Characterization of Human Urinary Metabolites of the Antimalarial Piperaquine

J. Tarning, Y. Bergqvist, N. P. Day, J. Bergquist, B. Arvidsson, N. J. White, M. Ashton, and N. Lindegårdh

Department of Pharmacology, Sahlgrenska Academy at Göteborg University, Göteborg, Sweden (J.T., M.A.); Dalarna University College, Borlänge, Sweden (Y.B.); Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (N.P.D., N.J.W., N.L.); Nuffield Department of Clinical Medicine, Centre for Tropical Medicine, University of Oxford, Oxford, United Kingdom (N.P.D., N.J.W., N.L.); and Department of Physical and Analytical Chemistry, Uppsala University, Uppsala, Sweden (J.B., B.A.)

ABSTRACT:

Five metabolites of the antimalarial piperaquine (PQ) (1,3-bis-[4-(7-chloroquinolyl)-4-piperazinyl]-1-propane) have been identified and their molecular structures characterized. After a p.o. dose of dihydroartemisinin-piperaquine, urine collected over 16 h from two healthy subjects was analyzed using liquid chromatography (LC)/UV, LC/tandem mass spectrometry (MS/MS), Fourier transform ion cyclotron resonance (FTICR)/MS, and H NMR. Five different peaks were recognized as possible metabolites [M1, 320 m/z; M2, M3, and M4, 551 m/z (PQ + 16 m/z); and M5, 567 m/z (PQ + 32 m/z)] using LC/MS/MS with gradient elution. The proposed carboxylic metabolite M1 has a theoretical monoisotopic molecular mass of 320.1166 m/z, which is in accordance with the FTICR/MS (320.1168 m/z) findings. The LC/MS/MS results also showed a 551 m/z metabolite (M2) with a distinct difference both in polarity and fragmentation pattern compared with PQ, 7-hydroxyipiperazine, and the other 551 m/z metabolites. We suggest that this is caused by N-oxidation of PQ. The results showed two metabolites (M3 and M4) with a molecular ion at 551 m/z and similar fragmentation pattern as both PQ and 7-hydroxyipiperazine; therefore, they are likely to be hydroxylated PQ metabolites. The molecular structures of M1 and M2 were also confirmed using H NMR. Urinary excretion rate in one subject suggested a terminal elimination half-life of about 53 days for M1. Assuming formation rate-limiting kinetics, this would support recent findings that the terminal elimination half-life of PQ has been underestimated previously.

Malaria, caused by the mosquito-borne protozoan parasite Plasmodium, is the most important parasitic disease in the world. The World Health Organization estimates that there are more than 400 million clinical cases every year with between 1 and 3 million deaths, mostly children below the age of 5. It is estimated that 90% of these deaths occur in sub-Saharan Africa. The development of drug resistance by Plasmodium falciparum has severely compromised our ability to treat the disease. The increasing drug resistance and widening geographic distribution of P. falciparum has highlighted the urgent need for the development of new antimalarial drugs (White, 2004). Piperazine (PQ) has recently received renewed interest as a suitable partner drug in artemisinin-based combination treatments. Recent randomized clinical studies on a fixed combination of PQ and dihydroartemisinin (DHA) in Cambodia, Vietnam, and Thailand indicate excellent tolerability and cure rates in multidrug-resistant P. falciparum malaria (Denis et al., 2002; Ashley et al., 2004, 2005; Hung et al., 2004; Karunajeewa et al., 2004; Tran et al., 2004; Tangpukdee et al., 2005).

PQ is considered a novel antimalarial drug even though it was synthesized for the first time nearly 50 years ago at Rhône-Poulenc’s research laboratories in France. PQ was then abandoned until developed and produced by the Shanghai Research Institute of Pharmaceutical Industry. PQ was deployed extensively as prophylaxis and as treatment in chloroquine (CQ)-resistant areas in China from 1978 to 1994. Efficacy deteriorated because of resistance development in the 1980s (Lan et al., 1989; Yang et al., 1992, 1999; Fan et al., 1998). It has not been widely used as monotherapy elsewhere.

