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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE METABOLISM OF PRIMAQUINE AND THE IDENTIFICATION OF A NEW MAMMALIAN METABOLITE

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SUMMARY

Using rats that had been dosed with 20 mg/kg of primaquine diphosphate (11.4 mg/kg free base), it was found that the drug underwent a metabolic oxidative deamination to give 8-(3-carboxy-1-methylpropylamino)-6-methoxyquinoline. The presence of this new mammalian metabolite was verified using high-performance liquid chromatographic, gas chromatographic, and mass spectral methods. A quantitative high-performance liquid chromatographic method for the determination of primaquine and the carboxylic acid metabolite in plasma using only 50 μl of whole blood from the rat was developed and the method could be used to detect levels as low as 0.05 μg/ml of the metabolite. Following intravenous administration of the drug, it was found that the plasma levels of primaquine fell very rapidly and after 30 min, the levels of the metabolite were much higher than those of primaquine.

INTRODUCTION

Primaquine (I) is often used in combination with other antimalarial drugs for prophylaxis in endemic areas. Primaquine has a fairly low therapeutic index and its use is frequently associated with hemolytic anemia, particularly in individuals with a deficiency in glucose 6-phosphate dehydrogenase [1]. In vitro studies using both normal and glucose 6-phosphate deficient erythrocytes

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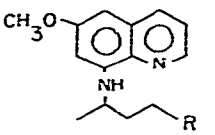
have indicated that this effect is caused by a primaquine metabolite rather than primaquine itself [2—4].

Earlier studies in these laboratories using microorganisms as models for mammalian metabolism had shown that 23 of 77 microorganisms studied could convert primaquine to II (Fig. 1) and/or III [5]. The primary objective of the present study was to develop analytical methods for these metabolites in plasma samples and to determine if these metabolites were formed in mammalian systems.



I $-CH_2NH_2$
II $-CH_2NHCOCH_3$
III $-CO_2H$
IV $-CO_2CH_3$

Fig. 1. Primaquine and metabolites.

EXPERIMENTAL

Reagents

Primaquine diphosphate was obtained from Aldrich (Milwaukee, WI, U.S.A.) and was used as received. The reference standards of 8-(4-acetamido-1-methylbutylamino)-6-methoxyquinoline (II) and 8-(3-carboxy-1-methylpropylamino)-6-methoxyquinoline (III) were prepared in these laboratories using a previously reported procedure [5]. All other materials were of reagent grade quality and were used as received.

Administration of primaquine and collection of blood samples

Primaquine diphosphate at a dosage of 20 mg/kg (11.4 mg/kg free base) was administered through the penile vein of male Wistar rats that had been anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneal). A single rat was used for the time—course study rather than using pooled data from several rats. However, the complete time—course study was repeated using a total of five rats. This method was made possible by the extremely small sample size required by the analytical procedure. Using 50-μl heparinized hematocrit tubes, blood samples were collected from the intraorbital sinus vein of a single rat at 1 min, 3 min, 15 min, 30 min, 1 h, 3 h, 6 h, 12 h, and 24 h after administration of primaquine diphosphate.

High-performance liquid chromatographic analysis of plasma samples

The 50-μl samples of whole blood were centrifuged for 2 min using a standard hematocrit centrifuge, then a 20.0-μl sample of the plasma was injected into the high-performance liquid chromatograph. A Waters Assoc. (Milford, MA, U.S.A.) Model 6000A pump, Model U6K injector, and Model 440 ultraviolet detector (254-nm and 280-nm dual detectors plumbed in series)

were used. A Whatman (Clifton, NJ, U.S.A.) C-18 guard column (with the first frit removed) was used between the injector and the analytical column. It was usually necessary to change the packing of the guard column every 20—30 injections.

