High-Performance Liquid Chromatographic–Ultraviolet Determination of Primaquine and Its Metabolites in Human Plasma and Urine

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Abstract □ A high-performance liquid chromatographic method was developed for the simultaneous determination of primaquine and its metabolites from plasma and urine samples obtained after oral administration of primaquine diphosphate. Following partial deproteinization with acetonitrile, samples were chromatographed by direct injection onto a cyano column with UV detection at 254 nm. Levels as low as 100 ng/mL per 20-μL injection were quantitated. Preliminary pharmacokinetic analysis is reported for two human volunteers after oral doses of 60 mg and 90 mg. Two apparent plasma metabolites and two possible urinary metabolites of primaquine are also reported.

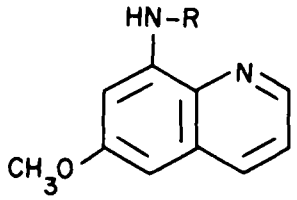
Keyphrases □ Primaquine—liquid chromatographic analysis, human plasma and urine, pharmacokinetics □ Liquid chromatography—primaquine, human plasma and urine, pharmacokinetics

Primaquine, 8-[(4-amino-1-methylbutyl)amino]-6-methoxyquinoline (I), is well tolerated in the therapeutic range except in individuals with glucose-6-phosphate dehydrogenase deficiency in whom it causes hemolytic anemia (1). The severity of hemolysis is thought to be related to either the concentration of primaquine or primaquine metabolite(s) and the duration of exposure (2). Nevertheless, primaquine is a true tissue schizonticide and is not only the drug of choice for radical cure of vivax malaria but also is a causal prophylactic agent against falciparum and vivax malaria. Early attempts to correlate primaquine blood levels with either antimalarial effects or toxicity, using a diazotization procedure, were unsuccessful (3).

Recently, several different techniques have been developed which are potentially selective and sensitive enough to measure human primaquine blood concentrations and to quantitate blood concentrations of human metabolites (4–6). The primary objective of this report is to describe the development of a high-performance liquid chromatographic (HPLC) method sensitive enough to measure primaquine concentrations in plasma from human subjects after a single oral dose of primaquine diphosphate. In addition, we report a preliminary pharmacokinetic analysis of human blood level curves, the probable identification of one of the two plasma metabolites, and two unidentified urinary metabolites.

EXPERIMENTAL SECTION

Reagents and Materials—The standards used in this assay were primaquine diphosphate (>99% pure)[1], 6-methoxy-8-aminoquinoline (II)[2], and 8-(3-carboxy-1-methyl propylamino)-6-methoxyquinoline (III)[3]. The chemicals used in the preparation of the mobile phase buffer were reagent-grade ammonium formate and formic acid (88% pure)[4]. Acetonitrile and methanol were UV grade[5]. All solutions containing I, II, and III were placed in amber glassware and refrigerated.



I, R = $-CH(CH_3)CH_2CH_2CH_2NH_2$
II, R = $-H$
III, R = $-CH(CH_3)CH_2CH_2COOH$

HPLC Conditions—The HPLC system consisted of a pump and a fixed-absorbance detector equipped with a 254-nm filter[6]. Samples were either injected manually using an injector with a manual signal recorder[7] or an automated injector[8] and integrator[9,10]. The separations were accomplished using a reverse-phase cyano column (30 × 0.39 cm i.d.; particle size 10 μm)[11]. The mobile phase consisted of a mixture of 0.1 M ammonium formate adjusted to pH 3.85 with formic acid and acetonitrile (89:11). It was filtered through a 0.45-μm filter[12] and deaerated under a house vacuum for 10 min. The flow rate of the mobile phase was 2 mL/min at ambient temperature. Deionized water was used in the final rinsing of glassware and in the preparation of the buffer and the standards[13].

Analytical Procedure—Blood samples were collected in EDTA containers[14] and centrifuged at 1900×*g* for 10 min to separate the plasma. The plasma was kept in aluminum foil covered glass tubes at 4°C until analysis.

The plasma (0.2 mL) was diluted 1:1 with acetonitrile and mixed well. The solution was incubated in the dark for 1 h and centrifuged at 1900×*g* for 10 min. Analyses by HPLC were carried out on 20-μL samples of the resulting supernatant.