Although PQ has been used for decades in China, published pharmacokinetic data and preclinical information are scarce. Only a few reports of the pharmacokinetic properties of PQ have been published, none of which have addressed metabolism. PQ exhibits multiphasic pharmacokinetics with a particularly long terminal elimination half-life (t1/2) of approximately 20 to 30 days, reminiscent of the 4-ami-

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ABBREVIATIONS: PQ, piperazine (1,3-bis-[4-(7-chloroquinolyl)-4-piperazinyl]-1-propane); DHA, dihydroartemisinin; CQ, chloroquine; 7-OHPQ, 7-hydroxyipiperazine; LC, liquid chromatography; MS/MS, tandem mass spectrometry; FTICR, Fourier transform ion cyclotron resonance; SPE, solid-phase extraction; M1, metabolite 1; M2, metabolite 2; M3, metabolite 3; M4, metabolite 4; M5, metabolite 5; P450, cytochrome P450; MAO, monoamine oxidase.
Materials and Methods

Chemicals. PQ and DHA-PQ (Artekin) were obtained from Holleykin (Guangzhou, China). A reference sample of 7-hydroxypiperazine (7-OHPQ) was obtained from BDH (Poole, UK). D2O (99.997%) was obtained from Dr. (Phillipsburg, NJ). Trifluoroacetic acid, formic acid, and acetic acid were high-performance liquid chromatography water were obtained from JT Baker (high-performance liquid chromatography grade), methanol (pro analysis), and was provided by Lt. Col. Dennis Kyle (Walter Reed Army Institute of Research and urine samples for metabolite pharmacokinetics were analyzed according to published methods (Lindegardh et al., 2003; Tarning et al., 2006). In brief, samples were buffered with phosphate buffer and centrifuged at 15,000g for 5 min, and the supernatant was loaded onto preconditioned solid-phase extraction (SPE) columns. SPE was carried out on an automated system consisting of an ASPEC XL (Gison, Middleton, WI) with a mobile phase of high pH (pH 7) was also used to investigate the polarity of possible metabolites during different conditions.

PQ and its metabolites were separated, and metabolite fractions (M1, M2, M3 and M4, and M5) were collected using gradient elution. Four metabolite fractions (metabolite 1, M1; metabolite 2, M2; metabolites 3 and 4, M3 and M4; and metabolite 5, M5) were collected in cycles and solvent-evaporated under a gentle stream of air at 65°C. Dry metabolite fractions were stored at 4°C until metabolite characterization experiments with FTICR/MS and H NMR.

LC. The LC system used was a LaChrom Elite system consisting of an L2130 LC pump, an L2200 injector, an L2300 column oven set at 25°C, and an L2450 DAD detector (Hitachi, Tokyo, Japan). The detector was set at 345 nm. Data acquisition was performed using LaChrom Elite software (VWR, Darmstadt, Germany). PQ and metabolites were separated on a Chromolith performance (100 × 4.6 mm) column (VWR) protected by a Chromolith guard column (5 × 4.6 mm i.d.) (VWR) either using an isocratic mobile phase or a gradient program.

PQ-spiked plasma, serum 3 h after dose, 16-h urine, and 7-OHPQ were analyzed with a mobile phase of acetone-trifluoroacetic acid buffer (pH 2.5; 0.1 M) (8:92, v/v) at a flow rate of 3 ml/min. An Xterra column (150 × 4.6 mm i.d.) (Waters, Milford, MA) with a mobile phase of high pH (pH > 8) and low pH (pH < 7) was also used to investigate the polarity of possible metabolites during different conditions.

PQ and its metabolites were separated, and metabolite fractions (M1, M2, M3 and M4, and M5) were collected using gradient elution with two mobile phases of 1% formic acid in acetone-trifluoroacetic acid (A) and of 1% aqueous formic acid (B) at a flow rate of 1 ml/min using the following gradient program: t = 0 min, 4% A; t = 9 min, 18% A; and t = 10 min, 4% A.

