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Title page

Characterization of human urinary metabolites of the antimalarial piperaquine

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Running title page

a) Running title:

Human urinary metabolites of the antimalarial piperaquine

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d) Abbreviations:

PQ: Piperaquine

7-OHPQ: 7-hydroxypiperaquine

DHA: Dihydroartemisinin

CQ: Chloroquine

M1: Metabolite 1

M2: Metabolite 2

M3: Metabolite 3

M4: Metabolite 4

M5: Metabolite 5

CYP450: Cytochrome P450

MAO: Monoamine Oxidase

ACT: Artemisinin based combination treatment

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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. Following an oral dose of dihydroartemisinin-piperaquine, urine collected over 16 hr from two healthy subjects was analysed using LC-UV, LC-MS/MS, FTICR-MS and H-NMR. Five different peaks were recognized as possible metabolites (M1:320m/z, M2:M3:M4:551m/z (PQ+16m/z), and M5:567m/z (PQ+32m/z)) using LC-MS/MS with gradient elution. The proposed carboxylic M1 has a theoretical mono-isotopic molecular mass of 320.1166 m/z, which is in accordance with the FTICR-MS (320.1168m/z) findings. The LC-MS/MS results also showed a 551m/z metabolite (M2) with a distinct difference both in polarity and fragmentation pattern compared to PQ, 7-hydroxypiperaquine and the other 551m/z metabolites. We suggest that this is due to N-oxidation of PQ. The results showed two metabolites (M3 and M4) with a molecular ion at 551m/z and similar fragmentation pattern as both PQ and 7-hydroxypiperaquine, they are therefore 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.

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Introduction

Malaria, caused by the mosquito-borne protozoan parasite *Plasmodium*, is the most important parasitic disease in the world. The World Health Organisation 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 *P. 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). Piperaquine (PQ) has recently received renewed interest as a suitable partner drug in artemisinin based combination treatments (ACT). 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; Hung et al., 2004; Karunajeewa et al., 2004; Tran et al., 2004; Ashley et al., 2005; Tangpukdee et al., 2005).

PQ, (1,3-bis-[4-(7-chloroquinolyl-4)-piperazinyl-1]-propane), is considered a novel antimalarial drug even though it was synthesized for the first time nearly fifty 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-resistant areas in China from 1978 to 1994. Efficacy deteriorated due to resistance development in

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the 1980s (Lan et al., 1989; Yang et al., 1992; Fan et al., 1998; Yang et al., 1999). It has not been widely used as mono-therapy 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 ($t_{1/2}$) of approximately 20-30 days, reminiscent of the 4-aminoquinoline chloroquine, which probably results from extensive tissue binding (Hung et al., 2004; Roshammar et al., 2006). The slow elimination of PQ limits the usefulness of *in vitro* assays and stresses the need for a different approach when investigating metabolism (i.e. enzyme involvement and metabolite characterization).

In earlier studies we have detected unknown peaks, thought to be metabolites in the plasma and urine of patients with malaria and a healthy volunteer after receiving DHA-PQ; each tablet (Artekin®) contains 40 mg DHA + 320 mg PQ phosphate (Lindegardh et al., 2005; Tarning et al., 2006). Substantial amounts of two unknown peaks were detected in urine for up to 123 days after a single oral dose of 3 tablets. The aim of this work was to isolate and characterize the main PQ metabolites in human urine collected after a single oral administration of the fixed combination (DHA-PQ).

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Materials and methods

Chemicals

PQ and DHA-PQ (Artekin®) were obtained from Holleykin (Guangzhou, China). A reference sample of 7-hydroxypiperaquine (7-OHPQ) was kindly provided by Lt.-Col. Dennis Kyle at the Walter Reed Army Institute of Research (Rockville, MD, USA). The structures are shown in Figure 1. Acetonitrile (HPLC-grade), methanol (pro analysis) and HPLC-water were obtained from JT Baker (Phillipsburg, USA). Trifluoroacetic acid, formic acid and acetic acid were obtained from BDH (Poole, UK). D_2O (99.997%) was obtained from Dr. Glaser GmBH (Basel, Switzerland).

Dosage and sampling

Two healthy male volunteers received a single oral dose of DHA-PQ (3 tablets each containing 40 mg DHA + 320 mg PQ phosphate) together with a fatty meal.

In one subject, urine was collected for 16 hours following the dose and one venous blood sample (i.e. serum) drawn at 3 hours post dose for metabolite structural characterization by LC-UV, LC-MS/MS, FTICR-MS and H-NMR.

In the other subject, urine samples were collected for 123 days and PQ pharmacokinetics evaluated as previously reported (Tarning et al., 2006). 24-h urine samples collected at 3, 4, 5, 8, 11, 15, 22, 31, 46, 64, 79, 93 and 123 days after drug administration were reanalyzed by LC-UV and evaluated with respect to the time-profile of metabolites. All samples were stored at –80°C until analysis.

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Analytical procedure

Serum and urine samples for metabolite identification and urine samples for metabolite pharmacokinetics were analyzed according to published methods (Lindegardh et al., 2003; Tarning et al., 2006). Briefly, samples were buffered with phosphate buffer, centrifuged at 15000×*g* for 5 minutes and the supernatant was loaded onto pre-conditioned solid phase extraction (SPE) columns. SPE was carried out on an automated system consisting of an ASPEC XL (Gilson, Middleton, WI, USA) using MPC-SD SPE cartridges (4mm, 1 mL) (3M Empore, Bracknell, UK). The SPE eluates were evaporated under a gentle stream of air at 65°C, reconstituted in phosphoric acid (0.05 M), and injected into the LC-UV and LC-MS/MS system.

PQ and its metabolites in the 16-h urine sample were extracted using the 96-well plate format as published for high-throughput quantification of PQ in plasma (Lindegardh et al., 2005). Metabolite fractions were then collected manually using LC-UV with gradient elution. Four metabolite fractions (metabolite 1: M1, metabolite 2: M2, metabolite 3 and 4: M3&4, 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.

Liquid chromatography (LC)

The LC system used was a LaChrom Elite system consisting of a L2130 LC pump, a L2200 injector, a L2300 column oven set at 25°C and a 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

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separated on a Chromolith™ Performance (100×4.6 mm) column (VWR, Darmstadt, Germany) protected by a Chromolith™ guard column (5×4.6 mm I.D)(VWR, Darmstadt, Germany) either using an isocratic mobile phase or a gradient programme.

PQ spiked plasma, serum 3 hours after dose, 16-h urine and 7-OHPQ were analyzed with a mobile phase of acetonitrile-phosphate buffer (pH 2.5; 0.1M) (8:92, *v/v*) at a flow rate of 3 mL/min. An Xterra column (150×4.6 mm I.D.) (Waters, Milford, USA) 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&4 and M5) collected using gradient elution with two mobile phases of 1% formic acid in acetonitrile (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; 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 mol/L)-acetonitrile (92:8, *v/v*) at a flow rate of 3 mL/min (Tarning et al., 2006).

