Research Article

Characterization of the Urinary Metabolites of Primaquine in Rats

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Following the synthesis of reference standards of the primaquine metabolites, a high-performance liquid chromatographic (HPLC) analytical method for carboxyprimaquine, its glycine conjugate, and its glucuronide conjugate in urine samples was developed. After the administration of primaquine, only trace quantities of primaquine and carboxyprimaquine (each less than 1% of dose) and no significant quantity of the two conjugates of carboxyprimaquine were excreted in the urine. When carboxyprimaquine was administered, only 0.3% of the dose was excreted in the urine. When carboxyprimaquine glycinate was administered, the compound was found in the 700- to 1300-μg/ml concentration range in the urine within the first few hours. Using ^{14}C-labeled primaquine, six new metabolites were found in the urine and partially characterized. The purported metabolites of primaquine (8-amino-6-methoxyquinoline, 5-hydroxyprimaquine, and 6-desmethyl-5-hydroxyprimaquine) were not found in the urine. A field screening test for the quantitation of primaquine metabolites in urine was also investigated. The inexpensive, but sensitive assay was found to give results that closely paralleled the more complex HPLC method and it was moderately well correlated with the total radioactivity in the urine samples.

KEY WORDS: glycine conjugate synthesis; glucuronide; field screening; carboxyprimaquine.

INTRODUCTION

Although the metabolism of primaquine has been studied over the last 35 years, remarkedly little is actually known about the metabolism of this very important antimalarial. Following an initial α-oxidation, one could predict that **2** and carboxyprimaquine (**3**) (Fig. 1) would be formed from primaquine. Baty *et al.* (1) reported the presence of **2** in urine samples but subsequent studies in rats (2), monkeys (3), and humans (4) have failed to detect this compound. However, it should be noted that **2** is commonly found as an impurity in commercial samples of primaquine. Carboxyprimaquine was first reported as a mammalian metabolite in rats (2), and it was subsequently found in monkeys (3) and humans (3–6). In rats, 22% of the primaquine was converted to the carboxyprimaquine, 35–83% conversion was found in monkeys, and 55% conversion was found in humans.

Although all of the above studies have found very high concentrations of carboxyprimaquine in the plasma of the subjects, only trace quantities of primaquine and carboxyprimaquine have been found in urine samples. Since the majority of the dose of primaquine was converted to carboxyprimaquine, it would seem likely that the reason that little carboxyprimaquine was found in the urine was that it was further metabolized to give either a glucuronide (**5**) or a glycine (**4**) conjugate. The major objectives of the present study were to prepare a reference standard of **4**, to develop an analytical method for **3**, **4**, and **5** using an internal standard, and to characterize the other urinary metabolites of primaquine.

EXPERIMENTAL

Chemistry

^1H (NMR) nuclear magnetic resonance spectra were obtained on a Varian Model EM390 (90-MHz) spectrometer, and ^{13}C NMR spectra on a JEOL JNM FX-60 (15.03-MHz) Fourier transform spectrometer. Infrared spectra were obtained on a Perkin–Elmer Model 281B spectrometer and electron-impact (70-eV) mass spectra were obtained on a Finnigan 3200 MS/DS system. Melting points were taken on a Mel-Temp apparatus and were uncorrected. Elemental analysis was performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Flash chromatography (7) columns were packed with Merck silica gel 60 (0.040–0.063 mm, Cat. No. 9385).

Ethyl 2-[1-(2-Oxo-5-hydroxy-6-methyl)pyrrolidinyl]-acetate (9A)

Method A. To a solution of levulinic acid (21 mmol, 2.4 g) in 10 ml of dry THF was added N,N^1-carbonyldiimidazole

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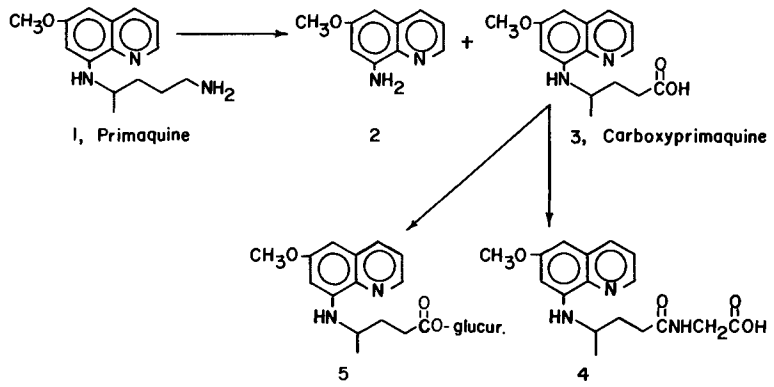

Fig. 1. Proposed metabolic pathway for the formation of the urinary metabolites of primaquine.

(21 mmol, 3.4 g), the mixture was stirred at room temperature for 30 min, and then ethyl glycinate (21 mmol, 2.1 g in $CHCl_3$) was added. After 10 hr, the solvent was evaporated under a stream of N_2 and the residue was purified by flash chromatography (elution with ethyl acetate) to give 1.84 g (44%) of a yellow transparent oil. IR (neat) 3350 (OH), 1750 ($-CO_2-$), 1675 cm^{-1} (Lactam); ^1H NMR ($CDCl_3$) δ 1.3 (t, 3, $-CH_2CH_3$), 1.5 (s, 3, C5 – CH_3), 2.3 (t, 2, C4 – H_2), 2.6 (t, 2, $-C3H_2$), 4.1 (d, 2, $-NHCH_2COO$), 4.3 (q, 2, $-COOCH_2-$); ^{13}C NMR ($CDCl_3$) 114.0 (q, CH_2CH_3), 26.0 (q, C5 – CH_3), 29.0 (t, C3), 35.0 (t, C4), 40.2 (t, $-NHCH_2-$), 61.3 (t, $-OCH_2$), 89.5 (s, C5), 169.8 (s, $-COO-$), 175.0 (s, C2); mass spectrum (70 eV), *m/e* (relative intensity) 201 (M^+, 0.1), 186 (17), 140 (10), 128 (14), 112 (20), 110 (15), 99 (100), 82 (14).

Method B (9A). A mixture of α-angelica lactone (6.5 mmol, 0.64 g) and ethyl glycinate free base (6.3 mmol, 0.65 g) in chloroform was stored at 0°C for 5 hr. Purification of the product by flash chromatography gave 0.99 g (78%) that had spectral data identical to those of 9A obtained by Method A.