Urine was collected in plastic urine specimen jars. The jars were capped and kept at 4°C in the dark until preparation for analysis. Urine was extracted by a modification of the method published by Baty *et al.* (4). Ten grams of resin[15] was suspended in methanol and put in a 1.8 × 25-cm column and washed with 100 mL of methanol followed by 200 mL of deionized water. Up to 100 mL of the urine was passed through the column at a rate of ~5 mL/min. The column was then washed with 100 mL of water. Primaquine and its metabolites were eluted from the column by 75–100 mL of methanol and then quantified by HPLC assay using 10-μL injections. The column was regenerated by washing with 100 mL of water, and then a new sample of urine was passed through the column and the process repeated.

Recovery Studies—Plasma standards containing known amounts of each of the three compounds were compared with standards in distilled water. The plasma standards were treated as in the analytical procedure and the water standards were injected directly. Other deproteinizing agents, *i.e.*, 15% trichloroacetic acid, 5% metaphosphoric acid, 6% perchloric acid, and copper sulfate–sodium tungstate, were also tested for recovery. Additionally, solutions of I and II in plasma and water were placed in membrane cones[16] and centrifuged at 12,000×*g* for 1 h. The concentrations of each compound were then determined for the plasma and water filtrates.

The recoveries were determined for urinary primaquine samples by passing a standard solution of primaquine through the column and analyzing the

[1] Aldrich Chemical Co., Inc., Milwaukee, Wis.
[2] ICN Pharmaceuticals, Plainview, N.Y. (no longer available).
[3] PQC was a gift from Dr. James D. McChesney, School of Pharmacy, University of Mississippi.
[4] Fisher Scientific, Fair Lawn, N.J.
[5] Burdick and Jackson, Muskegon, Mich.
[6] Model 6000A, Model 440; Waters Associates, Milford, Mass.
[7] Model U6K; Houston Instruments, Austin, Tex.
[8] WISP 710B; Waters Associates, Milford, Mass.
[9] Data module; Waters Associates, Milford, Mass.
[10] LC 9540; IBM Instruments, Danbury, Conn.
[11] Waters Associates, Milford, Mass.
[12] Type FG; Millipore Corp., Bedford, Mass.
[13] Milli-RO and Milli-Q deionizing system; Millipore.
[14] Becton, Dickinson & Co., Rutherford, N.J.
[15] Amberlite XAD2; Mallinckrodt, Paris, Ky.
[16] Centriflow membranes, 50,000 mol. wt. cut off; Amicon, Lexington, Mass.

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Table I—Recovery Values from Plasma Using Several Precipitating Agents

	Aceto-nitrile	15% Trichloro-acetic Acid	5% Meta-phospho-ric Acid	6% Perchlo-ric Acid	Copper Sulfate Sodium Tungstate
I (0.78 μg/mL)	98%	38%	38%	46%	0
II (0.34 μg/mL)	97%	30%	33%	53%	0
III (0.6 μg/mL)	98%	—		—	—

eluant for primaquine content. Recoveries of urine metabolites were determined by dividing urine samples into two parts. One part was passed through the column, eluted with methanol, and quantitated by HPLC. Four hundred microliters of the other part was added to an equal volume of acetonitrile, vortex mixed, and left for ≥30 min before being centrifuged. An aliquot of the supernatant was chromatographed, and the two chromatograms were then compared.

Calibration Curves—Calibration standards were prepared by adding known amounts of I and III (0.1 μg/mL–20 μg/mL and 0.23 μg/mL–0.96 μg/mL, respectively) to control plasma and urine samples. Solutions were then assayed by following the analytical procedure. The calibration curves for the quantitation of I and III were obtained by plotting peak height against concentration for each of the standard solutions. (Note: Aqueous solutions of primaquine are light sensitive and will begin to degrade within 30 min. Light protected, aqueous solutions showed no detectable changes after 1 week at −20°C and only slight changes after 1 month.)

Dosing and Sampling Procedure—After Institutional Review Board approval, 60 mg and 90 mg of primaquine diphosphate (calculated as the base) were administered with 118 mL of water to two healthy male volunteers. Three hours after the dose, volunteers were allowed to eat and drink *ad libitum*.

Venous blood was collected at 0, 0.5, 1, 2, 2.5, 3, 4, 5, 6, 7, 8, and 10 h for each subject. Urine was collected at time zero and at each void for up to 72 h.