Liquid chromatography – mass spectrometry (LC-MS/MS)

The 16-h urine was analyzed on a chromatographic system consisting of a Varian pump (Walnut Creek, CA, USA) and a manual injector (VICI, Schenkon, Switzerland). Analysis was performed on a Chromolith™ Flash (25×4.6 mm) column protected by a

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Chromolith™ guard column (5×4.6 mm I.D) (VWR, Darmstadt, Germany) 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; 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-iontrap mass spectrometer, QTRAP, (Applied Biosystems/MDS Sciex, Concord, Canada) equipped with a pneumatically assisted turbospray 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 (4,800 V). The collision energy was varied to achieve more information about the fragmentation pattern (20, 40, 60 and 80 V).

Fourier transform ion cyclotron resonance – mass spectrometry (FTICR-MS)

All mass spectra were acquired using a Bruker Daltonics (Billerica, MA, USA) BioAPEX-94e superconducting 9.4 T FTICR mass spectrometer in broadband mode. A home-built apparatus controlled the direct infusion of the samples. Helium gas at a pressure of 1.3 bar was used in order to infuse the sample through a 30 cm fused silica capillary with a 50 µm inner diameter. 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

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(Analytica of Branford, CT, USA) and a potential difference of 2.0-3.0 kV was applied across a distance of 3-5 mm between the spraying needle and the inlet capillary. Typically, 512 K data points were acquired, adding a minimum of 32 spectra.

Hydrogen Nuclear Magnetic Resonance (H-NMR)

PQ powder and evaporated metabolite fractions were reconstituted in D_2O and analyzed on a Varian Discovery 900 MHz NMR spectrometer equipped with a RT HCN-probe operating at a nominal signal:noise ratio of 540:1, as recorded on a 2 mM sucrose sample. Chemical shifts (δ) are reported relative to 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) at 25°C. Following a 90° excitation pulse, 1D FIDs comprising 32,768 data points, covering a spectral width of 25,039 Hz, were obtained and processed with a 90° shifted sine bell after zero-filling. A total recycle delay of 3.7 s (including the acquisition time) was used. Depending on sample concentration, 32 transients for PQ, M1, M2 and M3&4, and 55,000 transients for M5 were obtained.

Fischer esterification

All metabolites were exposed to a Fischer esterification in order 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 analysed with LC-UV. The Fischer esterification converts acids into their corresponding esters using alcohol and acid catalysis.

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Results

Liquid chromatography (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 result 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 settings used for fraction collection of the metabolites showed five unknown peaks, possibly metabolites.

Liquid chromatography – mass spectrometry (LC-MS/MS)

Six different peaks were recognized using LC-MS/MS (Figure 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 analysed to identify the fragmentation pattern of this compound. The fragmentation patterns with proposed structures for the different peaks can be seen in Figure 3.

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Fourier transform ion cyclotron resonance – mass spectrometry (FTICR-MS)

The most polar metabolite with a detected mass of 320 m/z was also collected using LC-UV, purified and analysed with FTICR-MS to obtain a precise estimate of its molecular mass. The proposed metabolite 1 (M1, Figure 4) has a theoretical mono-isotopic 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 PQ as reference and the molecular mass obtained for M1 was corrected using the internal error observed for PQ.

Hydrogen Nuclear Magnetic Resonance (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 to that of chloroquine, which has an identical aromatic structure and should provide the relative order of hydrogen signals in the aromatic structure (Figure 1). Based on the H-NMR spectra for chloroquine and the J-couplings detected for PQ, all hydrogens in PQ could be assigned (Table 1; Figure 5) (Maschke et al., 1997).

M1 showed no change in chemical shift in the aromatic structure compared to 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; Figure 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 to PQ.

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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 hydrogen signals still present, which would be expected with nitrogen oxidation in the aromatic structure (Table 1; Figure 5).

Metabolites 3 and 4 (M3 and M4), which were co-eluted 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; Figure 5). In order 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.

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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 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 was investigated and showed pure fractions of metabolites on the LC-UV which were stable and only to a small extent exhibit rearrangement to other structures during the evaporation phase (data not shown). The UV absorbance spectra for all metabolites were similar without any significant shifts in lambda max suggesting a strong structural resemblance between all metabolites and PQ.

Liquid chromatography – mass spectrometry (LC-MS/MS)

7-OHPQ powder was dissolved and analyzed using isolations at 551 m/z (7-OHPQ containing two 35-chloride isotopes) and 553 m/z (7-OHPQ containing one 35- and one 37-chloride isotope) to elucidate the fragmentation pattern. The two chloride atoms in the molecule and their isotope pattern can be used to evaluate where chloride is lost during fragmentation. The fragments without a chloride atom will display a single peak with the same m/z using isolation at 551 m/z and 553 m/z. The fragments containing one chloride atom will display a single peak using isolation at 551 m/z but double peaks 2 mass units apart using isolation at 553 m/z. The fragments containing two chloride atoms will

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display a single peak using isolation at 551 m/z and a single peak but with 2 m/z units higher using isolation at 553 m/z. All fragments at the 553 m/z isolation displayed double peaks, except for the 182 m/z fragment, thereby confirming that the molecule is initially spliced in the aliphatic bridge eliminating one of the chloride atoms. The 182 m/z fragment could be seen for PQ, 7-OHPQ, M2, M3, M4 and M5 when using a higher collision energy. PQ and 7-OHPQ showed similar fragmentation patterns except for a difference of 16 mass units in the first fragment. Evidently, a hydroxy group in the bridge does not influence the fragmentation pattern, in that the splicing still occurs at the same position in the molecules creating the discrepancy in mass for the first fragment. Surprisingly, the results did not show any typical water loss of 18 mass units for 7-OHPQ, M3 or M4 which could be expected for a hydroxylated molecule. These unexpected results were not dependent on the collision energy since the same results were obtained when increasing the energy. A radical fragment of 245 m/z was produced for PQ, 7-OHPQ, M2, M3, M4 and M5 at a high collision energy and was thus different from the expected fragment of 248 m/z that was seen for M1. Since all structures except M1 displayed this radical it is likely that this is a consequence of the adjacent carboxy group of M1.

Carboxylic metabolite (M1)

The LC-MS/MS results (i.e. isotope pattern) show that M1 contains one chloride atom, indicating a cleavage in the aliphatic 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 to PQ. The carboxylation in the aliphatic bridge of M1 would

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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 since almost all 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 (CYP450) and/or monoamine oxidase (MAO) by oxidative N-dealkylation at the alpha-carbon. Further oxidation to the carboxylic metabolite (M1) could be achieved by CYP450, aldehyde dehydrogenase and/or aldehyde oxidase (Watanabe et al., 1990; Watanabe et al., 1991). The CYP450 system is known to N-dealkylate xenobiotics and could possibly produce an unstable intermediate hydroxyl-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 CYP450 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 CYP450 system or MAO,

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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 carboxylic metabolite.

N-oxidated metabolite (M2)

A 551 m/z metabolite (M2) with a distinct difference in both polarity and fragmentation pattern, compared to the other 551 m/z metabolites (M3&4) was found. We propose that this is due to 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, since there was a change in chemical shift in one of the two aromatic parts of the molecule indicating an alteration in one of the aromatic parts of the structure. Deuterated water (D_2O) 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 to PQ. All 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. The only possible oxidation site in the aromatic part of the molecule is therefore the nitrogen position and it is therefore likely that this metabolite is a nitrogen-oxide PQ product.