Benzyl N-(4-Oxopentanoyl)glycinate (8B).

Benzyl glycinate was prepared from glycine and benzyl alcohol with thionyl chloride and isolated as the HCl salt (8). A mixture of benzyl glycinate free base (24 mmol, 4.0 g) and α-angelica lactone (24 mmol, 2.4 g) in chloroform was stirred at 0°C for 1 hr, then stored at 20°C for 10 hr. After purification by flash chromatography (elution with ethyl acetate) and evaporation *in vacuo,* 5.58 g (88%) of a yellow oil was afforded. Crystallization of the material from diethyl ether gave 8B (mp 44–45°C). IR (neat) 3280 (NH), 1760 (COO), 1740 (CO), 1700 cm^{-1} (CONH); ^1H NMR ($CDCl_3$) δ 2.15 (s, 3, CH_3), 2.5 (t, 2, $-COCH_2$), 2.8 (t, 2, CH_2CONH), 4.1 (d, 2, $NHCH_2$), 5.2 (s, 2, OCH_2), 6.35 (s, 1, NH), 7.3 (s, 5, Ar); ^{13}C NMR ($CDCl_3$) 29.4 (t), 29.7 (q, CH_3), 38.3 (t), 41.4 (t, $NHCH_2$), 66.9 (t, OCH_2), 128.3 (d, Ar), 128.4 (d, Ar), 128.6 (d, Ar), 135.5 (s, Ar), 170.0 (s, COO), 172.6 (s, CONH), 207.7 (s, CH_3CO); mass spectrum (70 eV), *m/e* (relative intensity) 236 (M^+, 3.6), 156 (20), 139 (10), 128 (13), 110 (11), 99 (100), 91 (79), 65 (10).

N-[4-N-(6-Methoxy-8-quinolinyl)amino]pentanoyl Glycine (4)

Using a literature procedure, 8-amino-6-methoxyquinoline was prepared from 4-methoxy-2-nitroaniline and nitrobenzene (9). A solution of the aminoquinoline (6.8 mmol, 1.79 g) in 1.0 ml of methanol was added to a mixture of 8B (11.7 mmol, 2.04 g) in 3.5 ml of methanol and 0.8 ml of 5 *N* methanolic HCl (4 mmol), followed by the addition of $NaBH_3CN$ (3.4 mmol, 0.213 g) and 10 g of molecular sieves (3A). The mixture was occasionally stirred and the reaction allowed to proceed at room temperature for 48 hr. After removal of the molecular sieves by filtration, the methanol was evaporated *in vacuo* to yield a dark oil, which was purified by flash chromatography (eluted with 20% CH_3CN in benzene) to give 1.77 g (62%) of 10B. ^1H NMR ($CDCl_3$) δ 1.25 (d, 3, $CHCH_3$), 2.0 (t, 2, $CHCH_2$), 2.4 (t, 2, $CHCH_2CO$), 3.9 (s, 3, OCH_3), 4.05 (d, 2, $NHCH_2$), 5.2 (s, 2, CH_2Ar), 6.4 (s, 2, C5 – H, C7 – H), 7.3 (m, 1, C3 – H), 7.4 (s, 5, Ar), 8.0 (dd, 1, C4 – H), 8.6 (dd, 1, C2 – H).

A solution of 10B (3.02 mmol, 1.27 g) in 5 ml of 10% KOH (in 1:4 water:ethanol) was heated in a steam bath for 10 min and then allowed to cool in room temperature. After evaporation of the solvent *in vacuo,* 5 ml of H_2O was added and the pH was lowered to 3.08 by adding drops of 85% H_3PO_4, then extracted with ethyl acetate (3 × 20 ml). The extract was evaporated *in vacuo* to give a residue that was then purified by flash chromatography (eluted with 20% CH_3OH in $CHCl_3$). The solvents of the combined fractions were evaporated and the residue was crystallized from anhydrous ethanol by the addition of drops of 85% phosphoric acid to give 0.735 g (57%) of 4 as yellow crystals, mp 178.0–178.5°C. IR (KBr) 1710 (COOH), 1630 cm^{-1} (NHCO); ^1H NMR (CD_3OD) δ 1.35 (d, 3, $CHCH_3$), 2.05 (t, 2, $CHCH_2$), 2.45 (t, 2, CH_2CH_2CO), 3.6–3.8 (CH and $OCNHCH_2$), 3.9 (s, 3, OCH_3), 6.45 (s, 1, C7 – H) (C5 – H exchanges with CD_3OD), 7.45 (dd, 1, C3 – H), 8.15 (dd, 1, C4 – H), 8.55 (dd, 1, C2 – H); ^{13}C NMR (CD_3OD) 20.2 (5′), 32.3 (3′)*, 32.31 (2′)*, 43.1 ($NHCH_2CO$), 47.8 (1′), 55.1 (OCH_3), 91.9 (5), 97.3 (7), 121.8 (3), 130.0 (4a), 135.1 (4), 135.1 (8a), 144.2 (2), 144.9 (8), 159.6 (6), 174.3 (4′), 176.2 (COOH); mass spectrum (70 eV), *m/e* (relative intensity) 331 (M^+, 3.6), 228 (12), 201 (100), 200 (42), 187 (33), 186 (36), 159 (53), 158 (24), 130 (20). *Anal.* ($C_{17}H_{24}N_3O_8P$) Calcd: C, 47.56: H, 5.63; N, 9.79. Found: C, 47.68; H, 5.94; N, 9.62.

4-(1-Naphthylamino)butyric Acid

To a solution of 1-aminonaphthalene (0.11 mol, 15 g) and ethyl 4-bromobutyrate (0.14 mol, 27 g) in 27 ml of

DMSO, crystals of KI (5 g) and 22 g of $NaHCO_3$ were added at room temperature. The reaction mixture was stirred at 90°C for 2 days, cooled, mixed with 400 ml diethyl ether, filtered, then extracted with H_2O (3 × 100 ml). The ether layer was evaporated *in vacuo*, then the residue was hydrolyzed in aqueous KOH. The hydrolysis mixture was adjusted to pH 4.0 with 4 *N* HCl, extracted with diethyl ether, dried, then evaporated to give a crystalline product. Recrystallization from MeOH/H_2O (1:1) gave 2.5 g (10%) of the final product as white crystals [mp 110–111°C; lit. (10) mp 105–107°C].