The urine samples collected over 123 days were analyzed according to a previously published method using an isocratic mobile phase of phosphate buffer (pH 2.5; 0.1 M) at a flow rate of 3 ml/min (Tarning et al., 2006).

LC/MS/MS. The 16-h urine was analyzed on a chromatographic system consisting of a Varian pump (Walnut Creek, CA) and a manual injector (VICI, Schenkon, Switzerland). Analysis was performed on a Chromolith flash (25 ×
4.6 mm) column protected by a Chromolith guard column (5 × 4.6 mm i.d.) (VWR) and eluted with 1% formic acid in acetonitrile (A) and 1% aqueous formic acid (B) using the following gradient program: t = 0 min, 4% A; t = 9 min, 18% A; and t = 10 min, 4% A. The flow rate was maintained at 0.5 ml/min. The LC system was coupled on-line to a quadrupole-ion trap mass spectrometer, QTRAP (Applied Biosystems/MDS Sciex, Concord, Canada), equipped with a pneumatically assisted turbo-spray ionization source. Data acquisition was performed using Analyst 1.3 software (Applied Biosystems/MDS Sciex). The QTRAP was operated in positive ion mode and was optimized for the temperature in the ion source (300°C), entrance potential (10 V), declustering potential (65 V), ion source gas (30), curtain gas (20), and the potential in the ion spray (4800 V). The collision energy was varied to achieve more information about the fragmentation pattern (20, 40, 60, and 80 V).

FTICR/MS. All the mass spectra were acquired using a Bruker Daltonics (Billerica, MA) BioAPEX-94e superconducting 9.4-T FTICR/MS in broadband mode. A home-built apparatus controlled the direct infusion of the sample. Helium gas at a pressure of 1.3 bar was used to infuse the sample through a 30-cm fused silica capillary with a 50 µm i.d. One end of the capillary was inserted into the sample, and the other one, coated by a conductive graphite/polymer layer “Black Dust,” was connected to ground functioning as a sheathless electrospray needle (Nilsson et al., 2001). No sheath flow or nebulizing gas was used. The flow rate was approximately 40 nl/min. The ion source was coupled to an Analytica atmosphere-vacuum interface (Analytica, Branford, CT), and a potential difference of 2.0 to 3.0 kV was applied or nebulizing gas was used. The flow rate was approximately 40 nl/min. The LC/UV results suggest the presence of two possible metabolites (M1 and M2) in urine and small amounts in serum when analyzed with an isocratic mobile phase. M1 elutes before PQ, and M2 elutes after PQ. However, when using a high pH in the mobile phase to resemble the physiological conditions found in biological fluids, the results show that both metabolites elute before PQ, implying that both of the metabolites would be more polar than PQ in vivo. Different gradient mobile phases were evaluated to investigate the presence of any peaks eluting in the solvent front and/or after PQ. The results used for fraction collection of the metabolites showed five unknown peaks, possibly metabolites.

LC/MS/MS. Six different peaks were recognized using LC/MS/MS (Fig. 2). The first peak eluted very early in the gradient and had a detected mass of 320 m/z. The following peaks consisted of the PQ peak with a detected mass of 535 m/z, and three additional metabolites with detected masses of 551 m/z (PQ + 16 m/z). The least polar metabolite eluted with a detected mass of 567 m/z (PQ + 32 m/z). The hydroxy-derivative of PQ (i.e., 7-OHPQ) was also analyzed to identify the fragmentation pattern of this compound. The fragmentation patterns with proposed structures for the different peaks can be seen in Fig. 3.

Fischer Esterification. All the metabolites were exposed to Fischer esterification to confirm the presence or absence of a carboxylic acid. LC-collected fractions of the metabolites were exposed to Fischer esterification using acidic methanol and ethanol under the influence of heat (i.e., 65°C) and analyzed with LC/UV. The Fischer esterification converts acids into their corresponding esters using alcohol and acid catalysis.