A 30 cm × 3.9 mm I.D. octadecyl reversed-phase analytical column (Waters Assoc., μBondapak C_{18}, 10 μm particle size) was used with a flow-rate of 1.0 ml/min. The mobile phase consisted of 6.6 g K_2HPO_4, 8.4 g KH_2PO_4, 4.0 g N,N-dimethyl-octylamine, 2.4 l of methanol, and 1.6 l of water. The retention characteristics of primaquine and its metabolites (Table I) were quite consistent when aged columns were used. When new columns were first put into service, III was observed to elute (retention time, 10 min) after primaquine. After the column had aged, III (retention time, 7.44 min) was observed to elute before primaquine. This aging process usually took 7—10 days after which the retention times were very stable.

Quantitation of primaquine and metabolites was based on a direct comparison of the height of the chromatographic peaks for the plasma samples and freshly prepared pure reference standards of the compounds. The sensitivity of the procedure was primarily limited by the presence of naturally occurring plasma constituents. The quantity of III in the sample shown in Fig. 2 was 0.39 μg/ml. The peak for III was easily distinguished from adjacent chromatographic peaks by the marked differences in the A_{254}/A_{280} ratios.

Gas chromatographic analysis using a nitrogen-selective detector

A 2.0-ml sample of plasma was extracted with 2 × 3 ml of ethyl acetate and then the combined extracts were evaporated under a stream of nitrogen. The extract was then treated with 1.0 ml of diazomethane in diethyl ether at room temperature for 30 min. The ether was removed with a stream of nitrogen and the residue was taken up in 30 μl of methanol. Then 1.0 μl of the extract was injected into a Perkin-Elmer Model 900 gas chromatograph equipped with dual rubidium-bead nitrogen-selective and flame ionization detectors. A 183 cm × 2 mm glass column packed with 3% OV-17 on 110—120 mesh Anachrom ABS support was used at 265°C with a flow-rate of 30 ml/min. The ratio of the response of the two detectors (response index) was measured using a previously published procedure [6] and was used as a means of characterizing the materials in addition to their retention times.

TABLE I

HPLC CHARACTERIZATION OF PRIMAQUINE AND METABOLITE REFERENCE STANDARDS

Compound	Retention time (min)	A_{254}/A_{280}*
III	7.44	3.79 (3.74)
Primaquine, I	8.40	4.41 (4.26)
II	12.5	4.18

*Values in brackets are the absorbance ratios observed for the compounds as detected in the plasma of the test animal.

Gas chromatography—mass spectroscopy of metabolites

The plasma of a rat that had been given primaquine and one that had not received any drugs other than the anesthetic agent were extracted and derivatized as indicated in the previous section. The two extracts were chromatographed on a 3% OV-17 column in a Finnigan Model 3200 mass spectrometer with an INCOS data system. Ionization was by electron impact at 70 eV. Because of coelution of the metabolite and compound common to the extracts of the treated and control animals, a mass spectrum of the metabolite could not be obtained that was completely free of the peaks associated with the interfering substance. The gas chromatographic—mass spectrometric (GC—MS) data files of the treated animal and control animal were then re-examined by reconstructing single ion chromatograms (Fig. 4) at *m/e* 159, 186, 201, 215, and 288 which are characteristic of IV [5].

RESULTS AND DISCUSSION

The high-performance liquid chromatograms of plasma of the rats that had been given the intravenous (I.V.) dose of primaquine (Figs. 2 and 3) clearly indicate the rapid disappearance of the drug and the formation of metabolite III. The chromatograms of the plasma of a rat that had not received primaquine showed peaks at 6.80, 9.28, 11.32, and 15.10 min, but did not exhibit peaks at the same retention times for III (7.44 min) or I (8.40 min). Analysis of plasma samples taken 3 min after the injection of primaquine (Fig. 2) showed that metabolite III was formed very rapidly and by 3 h (Fig. 3) the concentration of the metabolite far exceeded that of the drug. Other than I and III, the only peaks observed in the chromatograms corresponded to natural constituents also found in the control plasma.