Animal Studies

Male Wistar rats (380–500 g) were administered primaquine diphosphate (Aldrich Chemical Co.) by intraperitoneal injection (20 mg/kg). For the radiolabel studies, a 9:1 mixture of primaquine diphosphate and 2,4-[^{14}C]primaquine diphosphate (1.55 mCi/mmol, New England Nuclear Corp.) was administered to the rats. For collection of urine, a maximum of two animals was placed in each metabolism cage, which separated the urine from the feces. Tap water was provided *ad libidum*, but no food was given during the collection of the samples. After collection, the urine samples were stored in amber bottles at 0°C.

Analytical Methods

Determination of Carboxyprimaquine and the Glycine Conjugate of Carboxyprimaquine (4) in Urine Samples

A 1.0-ml sample of the urine was spiked with 5 μg of the internal standard [4-(1-naphthylamino)butyric acid] and mixed with 1.0 ml of a 0.2 *M*, pH 3.1, phosphate buffer, then the pH of the mixture was brought to 3.1 by the careful addition of microliter volumes of 85% phosphoric acid. The solution was extracted with 10 ml of ethyl acetate, the aqueous layer was discarded, then the ethyl acetate phase was back extracted with 5 ml of aqueous 0.045 *N* NaOH for 2 min. The aqueous layer was mixed with 5 ml of a 2 *M*, pH 3.1, phosphate buffer, then extracted with 10 ml of ethyl acetate. The ethyl acetate layer was evaporated under a stream of N_2 at room temperature and the residue was redissolved in 25 μl of CH_3OH for high-performance liquid chromatographic (HPLC) analysis. Using HPLC System C, 5 μl of the solution was chromatographed and the peak heights at 254 and 280 nm were determined. The procedure was calibrated by subjecting a sample containing 5 μg of carboxyprimaquine, 5 μg of **4**, and 5 μg of the internal standard in 1.0 ml of H_2O to the same back-extraction and chromatographic procedure, and the peak height ratios of the metabolite peak and the internal standard peak were then compared to the same ratio in the test sample.

HPLC Determination of Metabolite B in Urine Samples

A 10-μl sample of the unprocessed urine sample was injected into the HPLC system equiped with a 2 × 60-mm C-18 guard column using HPLC System A. The concentration of Metabolite B in the samples was obtained by comparison of the peak heights to those of a standard solution of *N*-(5,6-dihydroxy-8-quinolinyl)-5-methyl-pyrrolidin-2-one.

^{14}C Radiochromatograms of Urine Samples

Unprocessed urine samples (20 μl) obtained from the rats dosed with 20 mg/kg ^{14}C-labeled primaquine diphosphate were chromatographed using either HPLC System A or HPLC System B. The output of the column was divided into 1.5-ml fractions for scintillation counting in 15 ml of the cocktail (Insta-Gel, United Technologies Packard Co.). Scintillation samples of 20 μl of the unfractionated urine sample, 1.5 ml of the mobile phase, and a known quantity of the ^{14}C-labeled primaquine were also individually prepared and counted. After correction for counting efficiency (external standard ratio method) and subtraction of the blank, the corrected disintegrations per minute (dpm) for each HPLC fraction was converted to the quantity of metabolite present expressed both as a percentage of the ^{14}C label in the 20 μl of urine that was assayed and as a percentage of the dose that had been administered.

HPLC System A

A 3.9 mm × 30-cm octadecyl reversed-phase column (10-μm particle size, μBondapak C_{18}, Waters Associates) was used at a flow rate of 1.5 ml/min with dual 254- and 280-nm ultraviolet (UV) detectors (Model 440, Waters Associates), the mobile phase was prepared using 3.2 liters of H_2O, 32.8 g of sodium acetate, 4.0 g of *n*-octylamine, 0.8 g of disodium ethylenediaminetetraacetate, and the dropwise addition of glacial acetic acid to bring the pH to 5.0. After adjusting the pH, 800 ml of CH_3OH was added to the mixture and then degassed.

HPLC System B

The same reversed-phase column as used for System A was also utilized for this determination, except for a modification in the mobile phase. The mobile phase (1.5 ml/min) was prepared using 1.6 liters of CH_3OH, 2.4 liters of H_2O, 6.6 g of K_2HPO_4, 8.4 g of KH_2PO_4, and 4.0 g of *N,N*-dimethyloctylamine (Ethyl Corp.). The chromatograms were obtained either using a dual-wavelength UV detector (254 or 280 nm) or by liquid scintillation counting.

HPLC System C

The column and mobile phase utilized were identical to those in System B, except that 1.8 liters of CH_3OH and 2.2 liters of H_2O were used. The chromatograms were obtained using dual 254- and 280-nm UV detectors.

Determination of Carboxyprimaquine Glucuronide Conjugates

After the administration of primaquine diphosphate to the rats, 0.5 ml of the urine, 0.5 ml of phosphate buffer (pH 6.4, 0.075 *M*), and 0.5 ml of β-glucuronidase (2.3 mg/ml, Type IX from *Escherichia coli*, Sigma Chemical Co.) were incubated at 37°C for 2 hr in a shaker bath (100 osc/min). After incubation, 5 μg of the internal standard [4-(1-naphthylamino)butyric acid] was added and the mixture was assayed for carboxyprimaquine using the extraction, UV-HPLC method described above for the determination of carboxyprimaquine in urine.

In order to verify that the incubation conditions were

suitable for the β-glucuronidase hydrolysis to the aglycon, a 0.5-ml urine sample from a control rat was spiked with phenolphthalein glucuronide (0.0015 *M*), then the sample was incubated under the same conditions for the same time period. After quenching the reaction with 4.0 ml of glycine buffer (pH 10.4, 0.2 *M*), the absorbance at 550 nm was measured to determine the amount of phenolphthalein that was released.

Determination of Glucuronides by ^{14}C HPLC

After the administration of ^{14}C-labeled primaquine diphosphate (20 mg/kg, ip), the urine was collected over a 24-hr period. A 200-μl portion of the urine collected from 6 to 12 hr was mixed with 200 μl of phosphate buffer (0.075 *M*, pH 6.4) and 200 μl of β-glucuronidase (2.27 mg/ml), then incubated at 37°C for 2 hr. A 60-μl fraction of this mixture was chromatographed using HPLC System A and a second 60-μl fraction of the mixture was chromatographed using HPLC System B. The output of both systems was collected in 0.5-ml fractions, mixed with 15 ml scintillation fluid, then counted.