Hydroxylated metabolites (M3 and M4)

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PQ and 7-OHPQ showed similar fragmentation patterns. A hydroxy group in the bridge does not influence the fragmentation pattern, so it is likely that the isolates with similar fragmentation patterns to PQ and 7-OHPQ with detected masses of 551 m/z are mono-hydroxylated 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. non-hydroxylated 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. Since 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 551 m/z metabolites and it could therefore 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 to the other metabolites. There were indications in the H-NMR spectra

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that only one set of signals were present but, with a change in chemical shift compared to 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. This could therefore 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 and this needs to be studied further to elucidate the issue.

Pharmacokinetics of M1

Only M1 and M2 were detected in the analysed serum/plasma samples, and these are therefore the most clinically relevant metabolites. Blood and plasma was collected for up to 93 days and urine for 123 days and PQ pharmacokinetics evaluated as previously reported (Tarning et al., 2005; Tarning et al., 2006). Reanalysed 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 since 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 mid-point of collection interval can be seen in Figure 6 and suggests a terminal elimination half-life of 53 days. Assuming that the

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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 might vary since the samples analysed 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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Footnotes

a) This study was part of the Wellcome Trust-Mahidol University-Oxford Tropical Medicine Research Programme Thailand (077166/Z/05/Z) supported by the Wellcome Trust of Great Britain. We thank the Medicines for Malaria Venture and the Swedish International Development Cooperation Agency (SIDA) for economic support. The support by the Swedish Strategic Foundation (SSF), Swedish Research Council (621-2002-5261, 629-2002-6821) and Applied Biosystems/MDS Sciex are acknowledged.

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c) Numbered footnotes, using superscript numbers, beginning with those (if any) to authors' names and listed in order of appearance.

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Legends for figures

Fig 1. Molecular structures of A;piperaquine (PQ), B;7-hydroxypiperaquine (7-OHPQ), C;chloroquine (CQ) and D;dihydroartemisinin (DHA).

Fig 2. LC-MS/MS chromatogram of 16-h urine collected after a single oral administration of DHA-PQ (3 tablets each containing 40 mg DHA + 320 mg PQ phosphate) together with a fatty meal in a healthy male subject.

Fig 3. Fragmentation pattern and proposed structures for all 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 oral administration of DHA-PQ (3 tablets each containing 40 mg DHA + 320 mg 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.

Fig 4. Proposed metabolite pattern and molecular structure of PQ urinary metabolites detected in a healthy male subject.

Fig 5. H-NMR spectra and assignment for protons for A;PQ, B;M1, C;M2 and D;M3&4 in 16-h urine collected separately with LC-UV after a single oral administration of DHA-PQ (3 tablets each containing 40 mg DHA + 320 mg PQ phosphate) together with a fatty meal in a healthy male subject.

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Fig 6. Urinary excretion rate plot of metabolite 1 (M1) after a single oral administration of DHA-PQ (3 tablets each containing 40 mg DHA + 320 mg PQ phosphate) together with a fatty meal in a healthy male subject.

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Table 1. H-NMR results for piperaquine and its metabolites

Molecule	Proton	Multiplicity	δ (ppm)	*J* (Hz)
Piperaquine (aromatic region)	H-14 / H-34	D	8.48	6.8
	H-10 / H-30	D	7.99	9.1
	H-9 / H-29	D	7.94	1.9
	H-7 / H-27	Q	7.61	9.2 / 1.9
	H-15 / H-35	D	7.14	6.8
(aliphatic region)	H-1-H-3 / H-21-H-23	S	3.96	
	H-4-H-6 / H-24-H26	S	3.55	
	H-18 / H-20	T	3.24	8.3
	H-19	M	2.26	
Metabolite 1 (aromatic region)	H-14	D	8.47	6.7
	H-10	D	7.98	9.1
	H-9	S	7.93	
	H-7	D	7.61	9.0
	H-15	D	7.13	6.7

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(aliphatic region)	H-1-H-3	S	4.22	
	H-4-H-6	S	3.70	
	H-18	T	3.48	6.8
	H-19	T	2.85	6.7
Metabolite 2				
(aromatic region)	H-34	D	8.58	6.9
	H-14	D	8.49	6.8
	H-29	S	8.38	
	H-30	D	8.04	9.2
	H-10	D	8.00	9.2
	H-9	S	7.94	
	H-27	D	7.67	9.2
	H-7	D	7.62	9.2
	H-15	D	7.15	6.9
	H-35	D	7.08	7.0
(aliphatic region)	H-1-H-3 / H-21-H-23	S	3.70	
	H-4-H-6 / H-24-H26	S	3.50	
	H-18 / H-20	T	3.30	7.9
	H-19	M	2.28	

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Metabolite 3 & 4 (aromatic region)	H-14 / H-34	D	8.48	6.5
	H-10 / H-30	D	7.98	9.2
	H-9 / H-29	S	7.94	
	H-7 / H-27	D	7.61	8.6
	H-15 / H-35	D	7.14	7.0
(aliphatic region)	Multiple peaks			
Metabolite 5	Weak signal with multiple peaks			

S: singlet, D: doublet, T: triplet, Q: quadruplet, M: multiplet

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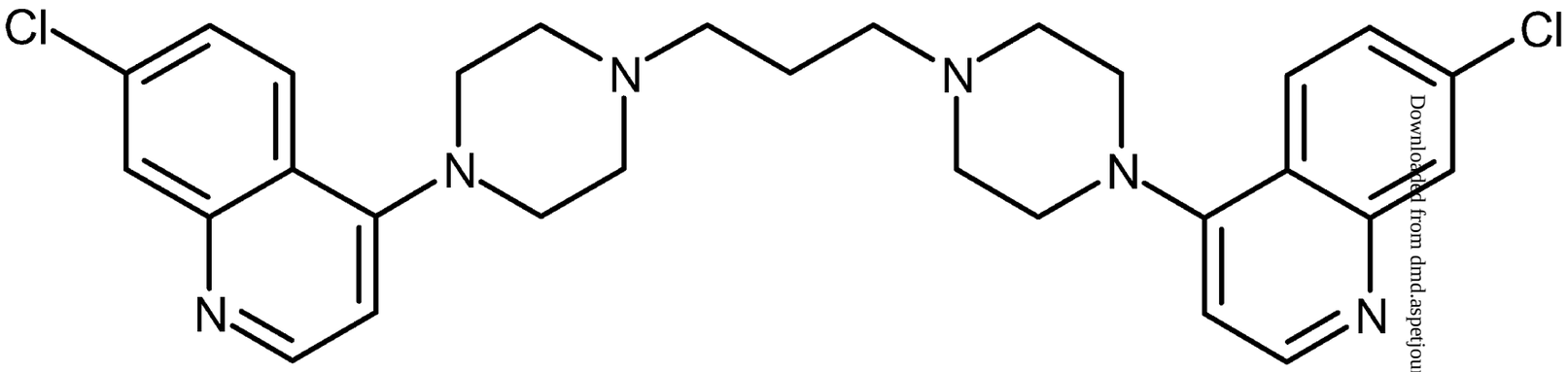
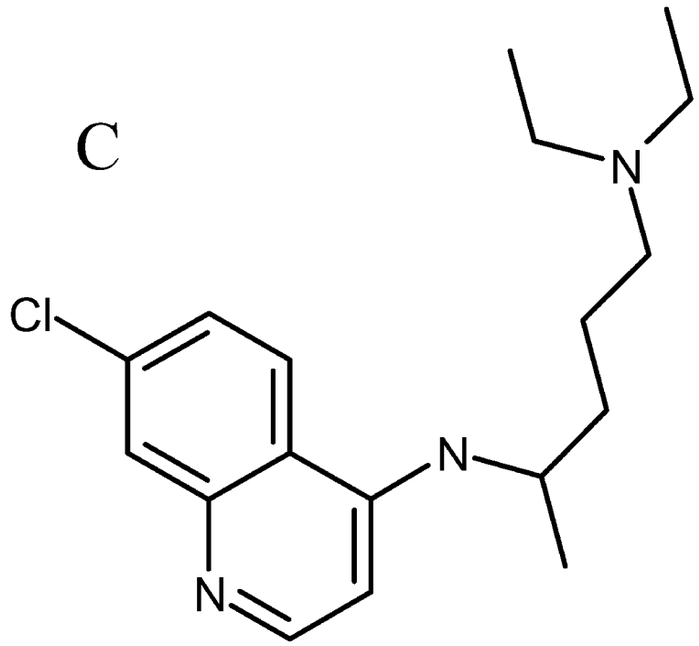
A



