Enantioselective Pharmacokinetics of Primaquine in Healthy Human Volunteers


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ABSTRACT

Primaquine (PQ), a racemic drug, is the only treatment available for radical cure of relapsing Plasmodium vivax malaria and blocking transmission of P. falciparum malaria. Recent studies have shown differential pharmacologic and toxicologic profiles of individual PQ enantiomers in rodent, dog, and primate animal models. This study was conducted in six healthy adult human volunteers to determine the plasma pharmacokinetic profile of enantiomers of PQ and carboxyprimaquine (cPQ), the major plasma metabolite. The individuals were orally administered PQ diphosphate, equivalent to 45-mg base, 30 minutes after a normal breakfast. Blood samples were collected at different time intervals, and plasma samples were analyzed for enantiomers of PQ and cPQ. Plasma PQ concentrations were low and variable for both parent enantiomers and peaked around 2-4 hours. Peak (−)-(R)-PQ concentrations ranged from 121 ng/ml to 221 ng/ml, and peak (+)-(S)-PQ concentrations ranged from 168 ng/ml to 299 ng/ml. The cPQ concentrations were much higher and were surprisingly consistent from subject to subject. Essentially all the cPQ detected in plasma was (−)-cPQ. The peak concentrations of (−)-cPQ were observed at 8 hours (range: 1104-1756 ng/ml); however, very high concentrations were sustained through 24 hours. (+)-cPQ was two orders of magnitude lower than (−)-cPQ, and in a few subjects it was detected but only under the limit of quantification. In vitro studies with primary human hepatocytes also suggested more rapid metabolism of (−)-PQ compared with (+)-PQ. The results suggest more rapid metabolism of (−)-PQ to (−)-cPQ compared with (+)-PQ. Alternatively, (−)-PQ or (+)-cPQ could be rapidly converted to another metabolite(s) or distributed to tissues. This is the first clinical report on enantioselective pharmacokinetic profiles of PQ and cPQ and supports further clinical evaluation of individual PQ enantiomers.

Introduction

The 8-aminoquinolines (8-AQs) are an important class of anti-infective drugs with promising utility in treatment of infections caused by parasitic protozoa and other emerging infectious disease organisms (Tekwani and Walker, 2006). Primaquine (PQ) is the only 8-AQ drug approved for clinical use for the treatment (radical cure) of relapsing Plasmodium vivax malaria (Tekwani and Walker, 2006; Vale et al., 2009; John et al., 2012). It is also used as a prophylactic drug against all major forms of human malaria and in combination with clindamycin for the treatment as well as prophylaxis of Pneumocystis pneumonia in human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) patients (Tekwani and Walker, 2006; Kim et al., 2009). The utility of PQ has also been shown in the prevention and blocking of transmission of Plasmodium falciparum malaria (Hill et al., 2006; White et al., 2012), and recently its use has been proposed as a key strategy in malaria control and elimination efforts (White, 2008; Fernando et al., 2011; John et al., 2012; Galappaththy et al., 2013). However, the therapeutic utility of PQ is limited because of its due to severe hemolytic toxicity in individuals with glucose 6-phosphate dehydrogenase deficiency (Youngster et al., 2010; Baird, 2012; Ganesan et al., 2012; Howes et al., 2013).

Synthesis and testing of many different 8-AQs in the 1940s led to the discovery of PQ, with its 4-amino-1-methylbutyl side chain (Fig. 1), which conferred the best combination of efficacy and tolerability (Edgcomb et al., 1950). This was further demonstrated by extensive structure-activity analysis of 8-AQs (McChesney, 1981; Schmidt, 1983; Nodiff et al., 1991). This side chain contains an asymmetric center at carbon 1, and thus two different configurations (enantiomers) are possible (Fig. 1). PQ synthetic methods yield a racemic mixture of these two enantiomers; when PQ was developed, enantiomeric separation of racemates was not available, and single enantiomers of racemic drugs were not the norm in drug development. Later, when the next generation of 8-AQs, namely, 8-AQ, 8-aminoquinoline; AUC, area under the curve; CL/F, apparent oral clearance; cPQ, carboxy primaquine; HPLC, high-pressure liquid chromatography; kₑ, elimination rate constant; LC-MS, liquid chromatography with mass spectrometry; PQ, primaquine; $T_{1/2}$, elimination half-life; $T_{max}$, time to maximum concentration; TOF, time-of-flight; TQ, tafenoquine.