A duplicate 200-μl sample of the 6- to 12-hr urine collection from the ^{14}C-primaquine-treated rat was mixed with 200 μl of phosphate buffer and 200 μl of H_2O, then incubated under the same conditions as the β-glucuronidase-treated urine samples. After the incubation, 60 μl was chromatographed using System B. The resulting ^{14}C chromatograms were then compared to the β-glucuronidase-treated samples to determine the presence of glucuronides.

Colorimetric Determination of Primaquine Metabolites

The C-18 extraction cartridges (Bond Elut C-18, 100 mg, Analytichem International) were first washed with CH_3OH (2 × 1.0 ml) and with H_2O (2 × 1.0 ml), then 1.0 ml of the urine sample was passed through the cartridge using centrifugation. After washing the cartridge with 1.0 ml of H_2O, the metabolites were eluted into a fresh conical collection tube using 1.5 ml of CH_3OH. The eluate was then mixed with 1.5 ml of phosphate buffer (0.075 *M*, pH 5.1) and 100 μl of 20 mg/mL fast violet B (6-benzamido-4-methoxy-*m*-toluidine, Sigma Chemical Co.). The coupling reaction mixture was allowed to stand at room temperature for 15 min, then the absorbance at 525 nm was determined. The procedure was calibrated by extracting and derivatizing a series of aqueous carboxyprimaquine standards, then comparing the observed 525-nm absorbance readings of the urine samples and the carboxyprimaquine standards.

RESULTS AND DISCUSSIONS

Chemistry

The initial attempts to prepare **4** directly from carboxyprimaquine and ethyl glycinate using the standard amino acid coupling reagents were not successful and the lactam (11) of carboxyprimaquine was found to be the major product. The second general approach to the synthesis was to couple the preformed side chain to the 8-aminoquinoline using a reductive alkylation (Fig. 2). It was found that the reaction of α-angelica lactone (**6**) (or 4-oxopentanoic acid with the amino acid coupling reagent carbonyldiimidazole) with the glycine ester gave either **9** (^1H NMR, CH_3 at 1.50 ppm; ^{13}C NMR, C_4 at 89.5 ppm) or **8** (^1H NMR, CH_3 at 2.15 ppm; ^{13}C NMR, C_4 at 207.7 ppm). As reported previously for similar reactions with α-angelica lactone (12–14), the ratio of the open-chain to the cyclic product was influenced by the reaction conditions and the nature of the substituents. In this specific case, the reaction of **6** and **7B** gave an 88% yield of the open-chain compound (**8B**), while the reaction of **6** with 7A gave mostly **9A**. The reductive alkylation of 8-amino-6-methoxyquinoline with **8B** (Fig. 2) gave a 62% yield of ester of the target compound (**10B**), which was hydrolyzed, then crystallized as the phosphate salt.

Analytical Methods

Carboxyprimaquine and the glycine conjugate of carboxyprimaquine (**4**) were difficult to extract from urine samples because of the amphoteric nature of both of these compounds. The pKa of the basic quinoline nitrogen would probably be close to the value for primaquine (pKa, 3.20) (15), while that of the carboxyl group (approx. pKa, 3.1) would be close to the value for *N*-glycylglycine. Experimentally it was found that the optimum pH for the extraction of the glycine conjugate was pH 3.1 and the recovery dropped off rapidly at higher or lower pH values (Fig. 3).

Because of the difficulty of the extraction of the conjugate, 4-(1-napthylamino)-butyric acid was synthesized and used as an internal standard for the assay because its amphoteric nature was fairly close to that of **4** and carboxyprimaquine. The extraction-pH profiles of all three compounds were found to be similar (Fig. 3), which enhanced the precision of the urine analysis. Using spiked urine samples, the absolute recoveries were 90 ± 18% for carboxyprimaquines, 81 ± 18% for the glycine conjugate, and 88 ± 22% for the internal standard (values determined from nine assays). Using spiked urine samples, the precision and accuracy of the working method (Table I) were found to be very satisfactory.

Excretion of Conjugates Following the Administration of Primaquine

Following the administration of primaquine, only trace quantities of carboxyprimaquine were found in the urine (Table II) and an average of only 0.58% of the dose was recovered in this form. It was also found that treatment of the urine with β-glucuronidase did not increase the amount of free carboxyprimaquine, while treatment of the same urine samples spiked with phenolphthalein glucuronic acid did release phenolphthalein. Thus it did not appear that glucuronic acid conjugation with carboxyprimaquine was a significant pathway. Analysis of the urine samples also failed to find concentrations of the glycine conjugate of carboxyprimaquine above the detection limit of the procedure (0.23 μg/ml, equivalent to 0.02% of the dose).

Excretion Following the Administration of Purported Metabolites

From previous studies of blood plasma metabolites, it had been found that a major portion of primaquine was converted to carboxyprimaquine (2–6). However as shown in Table II, an average of only 0.58% of the dose was actually

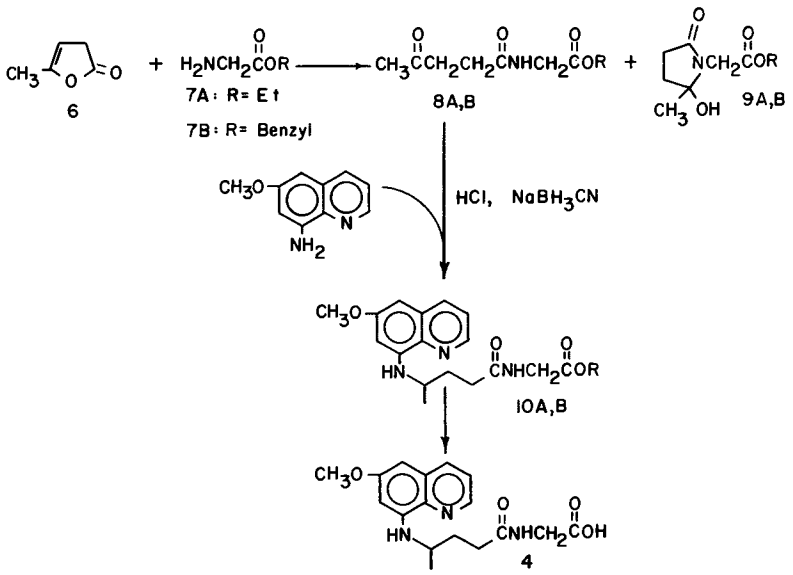

Fig. 2. Synthesis of the glycine conjugate of carboxyprimaquine.

excreted in the urine as carboxyprimaquine. In order to study further this metabolic pathway, carboxyprimaquine was administered directly to the rats and analysis of the urine (Table III) showed that only a very small fraction of the dose was excreted as carboxyprimaquine. It was also noted that the concentration of carboxyprimaquine in the urine was about the same irrespective of whether primaquine or carboxyprimaquine was administered to the animal.