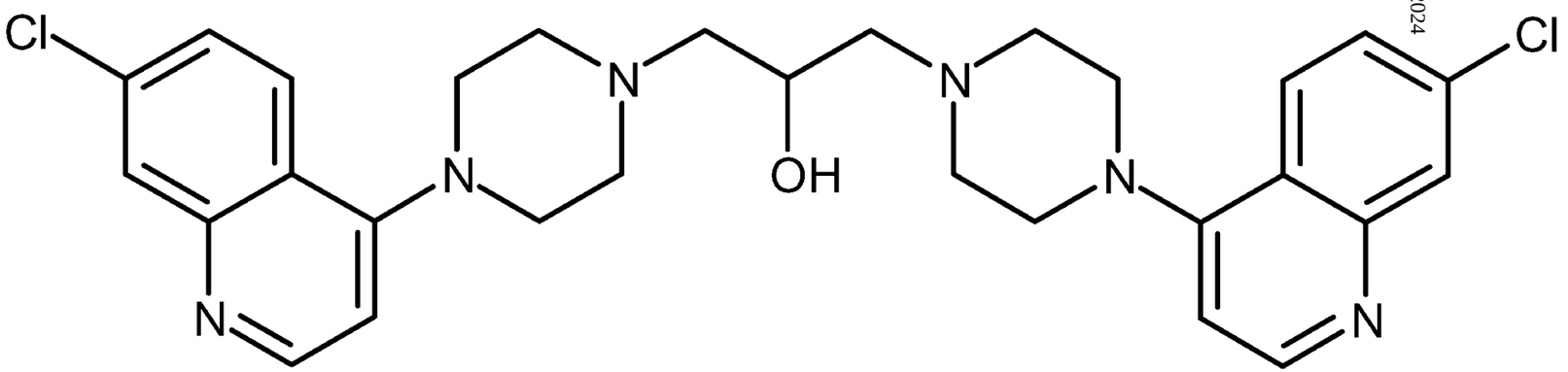
Piperaquine (PQ)

C



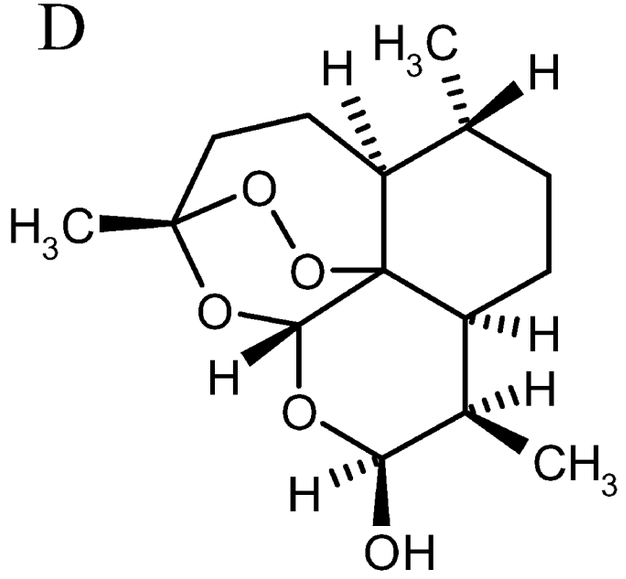
Chloroquine (CQ)

B



7-hydroxypiperaquine (7-OHPQ)

D



Dihydroartemisinin (DHA)

Figure 1.

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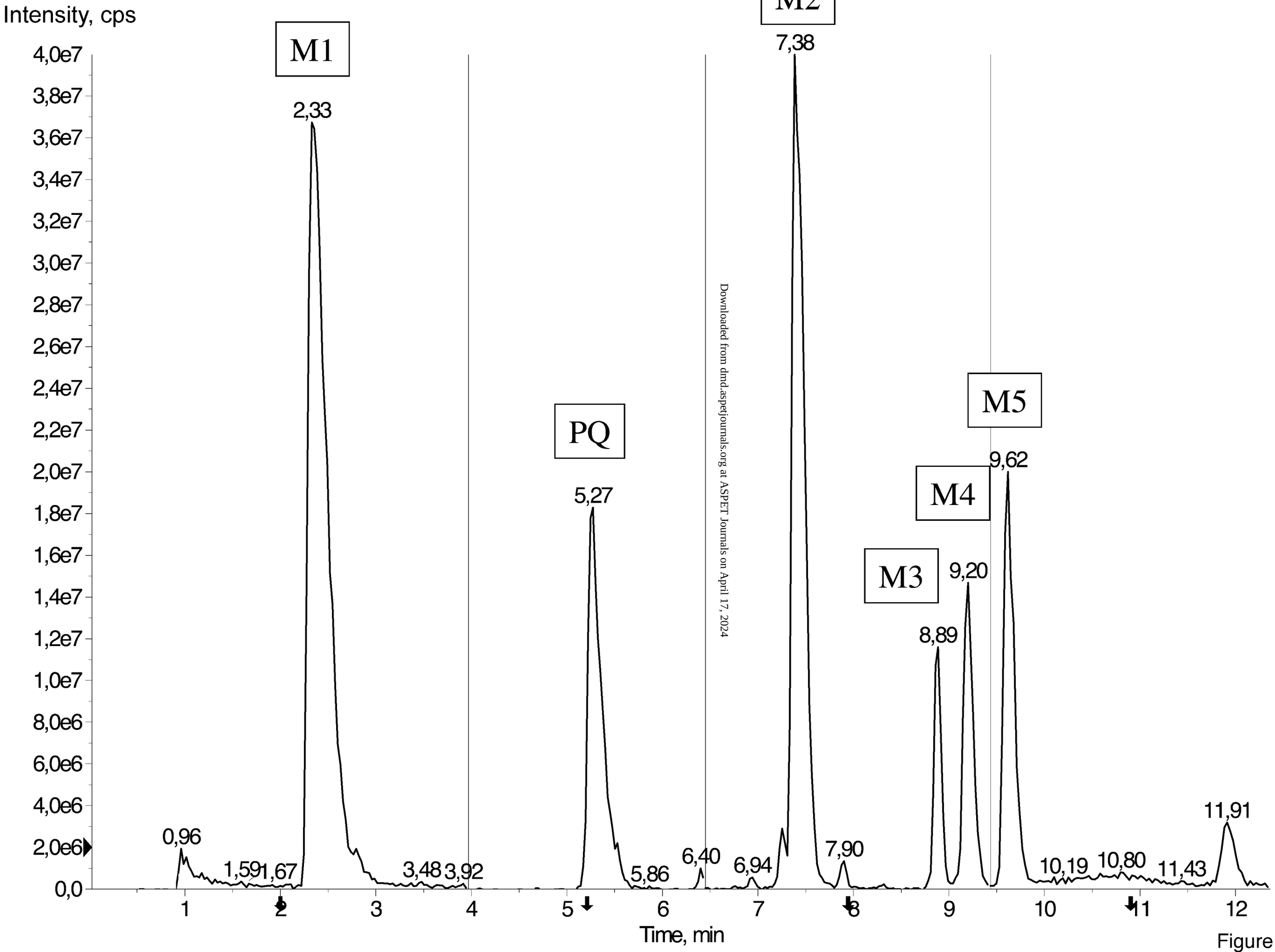