RESULTS AND DISCUSSION

Assay—To obtain suitable sample preparation from plasma samples for HPLC analysis with UV detection in the nanogram range, various deproteinization techniques were examined on the basis of a previous report (7). The inorganic agents and trichloroacetic acid resulted in nonquantitative and variable recoveries of I and II, Table I. Treatment with acetonitrile (1:1) yielded 98 + 2% recovery of I, II, and III from spiked plasma. Extensive protein binding of primaquine probably accounts for poor recovery with many agents. When solutions containing I and III in plasma and water were filtered through membrane cones, 99% of the compounds were recovered in the water filtrate, while neither compound was detectable in the plasma filtrate[17].

The mobile phase was chosen to achieve separation between I and III and still maintain desirable retention times. Under the HPLC conditions given, I, II, and III had retention times of 6.3 min (K', −4.2), 4.4 min (K', −2.8), and 10.9 min (K', −7.8), respectively.

Calibration curves were constructed for each compound in time zero plasma and urine specimens. In the range of concentrations tested, responses were linear and had coefficients of determination (r^2) >0.99. The detection limit using 20-μL injections was 100 ng/mL of primaquine, 500 ng/mL of III, and 50 ng/mL of II using a signal to noise ratio >3 in plasma and 100 ng/mL of primaquine in urine. Reproducibility of this method at the above detection limits was 98 ± 3.8% for primaquine (n = 10), 98 ± 4.2% for III (n = 10), and 97 ± 1.4% for II (n = 10), using separate determinations. Figure 1 shows

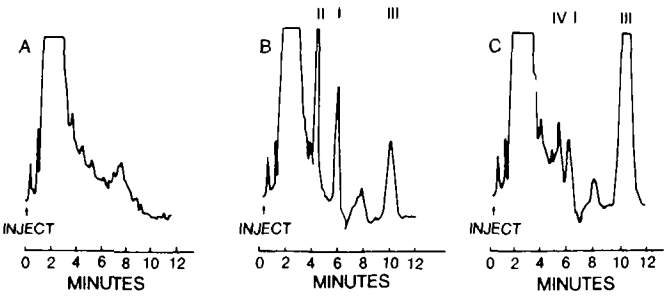

Figure 1—*Chromatograms of (A) time zero plasma; (B) time zero spiked plasma containing 200 ng/mL of 6-methoxy-8-aminoquinoline (II), 450 ng/mL of primaquine (I), and 1.2 μg/mL of primaquine metabolite (III); (C) plasma 2.5 h after a 90-mg oral dose of primaquine diphosphate (I) where IV is the more polar metabolite.*

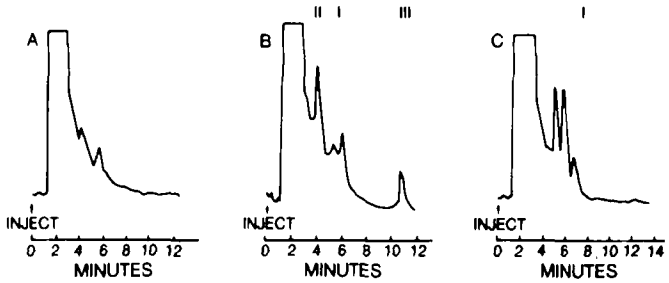

Figure 2—*Chromatograms of (A) time zero urine; (B) time zero spiked urine containing 1.0 μg/mL of 6-methoxy-8-aminoquinoline (II), 1.0 μg/mL of primaquine (I), and 2.0 μg/mL of primaquine metabolite (III); (C) urine 4 h after a 90-mg oral dose of primaquine diphosphate.*

chromatograms of plasma before and 2.5 h after primaquine administration. In addition, a time zero plasma sample spiked with primaquine, II, and III is shown in Fig. 1B. Chromatograms of urine samples taken at time zero, 4 h post primaquine administration, and time zero spiked with I, II, and III are shown in Fig. 2.

Metabolism and Pharmacokinetics—To demonstrate the utility of the present method, it was applied to plasma and urine samples obtained from the two volunteers who were part of an ongoing study (8). These data were fitted to an open one-compartment pharmacokinetic model (9) using a nonlinear least-squares regression program (NONLIN) (10). Oral absorption lag time was incorporated into the model. The unavailability of intravenous data precluded the individual estimation of volume of distribution and bioavailability; therefore, the ratio F/V was inserted in the model:

$$C = \frac{k_a \frac{F}{V} X_o}{(k_a - K)} (e^{-K(t-t_L)} - e^{-k_a(t-t_L)})$$

where t_L is the lag time.