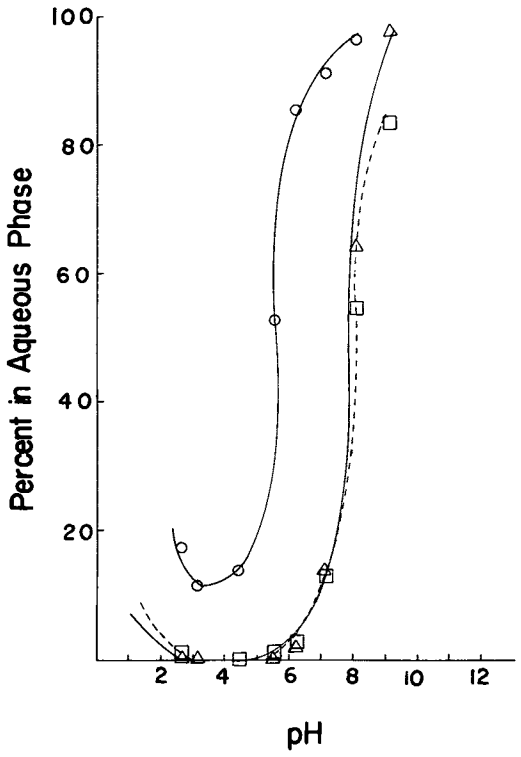

Fig. 3. Effect of pH on the extraction of metabolites from the aqueous phase into ethyl acetate. Carboxyprimaquine (□); glycine conjugate of carboxyprimaquine (○); 4-(1-naphthylamino)butyric acid (△).

When the glycine conjugate of carboxyprimaquine (4) was directly administered to the animals, very high concentrations of the conjugate were found in the urine (Table IV). The excretion was found to be very rapid, with approximately 90% of the output occurring within the first 3 hr. From a comparison of the very high urinary concentrations of the conjugate found after the administration of 4 (approx. 900 μg/ml; Table IV) with the very low urinary concentrations (less than 0.2 μg/ml; Table III) found after the administration of primaquine, it appeared that little, if any, of the carboxyprimaquine that was formed was conjugated with glycine.

Distribution of ^{14}C-Labeled Metabolites in Urine

Although the oxidative deamination of primaquine to carboxyprimaquine was the major metabolic pathway, the above studies had shown that little of the unchanged primaquine or unchanged carboxyprimaquine was excreted in the urine and the studies had shown that the conjugation of carboxyprimaquine with either glucuronic acid or glycine was not a major pathway. In the next series of experiments, ^{14}C-labeled primaquine was used to follow the pathway through carboxyprimaquine to secondary metabolites other than the simple conjugates that had already been investigated.

Table I. Determination of the Precision and Accuracy of the HPLC Assay for Carboxyprimaquine and the Glycine Conjugate of Carboxyprimaquine (4) in Rat Urine

	Apparent concentration[a] (μg/ml)	
Urine sample	Carboxyprimaquine	Glycine conjugate (4)
1	4.36	4.55
2	4.52	4.64
3	5.37	5.27
Average	4.75	4.82
SD	±0.54	±0.39

[a] Apparent concentration of the conjugate and the aglycone following the addition of 5 μg/ml of each to control urine.

Table II. Urinary Excretion of Carboxyprimaquine and Its Glycine and Glucuronide Conjugate (4) Following the Administration of 20 mg/kg of Primaquine Diphosphate

		Carboxyprimaquine		β-Glucuronidase[a]		Glycine conjugate (4)	
Animal	Time (hr)	μg/ml	Cum. excr. (%)[b]	μg/ml	Cum. excr. (%)	μg/ml	Cum. excr. (%)
A	0–12	11.9	0.93	9.56	0.75	<0.2[c]	<0.018
	12–24	3.12	1.17	1.90	0.89	<0.2	<0.036
	24–36	<0.1	1.17	—	—	<0.2	<0.048
B	0–12	1.15	0.17	3.72	0.56	<0.2	<0.034
	12–24	1.05	0.26	<0.1	0.56	<0.2	<0.054
	24–36	<0.1	0.26	<0.1	0.56	<0.2	<0.068
	36–48	0.1	0.26	<0.1	0.56	<0.2	<0.076
C	0–12	1.32	0.32	1.51	0.36	<0.2	<0.055
	12–24	<0.1	0.32	<0.1	0.36	<0.2	<0.13
	24–35	<0.1	0.32	<0.1	0.36	<0.2	<0.17
	36–48	<0.1	0.32	<0.1	0.36	<0.2	<0.28

[a] Carboxyprimaquine concentration after treatment with β-glucuronidase.
[b] Cumulative excretion as a percentage of the dose.
[c] Less than the detection limit of the assay.

Because of the very poor extractibility of primaquine metabolites, the ^{14}C high-performance liquid chromatograms (Figs. 4 and 5) were obtained through the direct injection of unprocessed urine samples. As had been found with the conventional HPLC analysis, the radio chromatograms showed that only small quantities of primaquine (Fig. 4) and carboxyprimaquine (Fig. 5) were excreted in the urine. In addition, six other transformation products (Metabolites A through F) were found in the urine. While 8-amino-6-methoxyquinoline (1) and 6-desmethyl-5-hydroxyprimaquine (16) have previously been reported as metabolites, a comparison of HPLC analysis of authentic reference standards of these materials failed to show a correspondence to the radiochromatograms of the urine samples. The HPLC retention index (17,18) of each of the six metabolites was also measured and compared to the available reference standards of the potential or purported metabolites of primaquine (Table V). Metabolite A (retention index, 538) and Metabolite B (retention index, 681) were found to be more polar than primaquine (retention index, 733) but neither metabolite coeluted with any of the hydroxylated reference standards. Metabolite C had a retention just slightly below the value for primaquine, but the two compounds could be chromatographically distinguished. It was also observed that the retention index of Metabolite F (retention index, 786) was not distinguishable from the index of the synthetic standard of 4 (retention index, 785) but the back-extraction HPLC assay (Table II) had clearly shown that 4 was not present in these urine samples.