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Figure 2.

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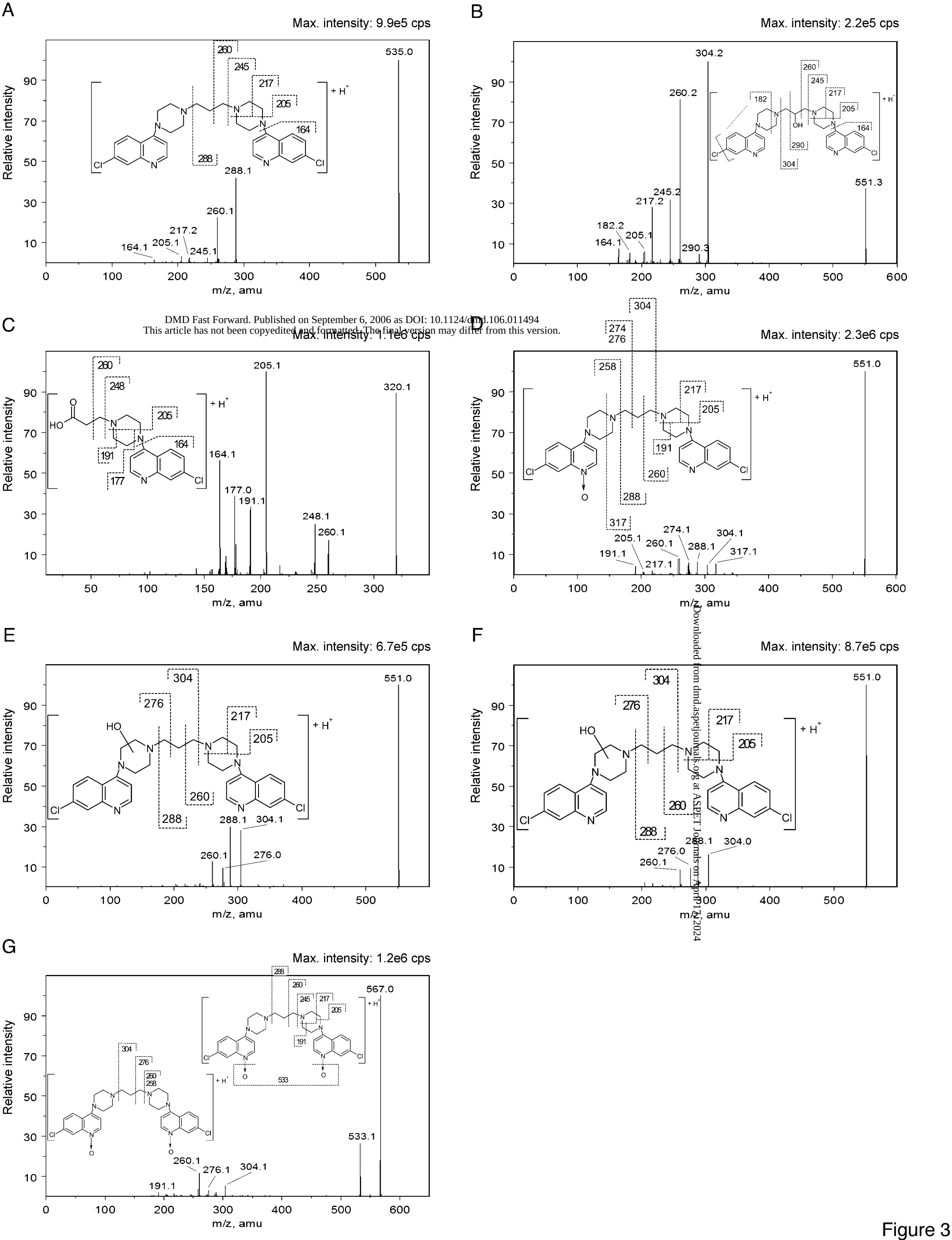