Results

LC. The LC/UV results suggest the presence of two possible metabolites (M1 and M2) in urine and small amounts in serum when analyzed with an isocratic mobile phase. M1 elutes before PQ, and M2 elutes after PQ. However, when using a high pH in the mobile phase to resemble the physiological conditions found in biological fluids, the results show that both metabolites elute before PQ, implying that both of the metabolites would be more polar than PQ in vivo.

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FTICR/MS. The most polar metabolite with a detected mass of 320 m/z was also collected using LC/UV, purified, and analyzed with FTICR/MS to obtain a precise estimate of its molecular mass. The proposed M1 (Fig. 4) has a theoretical monoisotopic molecular mass of 320.1166 m/z, which is in accordance with the result from the FTICR/MS (320.1168 m/z). The FTICR/MS result was calibrated with...
FIG. 3. Fragmentation pattern and proposed structures for all the metabolites (A, PQ; B, 7-OHPQ; C, M1; D, M2; E, M3; F, M4; G, M5) detected by LC/MS/MS of 16-h urine collected after a single p.o. administration of DHA-PQ (three tablets each containing 40 mg of DHA + 320 mg of PQ phosphate), together with a fatty meal in a healthy male subject. Relative intensity is denoted as the percentage of the maximum response within the mass spectra.
PQ as reference, and the molecular mass obtained for M1 was corrected using the internal error observed for PQ.

**H NMR.** The H NMR of the different metabolite fractions supported the proposed structures for M1 and M2 and gave inconclusive results about the other three metabolites. The J-couplings for the aromatic part of PQ was compared with that of CQ, which has an identical aromatic structure and should provide the relative order of hydrogen signals in the aromatic structure (Fig. 1). Based on the H NMR spectra for CQ and the J-couplings detected for PQ, all the hydrogens in PQ could be assigned (Table 1; Fig. 5) (Maschke et al., 1997).

M1 showed no change in chemical shift in the aromatic structure compared with that of PQ. A downfield chemical shift could be seen for the aliphatic part of M1, which was expected because of the carboxy group creating a different electron density for adjacent protons (Table 1; Fig. 5). A comparison of the integration of the M1 spectra and the PQ spectra provides additional support for the proposed M1 structure. As expected, the signal intensity from H-19 is increased relative to signals from the aromatic region in M1 compared with PQ.

M2 displayed two similar sets of proton signals for the aromatic part of the molecule. One set of signals was identical to PQ, suggesting that one half of the molecule would be identical to PQ. The other half of M2 showed a change in chemical shift with all the hydrogen signals still present, which would be expected with nitrogen oxidation in the aromatic structure (Table 1; Fig. 5).

M3 and M4, which were coeluted in the fraction collection from the LC/UV, showed no change in chemical shift in the aromatic part, indicating a hydroxylation in the aliphatic part (Table 1; Fig. 5). To elucidate the structures fully, more concentrated samples containing each individual metabolite will need to be analyzed.

M5 displayed a weak signal on the margin of resolution, which could suggest that the aromatic part of the structure is altered but symmetrical, possibly as a result of a double N-oxidation.

**Fischer Esterification.** M1 formed two esters with different polarity (i.e., delayed retention time in the LC) during Fischer esterification. M1 was the only metabolite considered to have a positive result based on an almost complete transformation of the metabolite into its less polar ester compound during esterification. None of the other metabolites showed any significant conversion, indicating the lack of carboxylic moieties.

**Discussion**

The presence of M1 and M2 in biological fluids has been observed earlier in LC analysis using an isocratic mobile phase (Lindegardh et al., 2005; Tarning et al., 2006). The present LC analysis with gradient elution revealed five metabolites of different polarities. Eluting with an isocratic mobile phase resembling physiological pH suggested all
the metabolites to be more polar than PQ, as would be expected for metabolites. The structural resemblance of the metabolites was evaluated by UV absorbance spectra of each individual metabolite, separated by a gradient elution (data not shown). Metabolite stability and purity were investigated and showed pure fractions of metabolites on LC/MS/MS. Metabolite stability and purity were investigated and showed pure fractions of metabolites on LC/MS/MS.