Most phase I metabolic transformations of xenobiotics give products that are more polar than the parent compound and the retention index of these products can be accurately estimated (17). Because most phase I transformations lead to increases in the polarity of the products, it would seem anomalous that the retention index of Metabolite D, Metabolite E, Metabolite F, and carboxyprimaquine all were *larger* than the value for primaquine. Although the retention of most compounds was governed by a simple lipophilicty mechanism, the addition of *N,N*-dimethyloctylamine to the mobile phase greatly increased the retention index of negatively charged molecules such as carboxyprimaquine. Thus it was possible that Metabolites D, E, and F could have been polar secondary metabolites of carboxyprimaquine or conjugates of an amino acid (other than glycine) with carboxyprimaquine, which would have a high retention index because of the ion-pair retention mechanism.

Table III. Urinary Excretion of Metabolites Following the Administration of Carboxyprimaquine[a]

		Carboxyprimaquine		Metabolite B	
Animal	Time (hr)	μg/ml	Cum. excr. (%)[b]	μg/ml	Cum. excr. (%)
D	0–3	0.96	0.021	<6[c]	<0.08
	3–6	2.1	0.085	<6	—
	6–12	1.5	0.179	<6	—
	12–24	1.7	0.337	<6	—
E	0–3	0.96	0.016	<6	<0.09
	3–6	2.9	0.064	<6	—
	6–12	2.3	0.106	<6	—
	12–24	1.7	0.274	<6	—

[a] 20 mg/kg, ip.
[b] Cumulative excretion as a percentage of the dose.
[c] Less than the detection limit of the assay.

Table IV. Urinary Excretion of Metabolites Following the Administration of 20 mg/kg of the Phosphate Salt of the Glycine Conjugate (4)

Animal	Time (hr)	Glycine conjugate (4) μg/ml	Glycine conjugate (4) Cum. excr. (%)
F	0–12	125.	15.6
	12–24	0.66	15.9
	24–36	<0.2	15.9
	36–48	<0.2	15.9
G	0–3	1283.	18.2
	6–12	79.0	20.3
H	0–3	742.	20.6
	3–6	7.8	20.8
	6–12	6.0	21.4

The time course of the ^{14}C chromatograms (Table VIA) indicated that primaquine, Metabolite B, and Metabolite C were excreted mostly in the 3- to 6-hr period, while Metabolite D was excreted mostly in the 6- to 24-hr period. This observation and the above retention index data both are consistent with the hypothesis that Metabolite D was a secondary metabolite of primaquine derived through carboxyprimaquine.

Glucuronide Conjugates

Although it was known that glucuronic acid conjugation of carboxyprimaquine was not a major pathway (Table II), the role of glucuronic acid conjugation with other primaquine metabolites was not known.

From a comparison of the ^{14}C HPLCs of the urine samples without enzyme treatment (Fig. 6) to those with β-glucuronidase treatment (Fig. 7), it appeared that Metabolite A was cleaved by the treatment, while Metabolite B was resistant to the enzyme. From previous studies with a larger number of other glucuronides, the HPLC retention index of the glucuronide would usually be 244 units lower than the parent aglycone (19). While primaquine (retention index, 733) and Metabolite A (retention index, 538) had approximately the correct relationship for an aglycone–glucuronide pair, the β-glucuronidase treatment did not show an increase in the primaquine peak corresponding to the decrease in Metabolite A.

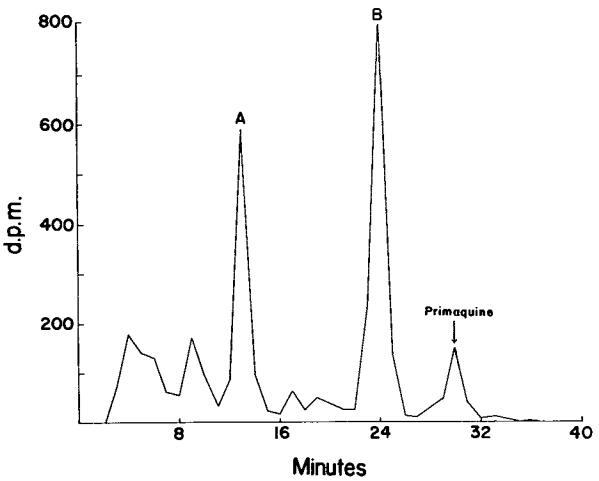

Fig. 4. ^{14}C high-performance liquid chromatogram of the 3- to 6-hr collection of an unextracted urine sample of a rat that had been administered 20 mg/kg of primaquine diphosphate. HPLC System A was utilized. Retention times of Metabolite A, Metabolite B, and primaquine are shown.

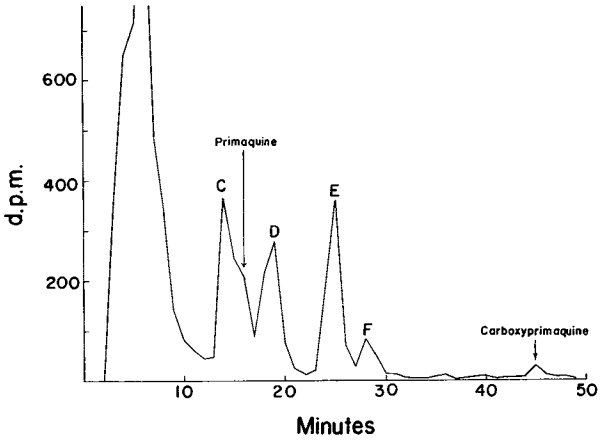

Fig. 5. ^{14}C high-performance liquid chromatogram of the 3- to 6-hr collection of an unextracted urine sample of a rat that had been administered 20 mg/kg of primaquine diphosphate. HPLC System B was utilized. Retention times of Metabolite C, Metabolite D, Metabolite E, Metabolite F, and carboxyprimaquine are shown. In some of the chromatograms in this series, the very small peak for primaquine could be completely resolved from Metabolite C, while as in this chromatogram, primaquine appeared as a shoulder on the preceding peak.

