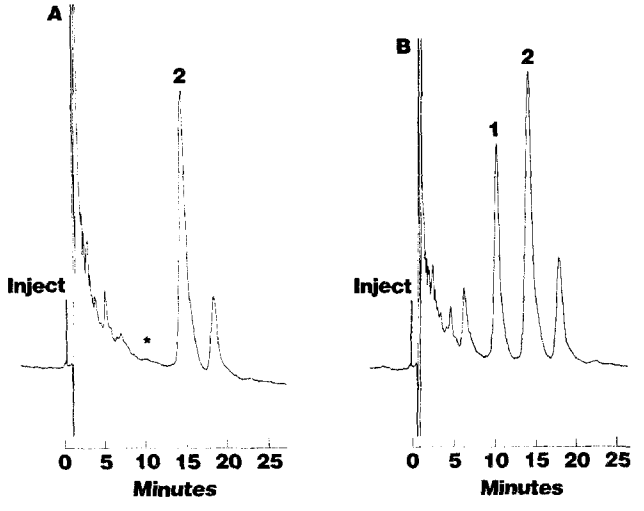

Figure 3—*HPLC analysis of clinical samples taken at the time of drug administration (A), with the retention time of 1 denoted by an asterisk, and 5 h after administration (B). Conditions as in Fig. 1B.*

Evidence for the existence of a major metabolite of **1** has been provided by the RIA method being developed concurrently in our laboratories. RIA analysis of HPLC fractions from several clinical samples demonstrated the presence of immunoreactive material more polar than the parent antimalarial. Chromatographic characteristics of this material are identical with **3**.

Conclusions

The HPLC method described herein allows quantitation of **1** in whole blood at therapeutic levels and should prove useful in future pharmacokinetic studies. The reproducibility and accuracy of the method have been demonstrated by analysis of intra- and interlaboratory whole blood samples to a lower limit of 50 ng/mL, although levels as low as 10 ng/mL can be successfully analyzed where background permits. Techniques to improve the specificity of the analysis relative to background and decrease the lower limit of quantitation to ≤10 ng/mL are currently being investigated.

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