### Table 1

<table>
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<th>Molecule</th>
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<td>Weak signal with multiple peaks</td>
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S, singlet; D, doublet; T, triplet; Q, quadruplet; M, multiplet.

The LC/MS/MS results (i.e., isotope pattern) show that M1 contains one chloride atom, indicating a cleavage in the aromatic bridge of PQ. The structure of M1 is further supported by H NMR, where no change in chemical shift can be seen in the aromatic part of the molecule compared with PQ. The carboxylation in the aromatic bridge of M1 would not affect the signals from the aromatic part but creates a downfield chemical shift in the aliphatic part of the molecule. The aliphatic part is not separated, as well as the aromatic part, from endogenous compounds (e.g., biological proteins) and noise, which makes for a more difficult analysis. Even so, the spectra show the same signals as PQ but with a change in chemical shift, which would be expected from carboxylation in the proposed position. The Fischer esterification performed also supports this metabolite being a carboxylic acid because almost all the metabolite is transformed to a more lipophilic substance during this reaction. The proposed structure of M1 is further supported by its theoretical mass being in agreement with the precise mass determination by FTICR/MS.

PQ may be metabolized by cytochrome P450 (P450) and/or monoamine oxidase (MAO) by oxidative N-dealkylation at the α-carbon. Further oxidation to the carboxylic metabolite (M1) could be achieved by P450, aldehyde dehydrogenase, and/or aldehyde oxidase (Watanabe et al., 1990, 1991). The P450 system is known to N-dealkylate xenobiotics and could possibly produce an unstable intermediate hydroxyx-metabolite. The resulting hydroxypiperaquine would undergo spontaneous cleavage into an aldehyde and an amine molecule, which could be further oxidized to the carboxylic acid. Although P450 is the most common metabolizing enzyme system of xenobiotics, the contribution of MAO is significant in the oxidative N-dealkylation of primary, secondary, and tertiary amines (Benedetti, 2001). A similar metabolic pattern, as proposed for PQ, can be seen for citalopram where an aldehyde is formed from the tertiary amine by MAO and further oxidized to the corresponding carboxylic acid by aldehyde oxidase (Rochat et al., 1998). The P450 system or MAO, or both, could be involved in the metabolism of PQ, as observed in the formation of the carboxy metabolite of the antimalarial primaquine (Constantino et al., 1999). Further studies are needed to determine the metabolic pathway for PQ to the carboxy metabolite.

**N-Oxidated Metabolite (M2).** A 551 m/z metabolite (M2) with a distinct difference in both polarity and fragmentation pattern, compared with the other 551 m/z metabolites (M3 and M4), was found. We propose that this is because of N-oxidation of piperaquine. N-
oxidation would influence the fragmentation pattern and polarity without affecting the mass weight of 551 \( m/z \). H NMR supported the proposed structure because there was a change in chemical shift in one of the aromatic parts of the molecule, indicating an alteration in one of the aromatic parts of the structure. Deuterated water (D\(_2\)O) was used as reconstitution media, and deuterated protons will easily replace hydroxyprotons in a hydroxylated molecule. In case of a hydroxylation at one of the carbon atoms, this hydroxyproton would be replaced by a deuterated proton and create an absent hydrogen signal compared with PQ. All the hydrogen signals were still present and J-couplings unaltered, indicating that no hydrogens were transformed or had undergone any reaction, thus excluding the possibility that this metabolite would be a hydroxylated PQ product. Therefore, the only possible oxidation site in the aromatic part of the molecule is the nitrogen position; thus, it is likely that this metabolite is a nitrogen-oxide PQ product.