In chromatograms of complex matrices such as these, identification of metabolites through comparison of retention times alone will lead to misidentifications fairly frequently. In the present study however, the components in the plasma were characterized by the retention time and by the A_{254}/A_{280} values for each compound (Table I). Using this approach, it was fairly easy to differentiate between the natural plasma constituent (retention time 6.80 min, $A_{254}/A_{280} \approx 120$) and the reference standard of metabolite III (retention time 7.44 min, $A_{254}/A_{280} = 3.74$) even though the retention times of the two were fairly close.

Since III was a new mammalian metabolite of primaquine, additional studies were conducted to verify the production of the metabolite. The gas chromatogram of a pure reference standard of III that had been derivatized to its methyl ester IV was found to contain a peak at 5.0 min and a nitrogen-detector response index of 0.27 was observed. When the pure reference standard of III was derivatized and injected, a second peak was also observed that is the lactam form of III that probably results from the on-column elimination of methanol and cyclization. The lactam form of III was observed to have a retention time of 6.1 min and a nitrogen-detector response index of 0.19 was observed. Previous studies [6] on the correlation of chemical structure and the nitrogen-detector response index have shown that secondary amines are usually observed to have an index value of 0.18—0.32 while amides have values of 0.08—0.17

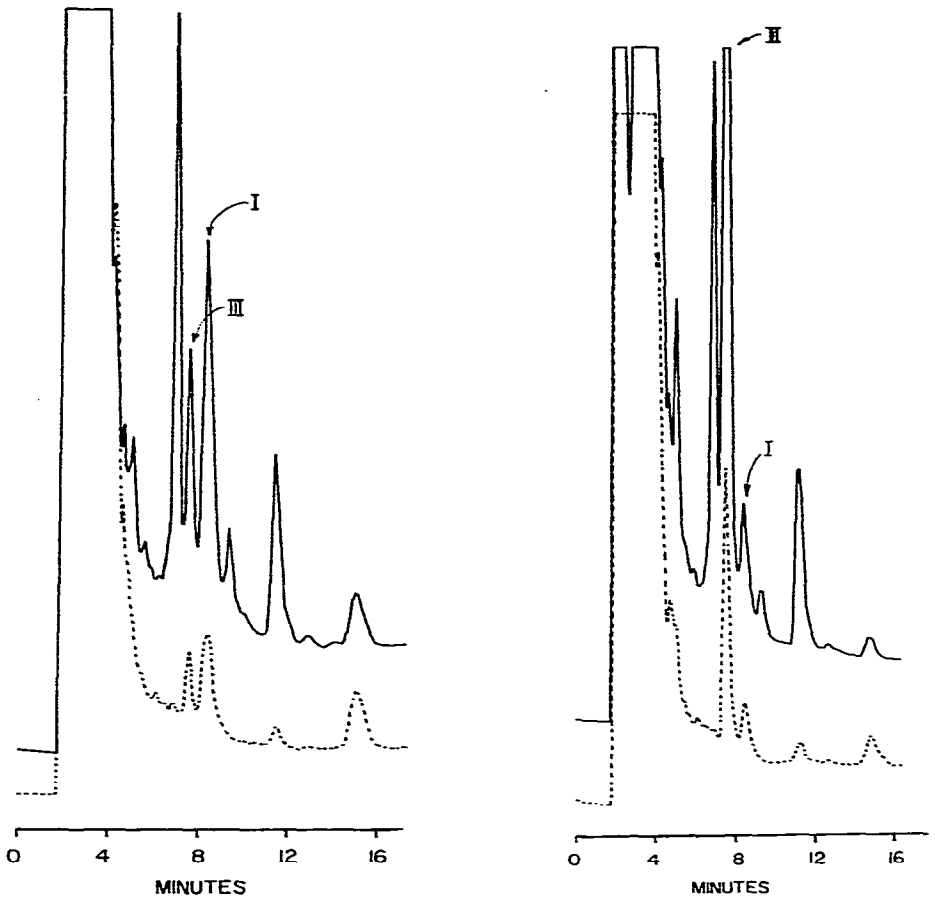


Fig. 2. High-performance liquid chromatogram of 3-min plasma sample. The solid line represents the response of the 254-nm UV detector (0.01 a.u.f.s.) and the dotted line represents the response of the 280-nm detector. The sample was found to contain 3.59 μg/ml I and 0.39 μg/ml III.