Table V. High-Performance Liquid Chromatographic Retention Indices and Detector Response Ratios for Primaquine Metabolites and Synthetic Reference Standards

Compound	Retention index	A_{254}/A_{280}
A. HPLC System A		
6-Desmethyl-5-hydroxyprimaquine	404	0.84
8-Amino-6-methoxyquinoline	441	1.3
8-Amino-5,6-dihydroxyquinoline	489	0.87
Metabolite A	538	—
6-Desmethylprimaquine	582	5.7
Metabolite B	681	6.9
Primaquine	733	5.0
B. HPLC System B		
8-Amino-5,6-dihydroxyquinoline	395	0.91
6-Desmethyl-5-hydroxyprimaquine	485	0.86
5-Hydroxyprimaquine	501	0.76
8-Amino-6-hydroxyquinoline	568	19.7
Carboxyprimaquine lactam	612	1.2
6-Desmethylprimaquine	615	5.6
8-Amino-6-methoxyquinoline	655	19.3
Metabolite C	710	—
Primaquine	721	5.1
Carboxyprimaquine amide	733	5.0
Metabolite D	744	—
Metabolite E	774	—
Glycylcarboxyprimaquine (4)	785	4.7
Metabolite F	786	—
Carboxyprimaquine (3)	839	4.3

Table VIA. Excretion of ^{14}C-Labeled Metabolites in Urine Compared with the HPLC Method and with the Colorimetric Method

Method	Quantity excreted as % of dose			
	0–3 hr	3–6 hr	6–12 hr	12–24 hr
Colorimetric[a]	1.46	2.35	0.93	0.36
HPLC-Metab. B[b]	3.33	6.09	4.40	0.76
Total ^{14}C label[c]	6.44	12.50	10.05	7.41
Metab. A[d]	0.97	1.28	1.44	0.67
Metab. B[d]	0.89	1.89	1.28	0.24
Metab. C[d]	0.86	1.47	0.77	0.37
Metab. D[d]	0.86	0.99	1.88	2.92
Metab. E[d]	0.39	1.06	0.77	0.53
Metab. F[d]	0.12	0.25	0.17	0.005
Primaquine[d]	0.30	0.44	0.16	0.12

[a] Determined by cartridge extraction and reaction with fast violet B.
[b] Determined by UV-HPLC analysis of unextracted urine.
[c] Total ^{14}C label in unprocessed urine.
[d] Determined by HPLC separation of ^{14}C label as shown in Figs. 4 and 5.

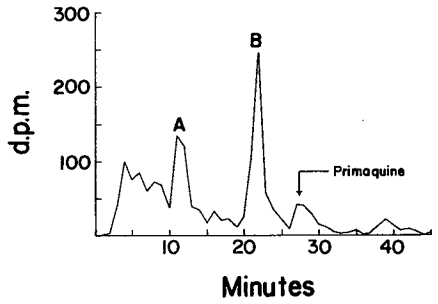

Fig. 6. ^{14}C HPLC (System A) of the 6- to 12-hr urine sample without β-glucuronidase treatment.

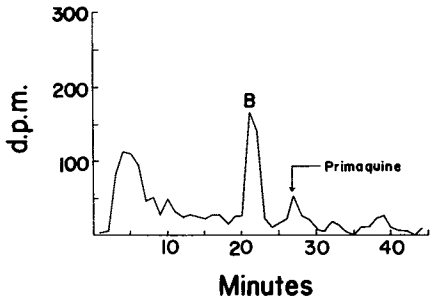

Fig. 7. ^{14}C HPLC (System A) of the 6- to 12-hr urine sample with β-glucuronidase treatment.

Metabolites D, E, and F were more polar than carboxyprimaquine, but the difference in their retention indices from carboxyprimaquine was much less than the 244 units (19) that would be expected for glucuronide conjugates. It was also found that there was essentially no difference between the β-glucuronidase treated urine sample (Fig. 9) and the untreated urine sample (Fig. 8), which also indicates that Metabolites C, D, E, and F were not glucuronides.

Detailed Studies of Metabolite B

Although primaquine and its metabolites had very strong UV chromophores, the presence of naturally occurring constituents in the urine with strong chromophores usually precludes the direct HPLC analysis of urine samples. As was the case in the present study for the HPLC analysis of 3, 4, and 5, lengthy purification procedures are usually needed before the HPLC analysis can be accomplished. However, in the case of Metabolite B, the HPLC peak detected by the radiochromatograms could also be distinguished from naturally occurring constituents using 254- and 280-nm UV detectors during the HPLC analysis of the urine samples. Using the UV-HPLC method of analysis, it was found that high concentrations of Metabolite B were found in the urine samples 3 to 12 hr after the administration of primaquine (Table VII). The average cumulative excretion of metabolite B was 8.9% of the dose, and 50% of that excretion occurred within 6 hr of the administration of primaquine. It was also observed that Metabolite B was not excreted in the urine following the administration of carboxyprimaquine (Table III).

Using preparative reversed-phase chromatography, a partially purified sample of Metabolite B was obtained and it was found to be free of the other metabolites and urine components as determined by UV-HPLC analysis. Primaquine, carboxyprimaquine, and the other 8-aminoquinoline derivatives have been found to undergo a diazo coupling reaction at the 5 position with fast violet B to give a colored product (20). The partially purified Metabolite B fraction was quantitatively assayed using the fast violet B reagent and it was found that the reagent did not react with the metabolite,

Table VIB. Correlation Matrix for Data Shown in Table VIA

	Colorimetric	HPLC	Total ^{14}C	Metab. A	Metab. B	Metab. C	Metab. D	Metab. E	Metab. F.	Primaquine
Colorimetric	1.000	0.873	0.632	0.515	0.874	0.984	−0.867	0.619	0.875	0.985
HPLC-Metab. B	0.873	1.000	0.805	0.865	0.995	0.931	−0.760	0.773	0.999	0.775
Total ^{14}C label	0.632	0.805	1.000	0.718	0.858	0.756	−0.265	0.998	0.813	0.543
Metab. A	0.515	0.865	0.718	1.000	0.846	0.624	−0.496	0.670	0.862	0.357
Metab. B	0.874	0.995	0.858	0.846	1.000	0.940	−0.710	0.831	0.996	0.780
Metab. C	0.984	0.931	0.756	0.624	0.940	1.000	−0.805	0.741	0.934	0.945
Metab. D	−0.867	−0.760	−0.265	−0.496	−0.710	−0.805	1.000	−0.229	−0.754	−0.844
Metab. E	0.619	0.773	0.998	0.670	0.831	0.741	−0.229	1.000	0.782	0.538
Metab. F	0.875	0.999	0.813	0.862	0.996	0.934	−0.754	0.782	1.000	0.778
Primaquine	0.985	0.775	0.543	0.357	0.780	0.945	−0.844	0.538	0.778	1.000

which indicates that the compound might be substituted at the 5 position.