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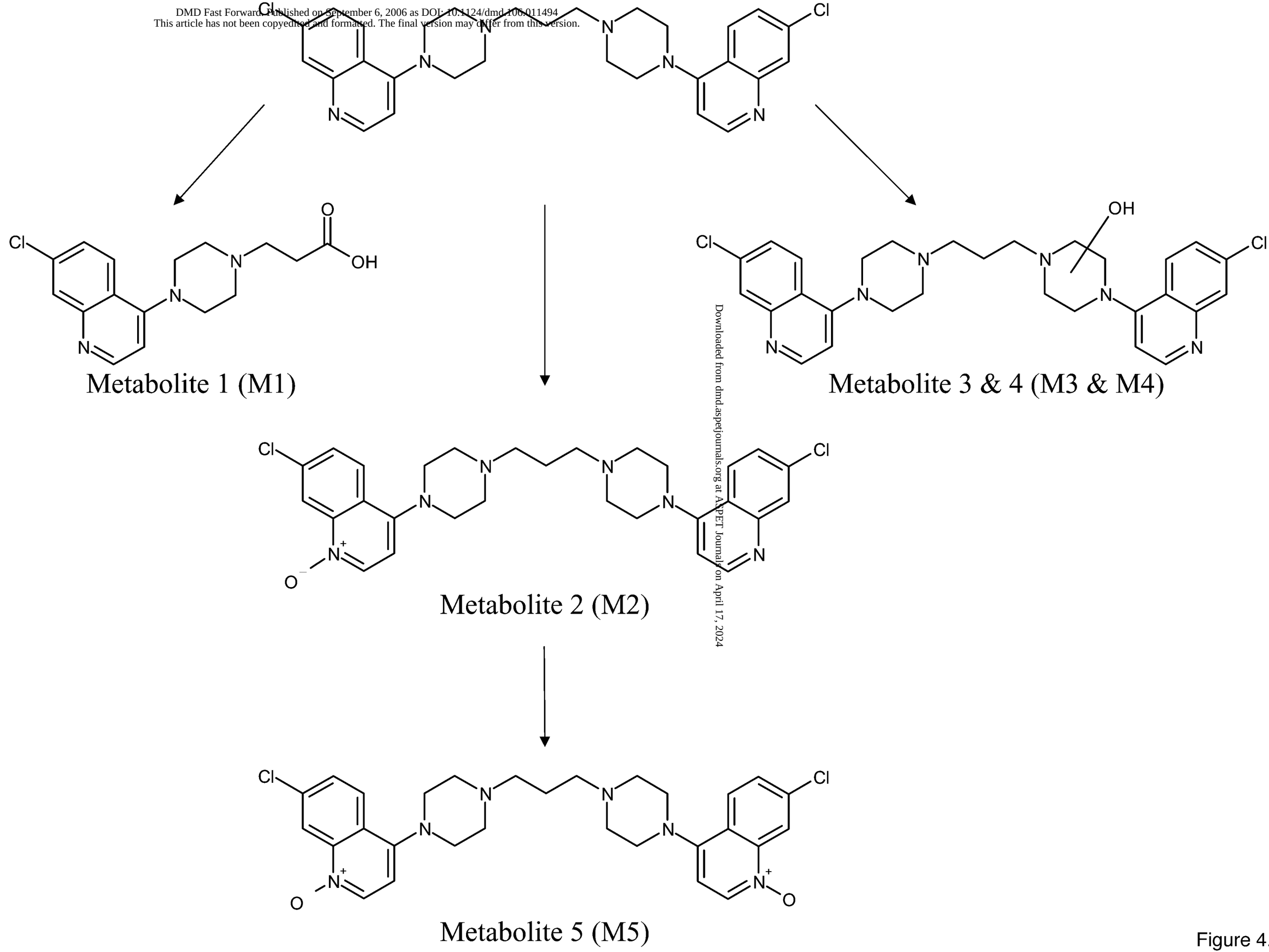

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Figure 4.

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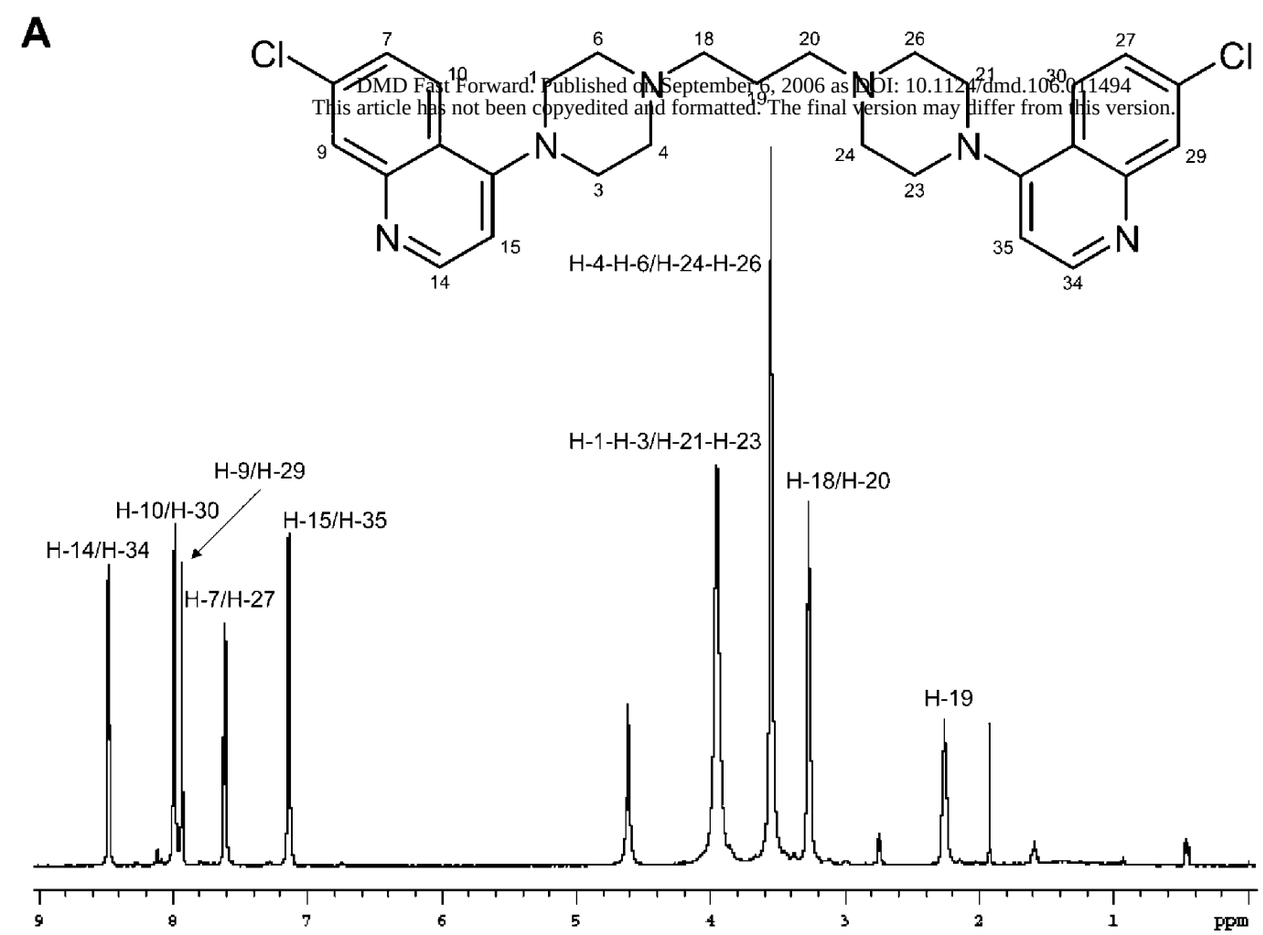