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bulaquine (Rajgor et al., 2003; Krudsood et al., 2006) and tafenoquine (TQ) (Crockett and Kain, 2007; Llanos-Cuentas et al., 2014), were developed, the same side chain was retained. Thus, PQ is still available as the racemate, and TQ is also being developed as a racemate (Llanos-Cuentas et al., 2014).

Previous as well as more recent findings suggest that the configuration of this side chain can dramatically impact metabolism, toxicity, and efficacy of PQ and that these can be differentially affected, depending on species and the test systems employed (Ward et al., 1987; Agarwal et al., 1988; Baker and McChesney, 1988). Actually, more than 37 years ago, Schmidt et al. (1977) provided early evidence about the enantioselective toxic effects of PQ in Rhesus monkeys (Schmidt et al., 1977). He tested hundreds of 8-AQs for efficacy in primates under the U.S. Army’s malaria drug development program (Schmidt, 1983). It was observed that though the enantiomers of PQ were equally efficacious in the Rhesus Plasmodium cynomolgi radical cure model, (−)-PQ was at least twice as toxic as (+)-PQ (Schmidt et al., 1977).

It should be pointed out that the toxicity tracked by Schmidt et al. (1977) in these studies was likely liver related. He did not report the hematologic parameters. However, if the toxicity differential holds for hemolytic potential as suggested by our other studies (Nanayakkara et al., 2014), it would be a key finding. It is suggested that if such a distinction held true in humans, a doubling of the clinical therapeutic index of PQ could be attained by simply separating the two enantiomers (Schmidt et al., 1977; Tekwani and Walker, 2006).

Recent studies at our laboratory have confirmed differential pharmacologic and toxicologic profiles of individual enantiomers of PQ in different rodent models and beagle dogs, where (+)-PQ was found to be more efficacious as well as hemotoxic compared with (−)-PQ (Nanayakkara et al., 2014). A more recent study in rhesus macaques (Macaca mulatta) has shown that treatment with (−)-PQ enantiomer caused greater methemoglobin toxicity than that seen for (−)-PQ. In combination with chloroquine, (−)-PQ was more effective in preventing P. cynomolgi relapse, a surrogate model for P. vivax relapse, compared with (+)-PQ (Saunders et al., 2014).

From regulatory, scientific, and humanitarian perspectives, clinical use of a chiral drug with confirmed enantioselective pharmacologic and toxicologic profiles should not be acceptable in racemic form. De novo development of a drug in enantiomerically pure form or a switch from an existing racemic drug to one of its isomers is the principal scenario in chiral drug development. The U.S. Food and Drug Administration now requires evaluation of enantiomers as well racemic mixtures of a chiral drug before its introduction into the clinics (FDA, 2014).

PQ was approved for clinical use in racemic form more than 60 years ago (Vale et al., 2009), at a time when the technologies for chiral chemical synthesis/separation were not well developed and the understanding of enantioselective pharmacology/toxicology of PQ was almost nonexistent. We have developed a fractional crystallization method for preparation of individual PQ enantiomers (Nanayakkara et al., 2014) and also an analytic method using liquid chromatography with mass spectrometry (LC-MS) with electrospray ionization (ESI) for the separation and identification of (−)-RS- and (+)-SS-PQ and its major plasma metabolite (−)-RS- and (+)-SS-carboxyPQ (cPQ) in plasma samples (Avula et al., 2011). This method quantifies the [M+H]+ ions of PQ, 4-methyl PQ (internal standard), and cPQ at m/z 260.1763, 274.1849, and 275.1396, respectively, in the positive ion mode with extractive ion monitoring (Avula et al., 2013). This method has been useful for investigating the enantioselective metabolism of PQ in rodent and primate animal models (Avula et al., 2011; Saunders et al., 2014). This method was further used to explore the pharmacokinetic and metabolic properties of the enantiomers of PQ in healthy human volunteers after administration of the racemic form of the drug.

**Materials and Methods**

**Chemicals and Materials.** Primaquine phosphate (Sanofi-Aventis U.S., Bridgewater, NJ) was obtained from a local pharmacy. Each tablet contained 15 mg of PQ base. High-pressure liquid chromatography (HPLC)-grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). Water for the HPLC mobile phase was purified in a Milli-Q system (Millipore, Bedford, MA). PQ diphosphate, ammonium formate, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO).