Figure 3 shows the results of the analysis of 11 plasma samples from each volunteer and the fitted curves obtained from these results. As would be expected, there appeared to be a considerable difference in absorption rates between the two subjects; however, the elimination rates were similar for both. Estimated pharmacokinetic parameters are summarized in Table II. Visual analysis of the chromatograms of plasma at the several sampling times after the ingestion of primaquine suggested the presence of primaquine metabolites. However, II, a human metabolite of primaquine metabolism reported by Baty *et al.* (4), was not observed to be present either by comparison of retention times or by addition of II to volunteer samples containing the presumed metabolites. Using our HPLC system, the presumed plasma metabolites were labeled as either more polar than primaquine or less polar than primaquine. They can be detected very shortly after primaquine ingestion and are detectable long after the primaquine concentration falls below detectable levels, Fig. 4. Authentic samples of III[18] (5) have the same retention time as the less polar metabolite in our HPLC system. In addition, in the samples from the two volunteers, the less polar metabolite was subsequently identified as III by mass spectrometry[19]. We conclude that the major primaquine plasma metabolite in humans is the same as that found in rats.

Analysis of the urinary data from the two volunteers indicated that only a small percent of primaquine was excreted unchanged. Values were 4.2% (subject 1) and 2.2% (subject 2) of the oral primaquine dose in 24 h. Chromatograms indicated the presence of two possible primaquine urinary me-

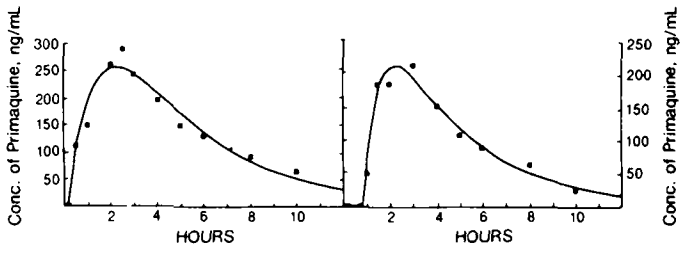

Figure 3—*Fitted blood level curves from two volunteers using a nonlinear least-squares regression program.*

[17] Unpublished results.

[18] Microbiological origin; provided by Dr. J. D. McChesney, School of Pharmacy, University of Mississippi, Oxford, Miss.

[19] Personal communication; Dr. J. D. McChesney.

Table II—Estimated Pharmacokinetic Parameters of Primaquine

	k_a, h^{-1}	Absorption Half-Life, h	k_e, h^{-1}	Elimination Half-Life, h	Lag Time, h	Ratio[a]	r^2
Subject 1[b]	0.81	0.85	0.257	2.7	0.18	7.3	0.946
Subject 2[c]	1.47	0.47	0.287	2.41	0.87	3.5	0.976

[a] Ratio of bioavailability/Vd. [b] After oral administration of 90 mg of primaquine diphosphate. [c] After oral administration of 60 mg of primaquine diphosphate.

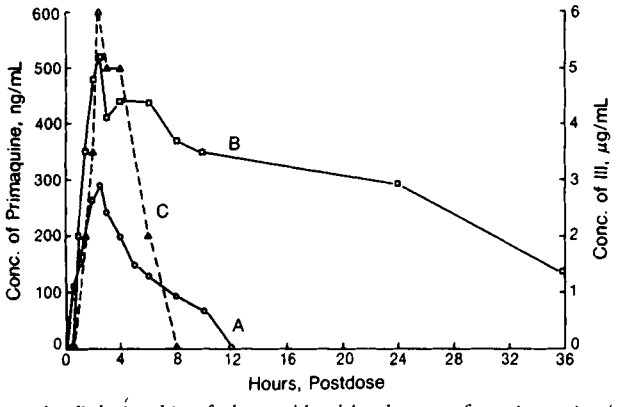

Figure 4—*Relationship of plasma blood level curves for primaquine (A), primaquine metabolite (B), and the more polar metabolite (C), reported as peak height in centimeters.*

tabolites, Fig. 2C. Both of these presumed urinary metabolite compounds are more polar than primaquine in our HPLC system. Neither urinary metabolite appeared to be II or III, but one appears to have a retention time which was the same as that of the more polar plasma metabolite.