Fig. 3. High-performance liquid chromatogram of 3-h plasma sample. Conditions were the same as in Fig. 1. The sample was found to contain 1.21 μg/ml I and 2.31 μg/ml III.

which is consistent with the peak assignments of the ester and lactam. The mass spectrum of the GC peak of IV gave a spectrum [288 (M^+, 8%), 256 (7%), 215 (17%), 201 (100%), 186 (26%), 159 (45%), and 115 (29%)] that was consistent with its structure. The mass spectrum of the GC peak for the lactam [256 (M^+, 12%), 228 (16%), 213 (16%), 200 (70%), 186 (50%), 159 (100%), and 115 (40%)] was also consistent with its structure.

The identification of metabolite III was also substantiated by a GC analysis of the plasma of a rat 4 h after it had been given a 20 mg/kg dose of primaquine diphosphate. In this analysis, the derivatized extracts of the test animal and a control animal plasma were examined using a gas chromatograph equipped with a nitrogen-selective detector and a flame ionization detector. The nitrogen-selective detector showed a very strong response at 5.0 min corresponding to the derivative of III and a smaller peak at 6.1 min corresponding to the lactam in the sample from the treated animal. The

nitrogen-detector response of the derivatized extract of the control animal did not show any response during the 3.0—9.0 min portion of the chromatogram. Unfortunately, the nitrogen-detector response index of the two chromatographic peaks could not be measured because the flame ionization detection—gas chromatograms of the test animals and the control animal showed a large number of peaks in the 3.0—9.0 min region. In order to measure the nitrogen-detector response index of a peak, the peak must be clearly identifiable with both the nitrogen-selective and flame ionization detectors. In this case the metabolite peaks were very easily identified using the nitrogen-selective detector, but the flame ionization detector peaks of the metabolites were completely obscured by a large number of naturally occurring components in the region where the metabolite would elute. The reconstructed total ion chromatogram (6th and 12th tracings in Fig. 4) of the derivatized extracts of the test animal and control animal plasma were outwardly identical and they both matched their flame ionization detection—gas chromatograms. The most direct method of demonstrating the presence of the metabolite in this complex mixture was through the single-ion monitoring technique. The ions that were selected as being most characteristic were *m/e* 159, 186, 201, 215 and 288 and the result of this analysis is shown in Fig. 4 for the test sample and the control sample. Each tracing in Fig. 4 represents the intensity observed for a specific ion as a function of time (scan number) during the GC analysis. The dotted vertical line in Fig. 4 represents the scan number in which IV appeared. In the test sample, all five ion monitoring channels showed a response at the appropriate retention time, while only one of the ion monitoring channels showed a significant response for the derivatized extract of the plasma of the control animal. It was also observed that the scan of the extract of the test animal (scan 104) contained all of the MS peaks associated with the pure sample of IV, but there were additional ions in the spectrum that came from some other material.

The identity of III as a metabolite of primaquine was thus established by comparing HPLC retention times, A_{254}/A_{280} ratios, GC retention times using a nitrogen-selective detector, and mass spectra of the metabolite and an authentic standard of III. For a pharmacokinetic analysis of the time—course of the formation of the metabolite, the HPLC method was found to be the most useful. In order to determine the precision of the HPLC method, a 1.0-ml plasma sample containing approximately 10 μg/ml III was subdivided and assayed four times giving a coefficient of variation of ±4.3%. Concentrations as low as 0.05 μg/ml of III were detected in plasma samples using the HPLC procedure. The detection of III was limited by the presence of a naturally occurring plasma constituent that eluted just before the metabolite. The signal-to-noise ratio of HPLC system was not a limiting factor in the detection of III.