The individual enantiomers of PQ were prepared by the fractional crystallization method described in Nanayakkara et al. (2014). PQ was resolved to (+)-SS- and (−)-RS-forms at NCNPR, their identity and purity were confirmed by spectral data (infrared, NMR and high-resolution MS), and their physical data (mp, [α]D) were compared with published values (Carroll et al., 1978). We prepared cPQ using the procedure reported by McChesney and Sarangan (1984).

This study was conducted at the Department of Student & Employees Health Services, University of Mississippi, under the supervision of Dr. Travis W. Yates, M.D., following a protocol approved by the institutional review board (University of Mississippi IRB file number 2010-0013).

**Subjects, Treatment, Samples Collection, and Processing.** The study was conducted with six healthy adult human volunteers (ages 26–51 years). The information on age, sex, and ethnicity of the individual human volunteers is provided in Supplemental Table 1. The individuals were orally administered three tablets of primaquine phosphate (equivalent to a total dose of 45 mg of primaquine base) (Sanofi-Aventis U.S.) 30 minutes after a normal breakfast. Blood samples were collected in 9-mL heparin Vacutainer tubes at different time intervals after administration of PQ.

The tubes with blood samples were immediately processed for centrifugation under refrigerated conditions (4°C) and for the separation of plasma and an erythrocytes pellet. The plasma samples from individual volunteers were divided into aliquots (500 μL) in cryovials, kept on dry ice, and transferred for storage at −80°C. The plasma samples were processed further and analyzed using LC-MS for enantiomers of PQ and cPQ as described herein.

**LC-ESI Time-of-Flight.** One part plasma (100 μL) was mixed with four parts methanol (400 μL). The drug was extracted by vortexing each sample for 30 seconds. After centrifuging at 10,000 rpm for 5 minutes, the supernatants were removed and filtered through 0.2-μm nylon membrane filters. An aliquot of 200 μL was transferred to the HPLC vials for analysis (Avula et al., 2011).

The details of the LC-MS analytic method and other conditions were described in an earlier publication (Avula et al., 2011). The LC system was an Agilent Series 1100 composed of the following modular components:
quaternary pump, a vacuum solvent microdegasser, and an autosampler with 100-well tray (Agilent Technologies, Palo Alto, CA). The MS analysis was performed on an LC-ESI time-of-flight (TOF) system (model G1969A; Agilent Technologies) equipped with an ESI source. The LC-ESI-TOF was calibrated using the Agilent tune mix. All acquisitions were performed under positive ionization mode with a capillary voltage of 3500 V. Nitrogen was used as the nebulizer gas (35 psig) as well as the drying gas (11 l/min, 350°C). The voltage of the photomultiplier tube, fragmentor, and skimmer was set at 850 V, 100 V, and 60 V, respectively. Full scan mass spectra were acquired from m/z 100–900.

Data acquisition and processing were performed using the Analyst QS software (Agilent Technologies). Separation was achieved on a Chiralcel OD-R: 250 × 4.6 mm i.d. and 10 µm particle size (Chiral Technologies, West Chester, PA). The column was equipped with a guard column (Chiral Technologies). A gradient LC method was used to separate PQ, cPQ enantiomers, and the internal standard from the matrix components, and to avoid ion suppression by the latter during quantification.

Linearity of the LC-MS method employed for analysis of human plasma samples ranged from 10–1000 ng/ml for mixture of (–)-PQ and (–)-cPQ concentrations in samples—that is, 5–500 ng/ml of each enantiomer. The lower limit of quantification for human plasma samples was 5 ng/ml for each enantiomer of PQ and 1 ng/ml for each enantiomer of cPQ. The lower limit of detection for the human plasma samples was 2 ng/ml and 0.5 ng/ml for each enantiomer of PQ and cPQ, respectively. The extraction recovery varied from 89% to 90% (with 0.2 ml of human plasma containing 10, 250, 500 ng/ml of PQ) and 91%–92% (with 0.2 ml of human plasma containing 10, 250, 500 ng/ml of cPQ).