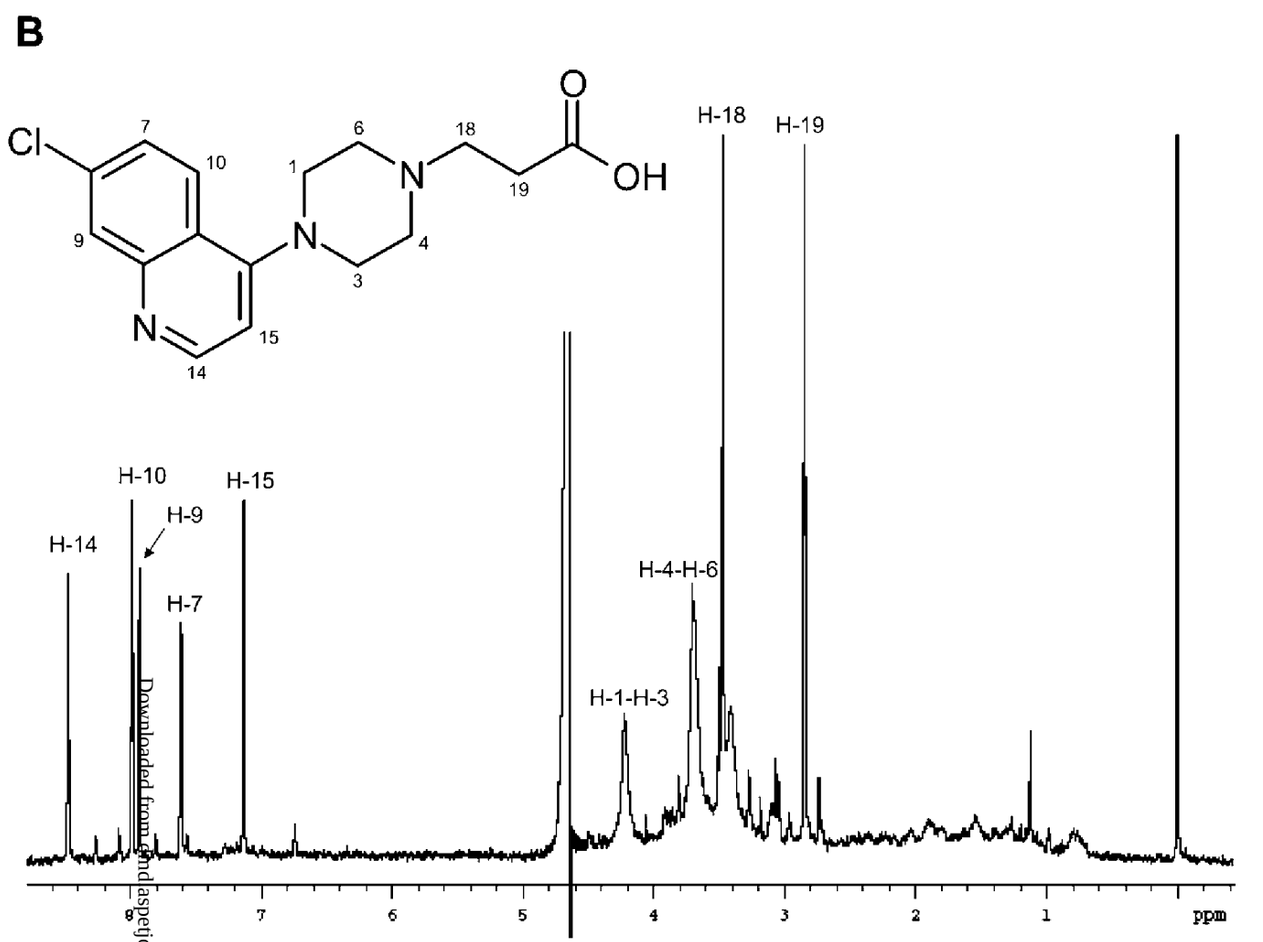

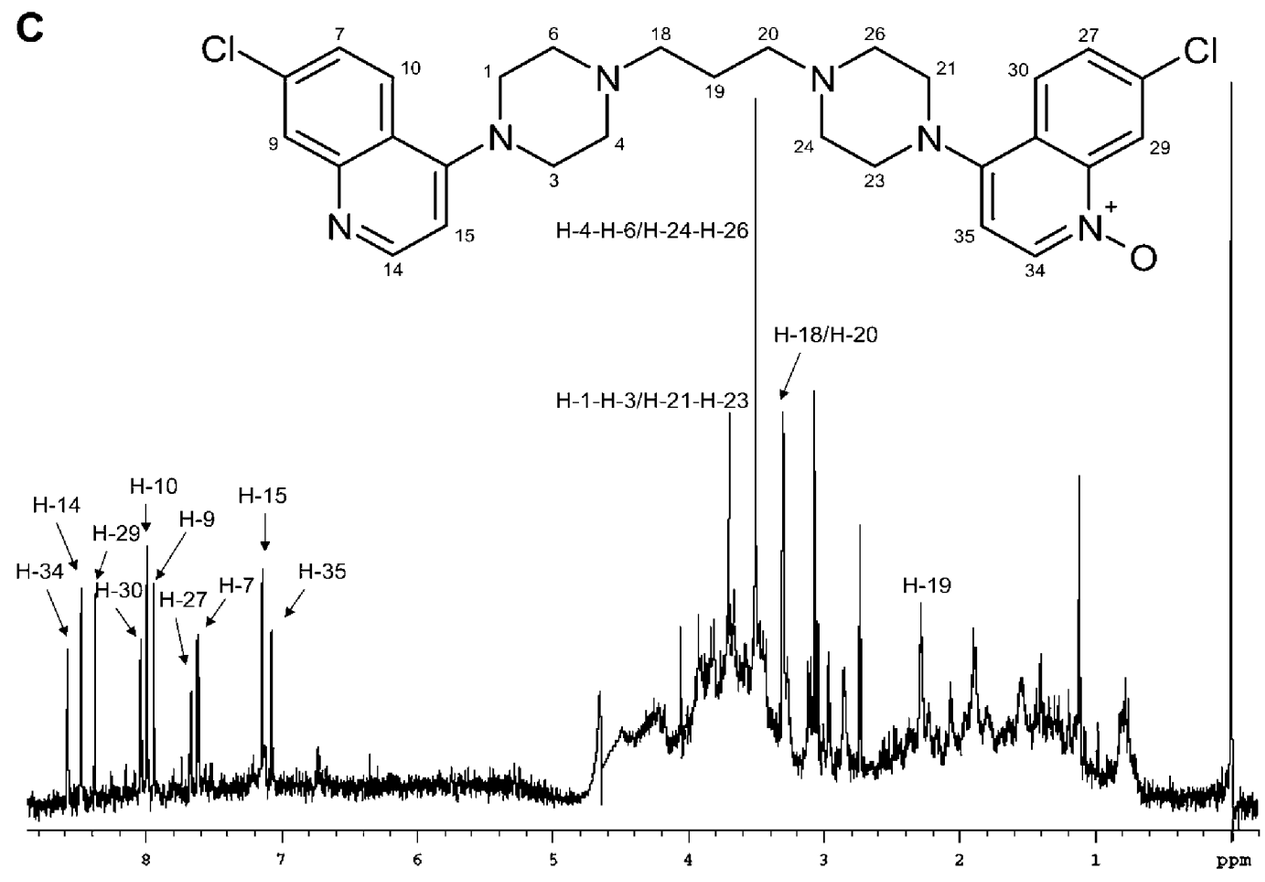

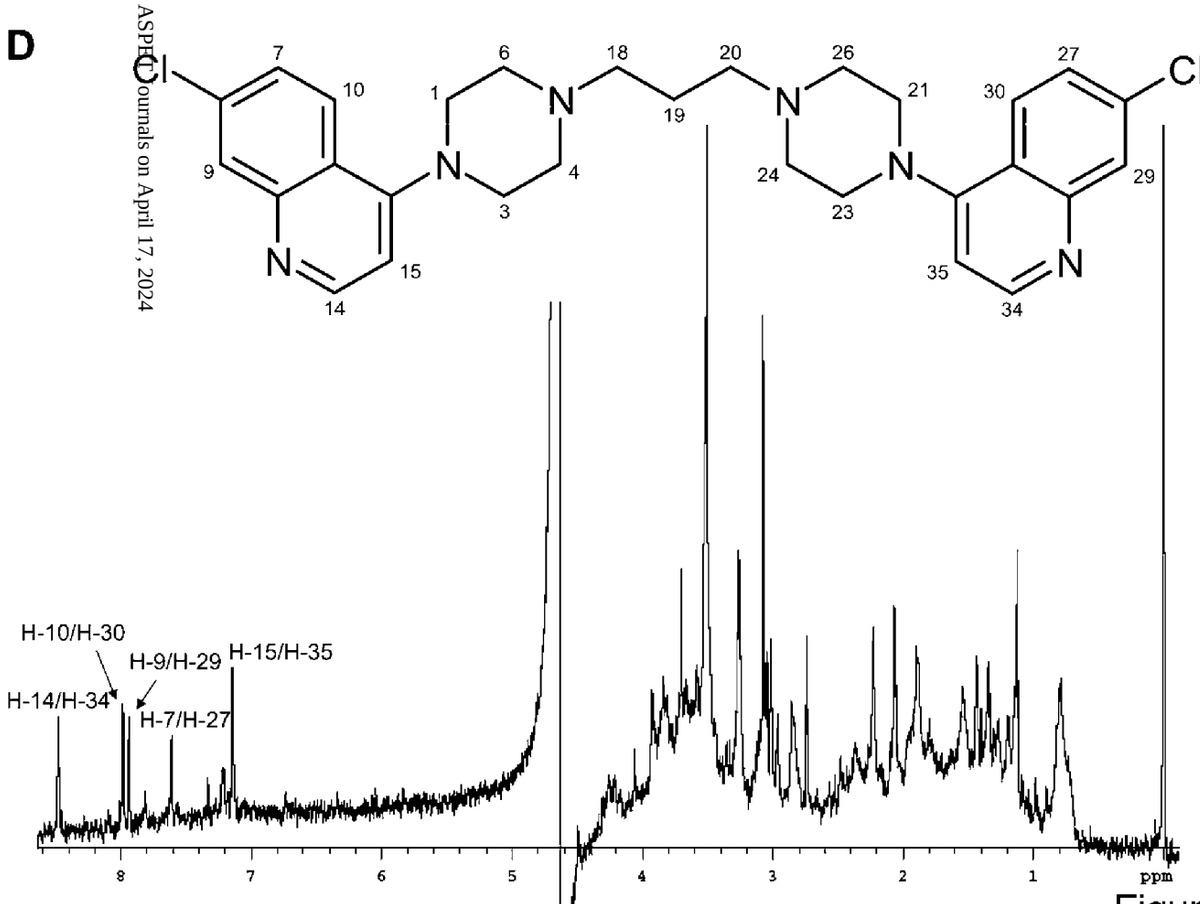