The present method utilizing HPLC with UV detection for the simultaneous determination of primaquine and its carboxy metabolite (III), is rapid and precise. The use of this method for plasma and urine samples of volunteers after oral administration of primaquine diphosphate indicates that absorption and metabolism is rapid, that there appear to be at least two plasma metabolites (one identified as an oxidative-deamination product of primaquine, III), and that there appear to be two as yet unidentified urinary metabolites of primaquine.

REFERENCES

(1) P. E. Carson, C. L. Flanagan, C. E. Ickes, and A. S. Alving, *Science*, **124**, 484 (1952).

(2) A. R. Tarlov, G. I. Brewer, P. E. Carson, and A. S. Alving, *Arch. Int. Med.*, **109**, 209 (1962).

(3) "Handbook of Experimental Pharmacology: Antimalarial Drugs," W. Peters and W. H. G. Richards, Eds., Springer-Verlag, New York, NY, 1984, chap. 3.

(4) J. D. Baty, D. A. Price-Evans, and P. A. Robins, *Biomed. Mass Spectrom.*, **2**, 304 (1975).

(5) J. K. Baker, J. D. McChesney, C. D. Hufford, and A. M. Clark, *J. Chromatogr.*, **230**, 69 (1982).

(6) T. G. Rajagopalan, B. Anjaneyulu, V. D. Shanbag, and R. S. Grewal, *J. Chromatogr.*, **224**, 265 (1981).

(7) J. Blanchard, *J. Chromatogr.*, **226**, 455 (1981).

(8) P. E. Carson, R. Hohl, M. V. Nora, G. W. Parkhurst, T. Ahmad, S. Scanlan, and H. Frischer, *Bull WHO*, **59** 427 (1981).

(9) M. Gibaldi and D. Perrier, "Pharmacokinetics," Marcel Dekker, Inc., New York, N.Y., 1975, p. 36.

(10) C. M. Metzler, G. L. Elfring, and A. J. McEwen, *Biometrics*, **30**, 3 (1974).

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Quantitation of Metronidazole in Pharmaceutical Dosage Forms Using High-Performance Liquid Chromatography

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Received June 6, 1983, from the *Department of Pharmaceutics, University of Houston, Houston, TX 77030.* Accepted for publication September 26, 1983.

Abstract □ A high-performance liquid chromatographic (HPLC) method for the quantitation of metronidazole in pharmaceutical dosage forms has been developed. The method is accurate and precise with an *RSD* of 0.68%, based on six readings. The excipients present in various dosage forms did not interfere with the assay procedure. A solution of metronidazole decomposed using heat showed 0% potency.

Keyphrases □ Metronidazole—HPLC, pharmaceutical dosage forms □ HPLC—pharmaceutical dosage forms of metronidazole

Metronidazole is extensively used in medicine as an antiprotozoal agent. The USP-NF method (1) for the quantitation of metronidazole in tablets is based on titration with perchloric acid, which requires a tedious extraction-purification procedure; moreover, other weak bases, impurities, and products of decomposition usually interfere with this type of volumetric titration. The quantitation of metronidazole in biological fluids was reviewed by Wood (2). The purpose of these investigations was to develop a stability-indicating assay procedure for the quantitation of metronidazole in pharmaceutical dosage forms based on HPLC.

EXPERIMENTAL SECTION

Chemicals and Reagents—All the chemicals and reagents were USP, NF, or ACS quality and were used without further purification. The USP quality powder of metronidazole[1] (2-methyl-5-nitroimidazole-1-ethanol; I) was used as received.

Apparatus—The high-performance liquid chromatograph[2] (HPLC) was equipped with a multiple-wavelength detector[3] and a recorder[4]. A semipolar column[5] (30 cm × 4 mm i.d.) was used. The average size of the particles was 10 μm.

[1] G. D. Searle & Co., Chicago, Ill.

[2] ALC202 equipped with U6K universal injector; Waters Associates, Milford, Mass.

[3] Spectroflow monitor SF770; Schoeffel Instruments Corp., Ramsey, N.J.

[4] Omniscribe 5213-12; Houston Instruments, Austin, Tex.

[5] μ-Bondapak phenyl; Waters Associates, Milford, Mass.

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