During storage at an ambient temperature (25°C), for 12 hours, the concentrations of the enantiomers of PQ and cPQ in plasma deviated less than ±10% from their calculated concentrations, showing that the samples were stable during the preparation and analytic processes. During storage of human plasma samples with individual enantiomers of PQ or cPQ at 4°C for 24 hours, the concentrations of the PQ or cPQ varied no more than ±10% of their calculated concentrations.

**Pharmacokinetic Analysis.** The pharmacokinetic parameters for PQ and cPQ were computed as described by Cuong et al. (2006). Maximum plasma drug concentration (Cmax) and time to maximum concentration (Tmax) were obtained from the plasma drug concentration–time curve (Binh et al., 2009). The elimination rate constant (kel) was estimated by least-squares regression analysis of the postabsorption and distribution log plasma drug concentration–time data using at least 4 points. The elimination half-life (T1/2) was calculated from the ratio ln2/kel. The area under the drug concentration–time curve from 0 to 24 hours (the last data point) (AUC0–24 h) was calculated by the linear trapezoidal rule from the beginning of PQ administration to the last data point. Apparent oral clearance (CL/F) was computed by (Dose/AUC0–24 h).

The results were statistically analyzed for significance by Student’s t test using GraphPad Prism (GraphPad Software, San Diego, CA).

In Vitro PQ–Primary Human Hepatocyte Incubation. Freshly isolated primary human hepatocytes (BD Biosciences, San Jose, CA) were used in these experiments. The hepatocytes used for in vitro PQ metabolism studies were from a 61-year-old Caucasian female donor with no history of any liver disease, who had tested negative for human immunodeficiency virus and for hepatitis B and C. BD-BioCoat, a fully defined serum-free hepatocyte culture medium without epidermal growth factor was used. The cells received in suspension were immediately centrifuged at 1000g for 15 minutes, washed with BioCoat, and resuspended in BioCoat at a cell density of 1 × 10⁶ cells/ml.

The metabolic reactions were set up in a clear cell culture-grade 24-well polystyrene plate. The in vitro incubation mixtures consisted of a 480-µl cell suspension and 20-µl medium or primaquine (1 mM) (to achieve a final PQ concentration of 20 µM). The plate was incubated in a CO2 incubator at 37°C with 5% CO2. The contents from individual wells (three replicates for each time point) were withdrawn at different time intervals and transferred to microcentrifuge tubes; we added 500 µl of prechilled HPLC grade methanol to each tube, which was then vortexed and stored overnight at −80°C. The samples were centrifuged at 10,000g. The clear supernatants were filtered through 0.2-µm nylon membrane filters, and 100-µl aliquots were transferred to a 10% of their calculated concentrations.