Following i.v. administration of primaquine diphosphate (Fig. 5), the plasma levels of the drug fall extremely rapidly. If one assumes that the dose was uniformly distributed in the blood at the moment of injection, a 200—400 μg/ml plasma level would be expected. Based on the observation of the plasma levels of nine rats 1 min following the injection, levels of only 2—10 μg/ml were observed and these values were rapidly falling. Studies still underway in these laboratories using ^{14}C-labeled primaquine have also shown that less than 1% of the dose remains in the blood when sampled just after the injection. The half-

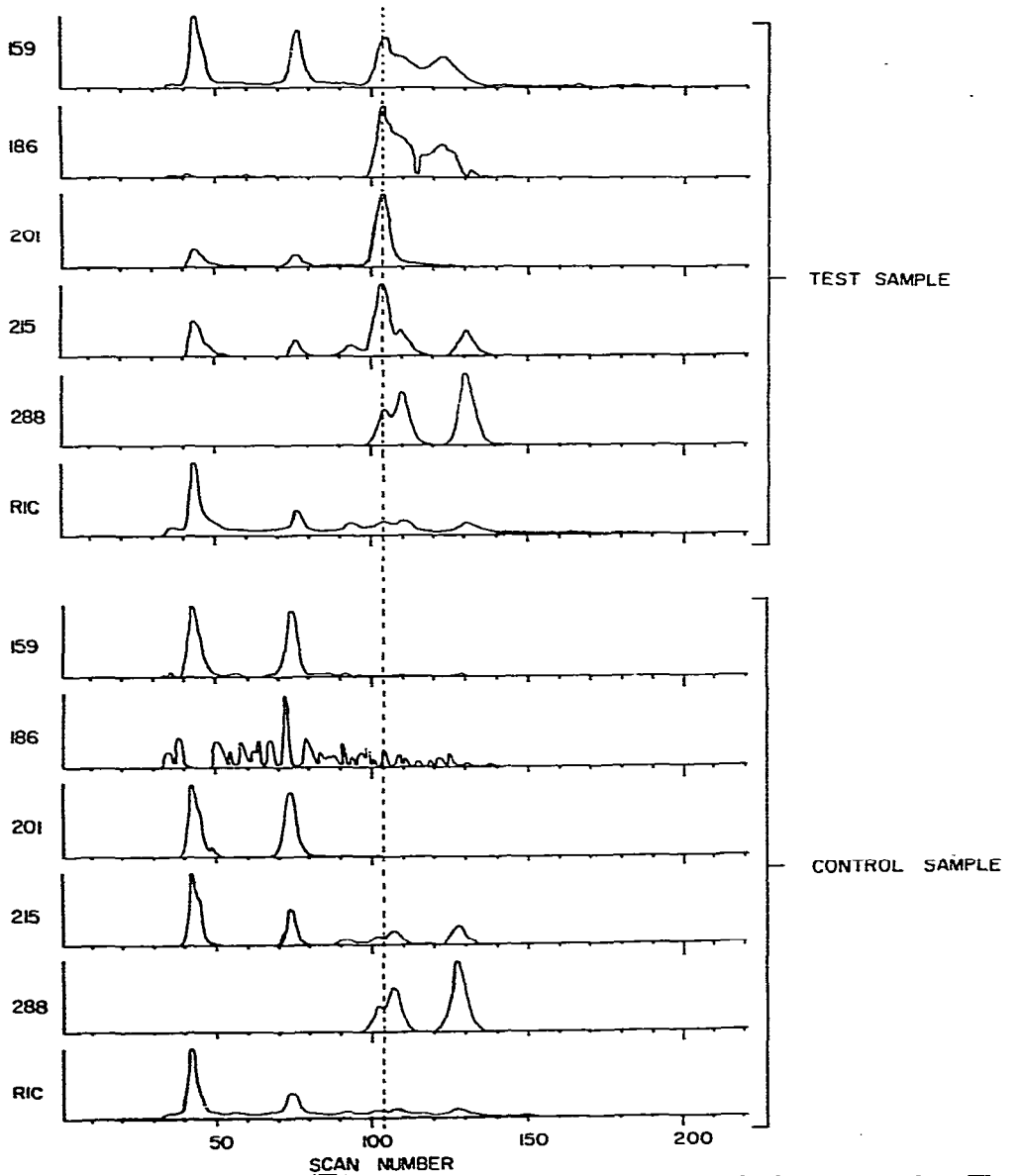


Fig. 4. Single-ion chromatograms of derivatized extracts of plasma samples. The upper 5 ion chromatograms were obtained from the derivatized extract of the plasma of a rat 4 h after it had received a 20 mg/kg i.v. dose of primaquine diphosphate. The lower 5 ion chromatograms were obtained from an animal that had not been given the drug. The dotted line represents the retention time obtained for an authentic standard of the methyl ester of III.

life of this rapid elimination would appear to be less than 1—2 min. During the 1—4.0 h period, the plasma level continues to fall with a half-life of 1.4—2.4 h, but the rate of decline appears to slow down after this period. The level of the metabolite (III) rises very rapidly (Fig. 5) and significant levels were observed 3.0 min following the injection (Fig. 2). From 2 to 4 h following