**Hydroxylated Metabolites (M3 and M4).** PQ and 7-OHPQ showed similar fragmentation patterns. A hydroxy group in the bridge does not influence the fragmentation pattern; therefore, it is likely that the isolates with similar fragmentation patterns to PQ and 7-OHPQ with detected masses of 551 \( m/z \) are monohydroxylated PQ molecules. M3 and M4 showed an almost identical fragmentation pattern. The hydroxy metabolites are likely to be spliced in the same molecular positions as PQ and 7-OHPQ, resulting in fragments identical to those seen for PQ (i.e., nonhydroxylated part) and in fragments with an addition of 16 mass units (i.e., oxygen). The small discrepancy in polarity between the two metabolites can be explained by the metabolites being hydroxylated in different positions in the molecular structure. The H NMR showed no alterations in the aromatic part of the molecule, suggesting that the hydroxylation had occurred in the aliphatic part of the molecule. Because only low intensity signals with a noisy background could be seen for the aliphatic part, it was not possible to propose an exact structure. An N-oxidation in the aliphatic part of the molecule cannot be excluded even though it is unlikely, considering the fragmentation pattern’s strong resemblance to those of PQ and 7-OHPQ.

**Double N-Oxidated or Hydroxylated Metabolite (M5).** A peak with a detected mass of 567 \( m/z \) (M5) was found, which is likely to represent an addition of two oxygen atoms to the PQ molecule, either as hydroxylation or N-oxidation. The fragmentation pattern of this metabolite had similarities to all the 551 \( m/z \) metabolites; therefore, it could be a combination of both hydroxylation and nitrogen-oxidation. This metabolite was the least abundant metabolite and displayed the weakest signal in the H NMR compared with the other metabolites. There were indications in the H NMR spectra that only one set of signals was present but with a change in chemical shift compared with
the aromatic part of PQ, suggesting a symmetrical molecule with an altered aromatic structure. The chemical shift and J-couplings for this metabolite showed similarities with the signals seen for the altered aromatic part of M2. Therefore, this could represent a double N-oxidated molecule in the same position as M2. The amount needed for H NMR is large relative to that obtainable by manual fraction collection of LC-injected samples, and the information presented in the results does not satisfactorily exclude one or another. This needs to be studied further to elucidate the issue.

**Pharmacokinetics of M1.** Only M1 and M2 were detected in the analyzed serum/plasma samples; therefore, these are the most clinically relevant metabolites. Blood and plasma were collected for up to 93 days and urine for 123 days, and PQ pharmacokinetics was evaluated as previously reported (Tarning et al., 2005, 2006). Reanalyzed urine contained substantial amounts of M1 and M2. M1 was present as larger peaks than PQ and could easily be detected in urine 123 days after administration. M2 pharmacokinetics was not evaluated because it eluted later in the chromatogram and was present in much lower concentrations (i.e., close to the limit of detection) than M1. As there is no reference compound for M1, a PQ/IS calibration curve was used to approximate the urine concentrations of M1, based on the response ratio of M1/IS (i.e., M1 concentrations expressed as PQ equivalents).

The excretion rate of M1 against time midpoint of collection interval can be seen in Fig. 6 and suggests a terminal elimination half-life of 53 days. Assuming that the metabolite formation limits the elimination (i.e., formation rate limiting kinetics), this would support recent findings that the previously reported elimination half-life of PQ might be underestimated (Tarning et al., 2005). Further studies in larger series are needed to investigate the pharmacological and pharmacokinetic properties of this metabolite.

In conclusion, we report for the first time the molecular structures of the two principal metabolites of PQ found in human serum and urine. The major metabolites are a carboxyl acid cleavage product, the other a mono-N-oxidated PQ product. The results also suggest two possible human urinary monohydroxylated metabolites and a di-N-oxidated metabolite. The relative amounts of metabolites may vary because the samples analyzed in this study are derived from only one healthy volunteer, and the proposed structures need to be confirmed in patients with malaria. The clinical relevance of PQ metabolites needs to be investigated with respect to pharmacokinetics, antimalarial activity, and possible toxicity to characterize further the safety of PQ as an antimalarial drug.

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**References**


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**Address correspondence to:** Niklas Lindegardh, Wellcome Unit, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Bangkok 10400, Thailand. E-mail: niklas@tropmedres.ac