**TABLE 1**

<table>
<thead>
<tr>
<th>Enantiomer</th>
<th>Mean</th>
<th>Maximum</th>
<th>Minimum</th>
<th>S.D.</th>
<th>P value</th>
</tr>
</thead>
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<td>(–)-PQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>163.35</td>
<td>221.60</td>
<td>121.30</td>
<td>37.64</td>
<td>0.017 (S)²</td>
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<tr>
<td>Tmax (h)</td>
<td>3.00</td>
<td>4.00</td>
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<td>1.09</td>
<td>0.50 (N.S.)²</td>
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<tr>
<td>AUC0–24 h (ng.hr/ml)</td>
<td>2231.00</td>
<td>2778.60</td>
<td>1768.70</td>
<td>374.64</td>
<td>0.022 (S)²</td>
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<tr>
<td>kel</td>
<td>0.085</td>
<td>0.043</td>
<td>0.114</td>
<td>0.025</td>
<td>0.782 (N.S.)²</td>
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<tr>
<td>T1/2 (h)</td>
<td>9.00</td>
<td>16.00</td>
<td>6.05</td>
<td>3.65</td>
<td>0.991 (N.S.)²</td>
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<tr>
<td>CL/F (l/h)</td>
<td>168.1</td>
<td>212.0</td>
<td>135.0</td>
<td>28.3</td>
<td>0.010 (S)²</td>
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<td>(+)-PQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>234.66</td>
<td>299.2</td>
<td>168.8</td>
<td>48.9</td>
<td></td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>2.67</td>
<td>4.00</td>
<td>2.00</td>
<td>1.03</td>
<td></td>
</tr>
<tr>
<td>AUC0–24 h (ng.hr/ml)</td>
<td>3221.55</td>
<td>4546</td>
<td>2359.5</td>
<td>813.9</td>
<td></td>
</tr>
<tr>
<td>kel</td>
<td>0.081</td>
<td>0.056</td>
<td>0.102</td>
<td>0.019</td>
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</tr>
<tr>
<td>T1/2 (h)</td>
<td>8.98</td>
<td>12.32</td>
<td>6.82</td>
<td>2.36</td>
<td></td>
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<tr>
<td>CL/F (l/h)</td>
<td>116.4</td>
<td>158.9</td>
<td>82.5</td>
<td>28.6</td>
<td></td>
</tr>
<tr>
<td>Total PQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>394.93</td>
<td>520.8</td>
<td>292.0</td>
<td>85.14</td>
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<tr>
<td>Tmax (h)</td>
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<td>2.00</td>
<td>1.03</td>
<td></td>
</tr>
<tr>
<td>AUC0–24 h (ng.hr/ml)</td>
<td>5402.55</td>
<td>7324.6</td>
<td>4021.2</td>
<td>1233.12</td>
<td></td>
</tr>
<tr>
<td>kel</td>
<td>0.079</td>
<td>0.043</td>
<td>0.099</td>
<td>0.0201</td>
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</tr>
<tr>
<td>T1/2 (h)</td>
<td>9.39</td>
<td>15.90</td>
<td>7.00</td>
<td>3.31</td>
<td></td>
</tr>
<tr>
<td>(–)-cPQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>1398.66</td>
<td>1756</td>
<td>1104</td>
<td>277.97</td>
<td>&lt;0.001 (S)⁶</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>13.33</td>
<td>1440</td>
<td>480</td>
<td>495.74</td>
<td>0.406 (N.S.)⁶</td>
</tr>
<tr>
<td>AUC0–24 h (ng.hr/ml)</td>
<td>28,033.96</td>
<td>33,827.7</td>
<td>23,573.1</td>
<td>4502.43</td>
<td>0.022 (S)⁶</td>
</tr>
<tr>
<td>(+)-cPQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>234.66</td>
<td>299.2</td>
<td>168.8</td>
<td>48.9</td>
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<tr>
<td>Tmax (h)</td>
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<td>4.00</td>
<td>2.00</td>
<td>1.03</td>
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<tr>
<td>AUC0–24 h (ng.hr/ml)</td>
<td>3221.55</td>
<td>4546</td>
<td>2359.5</td>
<td>813.9</td>
<td></td>
</tr>
<tr>
<td>Total cPQ</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>1396.16</td>
<td>1771.8</td>
<td>1069.2</td>
<td>302.47</td>
<td></td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>800</td>
<td>1440</td>
<td>480</td>
<td>495.74</td>
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<tr>
<td>AUC0–24 h (ng.hr/ml)</td>
<td>28,283.76</td>
<td>34,026.1</td>
<td>23,755.1</td>
<td>4461.47</td>
<td></td>
</tr>
</tbody>
</table>

* (+)-PQ versus (–)-PQ.

b (–)-cPQ versus (+)-cPQ.
Results

PQ (45 mg) was administered as three tablets of PQ diphosphate with a glass of water to each individual after a regular breakfast. This dose was well tolerated by all the participants, and no adverse reactions were observed in any of the six individuals who participated in this study. The plasma concentrations of the enantiomers of PQ and cPQ were analyzed by the chiral LC-MS method and were quantitated against a calibration curve prepared with authentic standards. The concentrations of enantiomers of PQ and cPQ in individual subjects at the different time intervals are presented in Supplemental Tables 2, 3, and 4. The computed pharmacokinetic parameters for plasma PQ and cPQ enantiomers for each individual subject are presented in Supplemental Tables 5 and 6, and the average pharmacokinetics data are presented in Table 1.

In five of the volunteers, the concentrations of (+)-PQ as well as (−)-PQ enantiomers were first detected at 60 minutes after administration of the drug (Fig. 2). In one subject, (+)-PQ and (−)-PQ were detected as early as 30 minutes after administration of racemic PQ (Supplemental Table 2). The plasma concentrations of (−)-PQ ranged from 121.3 ng/ml to 221.6 ng/ml and peaked between 2 and 4 hours. The plasma concentrations of (+)-PQ ranged between 168.8 ng/ml and 299.2 ng/ml. The (+)-PQ concentrations also peaked during 2 to 4 hours.