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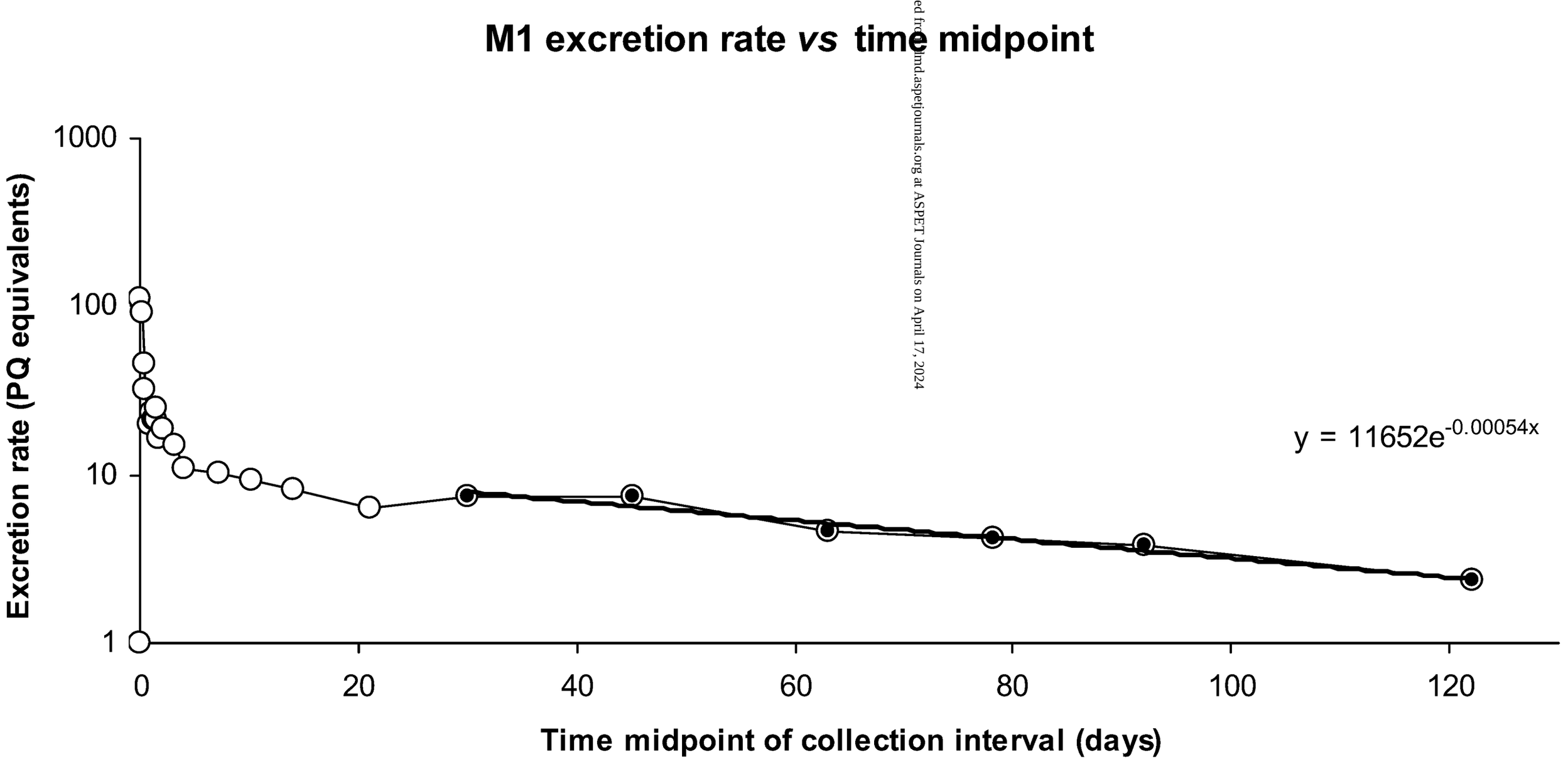

Figure 6.