Field Screening Tests

There has been a continuing interest in the development of a simple, low-cost method for detecting the presence of primaquine metabolites in urine samples. Because Metabolite B could be detected in urine assay by HPLC method that did not require any sample workup, the method might be useful for field screening urine samples for drug compliance measurement or determining individual-to-individual variations in drug response. A simple colorimetric method using a solid phase cartridge extraction and derivatization with fast violet B that was recently developed which might also be useful for field screening work.

A number of rats were administered ^{14}C-labeled primaquine diphosphate, then the colorimetric method and the HPLC method for Metabolite B were compared with the recovery of ^{14}C label in the urine (Table VIII). The colorimetric method was selective for metabolites that were not substituted at the 5 position of the quinoline ring and which were either acidic or neutral in their extraction characteristics (19). On average, the colorimetric method registered only one-seventh of the total ^{14}C label excreted in the urine (Table VIII), which was consistent with the high structural selectivity of the method. The excretion of this class of metabolites was rather slow, with the greatest output being in the 3- to 6-hr period or the 6- to 12-hr period. Although the colorimetric method detected only a small portion of the metabolites that were excreted, it was correlated well enough (r = 0.689) with the total output of radiolabel that the colorimetric method appears to be useful as a field screening procedure.

Metabolite B did not react with fast violet B, therefore it would not have been detected by the colorimetric method. The maximum output of metabolite B was found to occur during the 3- to 6-hr collection period for each of the test animals. It was also found that this method of measuring metabolite output was also fairly well correlated (r = 0.77) with the total output of ^{14}C-labeled metabolites.

The time courses of the colorimetric method and the UV-HPLC method were also correlated with the time

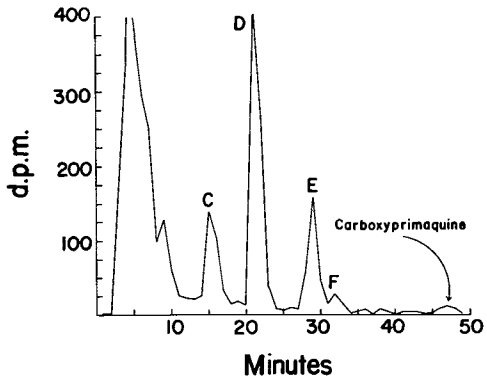

Fig. 8. ^{14}C HPLC (System B) of the 6- to 12-hr urine sample without β-glucuronidase treatment.

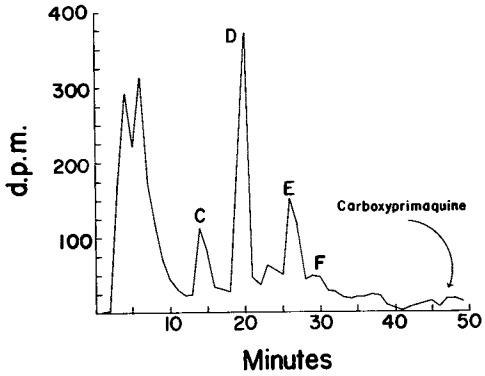

Fig. 9. ^{14}C HPLC (System B) of the 6- to 12-hr urine sample with β-glucuronidase treatment.

courses of the ^{14}C-HPLC of the six individual metabolites (Tables VIA and B). As would be expected, there was a high correlation (r = 0.995) between the UV-HPLC and the ^{14}C HPLC methods for measuring Metabolite B. It was also found that the UV-HPLC method for Metabolite B was moderately well correlated with the time course for the other metabolites, with the exception of Metabolite D. The output of Metabolite D was found not to be correlated with any of the other measures of metabolite production because of its unique time course (Tables VIA and B).

The time course of the colorimetric method was most highly correlated (r = 0.984) with the time course of Metabolite C. Using the ^{14}C HPLC method, the total output of Metabolite C was 3.47% of the administered dose (data from Table VIA), which would account for most of the total

Table VII. Determination of Metabolite B in Urine by Direct HPLC Analysis

Animal	Time (hr)	μg/ml	Cum. excr. (%)[a]
I	0–3	25	0.77
	3–6	106	5.63
	6–12	27	7.85
	12–24	<2	7.85
J	3–6[b]	104	2.86
	6–12	44	9.92
	12–24	<2	9.92
K	0–3	31	1.54
	6–12	51	6.99
	12–24	<2	6.99
L	6–9[b]	90	9.34
	9–12	14	9.91
	12–24	<2	9.91
M	0–6	27	2.74
	6–12	29	9.61
	12–15	<2	9.61
N	6–9[b]	100	9.12
	9–12	17	9.73
O	6–9[b]	109	7.17
	9–12	523	8.72
Average total excretion			8.96

[a] Cumulative excretion as a percentage of the administered dose.
[b] No measurable amount of urine was excreted during the preceding period.

Table VIII. Comparison of Field Screening Methods for the Detection of Primaquine Metabolites in Urine Reported as a Percentage of the Dose

Animal	Time (hr)	Colorimetric method	UV-HPLC method for Metabolite B	Total ^{14}C label in unextracted urine
P	0–3	1.46	3.33	6.44
	3–6	2.35	6.09	12.50
	6–12	0.93	4.40	10.05
	12–24	0.36	0.76	7.41
Total excr.		5.10	14.58	36.41
Q	0–3	0	0	0.005
	3–6	0.58	3.25	10.02
	6–12	2.24	2.46	7.90
	12–24	0.38	3.42	7.70
Total excr.		3.20	9.13	26.62
R	0–3	1.78	1.94	4.74
	6–12	3.57	5.43	21.92
	12–24	1.19	2.82	12.56
Total excr.		6.54	10.19	39.22
Avg. total excr.		4.95	11.30	34.08

Correlation matrix for above data

	Colorimetric	HPLC	^{14}C label
Colorimetric	1.000	0.601	0.689
HPLC	0.601	1.000	0.770
^{14}C label	0.689	0.770	1.000

output as measured by the colorimetric method (5.1% of the dose; data from Table VIA). Metabolite F appeared to contribute the remaining portion of the colorimetric method.

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