The plasma half-life ($T_{1/2}$) for (−)-PQ and (+)-PQ was not statistically significantly different. However, the $C_{max}$ for (+)-PQ was statistically significantly higher than for (−)-PQ ($P = 0.017$). The AUC$_{0-24\,h}$ value was about 1.5-fold higher for (+)-PQ than for (−)-PQ. This difference in AUC values for (−)-PQ and (+)-PQ was statistically significant ($P = 0.022$). The higher AUC value to (+)-PQ compared with (−)-PQ resulted in a significantly lower CL/F for (+)-PQ than for (−)-PQ.

In all subjects except one, cPQ first appeared at 60 minutes after administration of racemic PQ (Fig. 3). The key finding in this study was that nearly all the cPQ detected in plasma was (−)-cPQ, yielding a peak plasma concentration 60 times higher than that observed for (+)-cPQ; this was consistent with the kinetics of the parent enantiomers, with a $T_{max}$ 4 hours longer than for (+)-PQ. The peak plasma concentrations of (−)-cPQ were observed at 8 and 24 hours as being in the range of 1104–1756, with an average peak plasma cPQ concentration of 1399 ng/ml. However, very high concentrations of plasma (−)-cPQ were still present at 24 hours. The concentration of (−)-cPQ only marginally declined during 8–24 hours; in two subjects, the peak (−)-cPQ concentration was detected at 24 hours.

These observations also indicate a long plasma half-life of (+)-cPQ. The AUC$_{0-24\,h}$ values computed for (−)-cPQ were in the range 5236–9879, whereas for (+)-cPQ the AUC values were about 23,573 to 33,827 ng.hr/ml. The PQ-treated individuals therefore received more than 15-fold higher exposure to plasma cPQ concentrations than PQ concentrations. The concentrations of (+)-cPQ were two orders of magnitude lower than for (−)-cPQ. In one subject, (+)-cPQ was only detected under the limit of quantification throughout the period of the
The key finding in our study was that essentially all the cPQ detected in plasma was (+)-cPQ. We found that (+)-cPQ was two orders of magnitude lower than (-)-cPQ, and in most samples it was only detected under the limit of quantification. Very high concentrations of cPQ, mostly (-)-cPQ, were still present at 24 hours. Therefore, the half-life for cPQ enantiomers could not be computed from this experiment. However, the persistence of (+)-cPQ is consistent with earlier published studies on pharmacokinetics of racemic PQ (Ward et al., 1985; Bhatia et al., 1986; Singhasivanon et al., 1991; Bangchang et al., 1994; Kim et al., 2004) and PQ in combination with chloroquine (Pukrittayakamee et al., 2014) and artemisinin combinations (ACT) therapies (Hanboonkunupakarn et al., 2014; Jittamala et al., 2015). The computed elimination half-lives of (-)-cPQ, (+)-cPQ, and (+)-PQ were similar. Thus, in the event of future enantioselective utility of PQ, dosing frequency with enantiomers may not be different for the racemic mixture.

Discussion
Most of the antimalarials—namely, chloroquine, PQ, mefloquine, halofantrine, tafenoquine, and lumefantrine—are chiral but are used as racemates (Brocks and Mehvar, 2003). For antimalarials, stereoselectivity has been mainly noted in their ability to cause adverse effects. Development of analytic methods capable of measuring the individual enantiomers of these antimalarials has shown that almost all antimalarial drugs display stereoselectivity in their pharmacokinetics (Brocks and Mehvar, 2003).

Our group has reported recently on the enantioselective pharmacologic, pharmacokinetic, and toxicologic properties for PQ in mice, dogs, and primates (Nanayakkara et al., 2014; Saunders et al., 2014). These studies added to a body of literature that suggested important differences in these biologic activities for PQ enantiomers. No stereoselectivity was noticed in the metabolism of (+)-(S) and (-)-(R) isomers of PQ in vitro by isolated perfused liver (Nicholl et al., 1987) and rat liver microsomes (Ward et al., 1987), but when racemic PQ was administered to rats the majority of residual PQ excreted in urine was found to be the (+)-isomer (Baker and McChesney, 1988). The (+)-isomer of PQ caused a significantly higher generation of methemoglobin in erythrocytes from a glucose 6-phosphate-deficient individual than the (-) isomer (Agarwal et al., 1988, 1991).