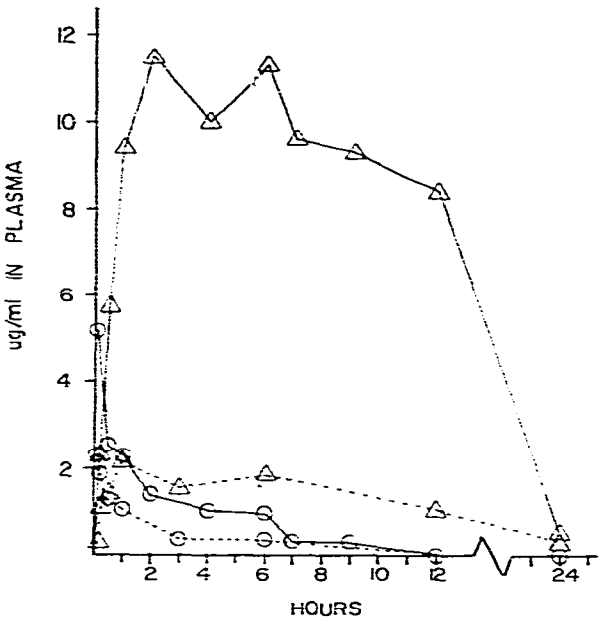


Fig. 5. Plasma concentration of primaquine diphosphate and III following a 20 mg/kg intravenous dose. ○, Primaquine diphosphate, △, III. The dotted lines were obtained using a second test animal.

the injection, the levels of III remained fairly constant and much higher than primaquine diphosphate. Even after 24 h, 0.2—0.5 μg/ml levels of the metabolite were still observed.

As can be seen in data for the two rats given the i.v. dose (Fig. 5), there was a considerable variation in the amount of III formed. More extensive pharmacokinetic studies with a larger number of animals are still in progress and will be reported at a later date. The data for the two animals shown in Fig. 5 represent the extremes of the data that have been observed to date.

In summary, for the first time in mammals, primaquine has been found to be metabolized to III and the concentration of this metabolite in the plasma is considerably higher than that of the parent drug except for the first few minutes after administration. In contrast to the metabolism by fungi, the N-acetyl derivative (II) was not found to be a metabolite of primaquine in rat. The HPLC method that was developed requires only 50 μl of whole blood for the analysis of primaquine and the metabolite. The low limit of detectability of III is about 0.05 μg/ml and this limit arises primarily because of a naturally occurring peak that elutes just before the metabolite.

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