Schmidt et al. (1977) examined the relative curative and toxic activities of PQ and its (+)- and (-)-isomers in mice and rhesus monkeys. They confirmed their earlier report that (+)-PQ was approximately 4 times more toxic as compared with the (-)-form and at least twice more toxic than racemic PQ in mice; they also indicated that the opposite is true in the Rhesus monkey, in which the (+)-PQ was 3 to 5 times more hepatotoxic as compared with (+)-PQ and at least twice more toxic than racemic PQ. More importantly, all three forms of PQ, the (+)-PQ and (-)-PQ and (+)-PQ forms, showed similar curative potencies against sporozoite-induced P. cynomolgi infections.

In several studies on the metabolism of (+)- and (-)-isomers, it was shown that the metabolic rates for these isomers were different under most conditions (Nicholl et al., 1987; Baker and McChesney, 1988). However, this is the first ever study in humans to provide conclusive evidence of differential pharmacokinetic profiles of PQ enantiomers.

The results obtained with human pharmacokinetic studies are further supported by the enantioselective metabolism of PQ in vitro by primary human hepatocytes.

Generally, there was rapid absorption of PQ after the single oral administration of PQ, with mean peak blood concentrations attained within less than 3 hours (160 minutes). This was followed by a less rapid but steady fall in plasma concentration with a mean elimination half-life of 9.39 hours. The extent of absorption of individual PQ enantiomers was not captured in this study, but early pharmacokinetic studies with (+)-PQ reported that PQ is almost completely absorbed in humans after oral administration (96% bioavailability) (Mihaly et al., 1985).

Fig. 4. In vitro metabolism of primaquine (PQ) and formation of carboxyprimaquine (cPQ) with primary human hepatocytes. (A) Enantioselective depletion/metabolism of PQ measured as depletion of (−)-PQ and (+)-PQ. (B) Enantioselective formation of cPQ measured as formation of (−)-cPQ and (+)-PQ. Each data point represents the mean ± S.D. of at least three observations.

Study. The peak concentrations of (+)-cPQ ranged between below the limit of quantification to 36.8 ng/ml.

The in vitro studies on metabolism of PQ with primary human hepatocytes also showed results similar to those observed with human pharmacokinetics studies. Incubation of racemic PQ with human hepatocytes showed a more rapid depletion of (−)-PQ (78% in 4 hours) compared with (+)-PQ (22% in 4 hours) (Fig. 4A). Concomitant formation of cPQ was observed on in vitro incubation of PQ with primary human hepatocytes (Fig. 4B). During the initial 2 hours of incubation, 96.1% of the total cPQ formed with human hepatocytes was (+)-cPQ. Very slow formation of (+)-cPQ was observed after 4 hours. Still, (+)-cPQ represented only 13% of the total cPQ formed at 16 hours (Fig. 4B).

Discussion
Most of the antimalarials—namely, chloroquine, PQ, mefloquine, halofantrine, tafenoquine, and lumefantrine—are chiral but are used as racemates (Brocks and Mehvar, 2003). For antimalarials, stereoselectivity has been mainly noted in their ability to cause adverse effects. Development of analytic methods capable of measuring the individual enantiomers of these antimalarials has shown that almost all antimalarial drugs display stereoselectivity in their pharmacokinetics (Brocks and Mehvar, 2003).

Our group has reported recently on the enantioselective pharmacologic, pharmacokinetic, and toxicologic properties for PQ in mice, dogs, and primates (Nanayakkara et al., 2014; Saunders et al., 2014). These studies added to a body of literature that suggested important differences in these biologic activities for PQ enantiomers. No stereoselectivity was noticed in the metabolism of (+)-(S) and (-)-(R) isomers of PQ in vitro by isolated perfused liver (Nicholl et al., 1987) and rat liver microsomes (Ward et al., 1987), but when racemic PQ was administered to rats the majority of residual PQ excreted in urine was found to be the (+)-isomer (Baker and McChesney, 1988). The (+)-isomer of PQ caused a significantly higher generation of methemoglobin in erythrocytes from a glucose 6-phosphate-deficient individual than the (-) isomer (Agarwal et al., 1988, 1991).

Schmidt et al. (1977) examined the relative curative and toxic activities of PQ and its (+)- and (-)-isomers in mice and rhesus monkeys. They confirmed their earlier report that (+)-PQ was approximately 4 times more toxic as compared with the (-)-form and at least twice more toxic than racemic PQ in mice; they also indicated that the opposite is true in the Rhesus monkey, in which the (+)-PQ was 3 to 5 times more hepatotoxic as compared with (+)-PQ and at least twice more toxic than racemic PQ. More importantly, all three forms of PQ, the (+)-PQ and (-)-PQ and (+)-PQ forms, showed similar curative potencies against sporozoite-induced P. cynomolgi infections.

In several studies on the metabolism of (+)- and (-)-isomers, it was shown that the metabolic rates for these isomers were different under most conditions (Nicholl et al., 1987; Baker and McChesney, 1988). However, this is the first ever study in humans to provide conclusive evidence of differential pharmacokinetic profiles of PQ enantiomers. The results obtained with human pharmacokinetic studies are further supported by the enantioselective metabolism of PQ in vitro by primary human hepatocytes.

Generally, there was rapid absorption of PQ after the single oral administration of PQ, with mean peak blood concentrations attained within less than 3 hours (160 minutes). This was followed by a less rapid but steady fall in plasma concentration with a mean elimination half-life of 9.39 hours. The extent of absorption of individual PQ enantiomers was not captured in this study, but earlier pharmacokinetic studies with (+)-PQ reported that PQ is almost completely absorbed in humans after oral administration (96% bioavailability) (Mihaly et al., 1985).

The key finding in our study was that essentially all the cPQ detected in plasma was (+)-cPQ. We found that (+)-cPQ was two orders of magnitude lower than (-)-cPQ, and in most samples it was only detected under the limit of quantification. Very high concentrations of cPQ, mostly (-)-cPQ, were still present at 24 hours. Therefore, the half-life for cPQ enantiomers could not be computed from this experiment. However, the persistence of (+)-cPQ is consistent with earlier published studies on pharmacokinetics of racemic PQ (Ward et al., 1985; Bhatia et al., 1986; Singhasivanon et al., 1991; Bangchang et al., 1994; Kim et al., 2004) and PQ in combination with chloroquine (Pukrittayakamee et al., 2014) and artemisinin combinations (ACT) therapies (Hanboonkunupakarn et al., 2014; Jittamala et al., 2015). The computed elimination half-lives of (-)-cPQ, (+)-cPQ, and (+)-PQ were similar. Thus, in the event of future enantioselective utility of PQ, dosing frequency with enantiomers may not be different for the racemic mixture.
The attainment of the peak plasma concentration of a drug often reflects the net balance between the rates of absorption, tissue distribution, and elimination. Several factors can be attributed to the observed significantly higher peak plasma concentration of (+)-PQ in comparison with (−)-PQ. Variation in enantioselective susceptibility to presystemic metabolism including intestinal efflux activities may result in varying blood concentrations. These factors are more likely to be responsible in this case, as supported by the delayed $T_{\text{max}}$ observed with (−)-PQ in addition to its lower $C_{\text{max}}$.

Alternatively, discrepancies in the enantiomers’ affinity for tissue penetration and protein binding might affect the measurable concentrations in the blood. Thus, if (−)-PQ is more distributed in the tissues, a lessened blood concentration will be expected. Analysis of PQ and cPQ in tissues is beyond the scope of this study, as extravascular concentrations of the enantiomers are not captured. However, in vitro studies also showed more rapid metabolism of (−)-PQ compared with (+)-PQ by primary human hepatocytes, which was further reflected in terms of more rapid formation of (−)-cPQ compared with (+)-cPQ. Another possible reason for varying the $C_{\text{max}}$ of the enantiomers may be the variation in systemic clearance of individual PQ enantiomers.

In conclusion, the results presented here suggest a markedly more rapid metabolism of (−)-PQ to (−)-cPQ than (+)-PQ to (+)-cPQ. Alternatively, the (−)-PQ or (+)-cPQ could be rapidly converted to another metabolite(s) or distributed to the tissues. This study confirms the enantioselective pharmacokinetic and metabolite profiles of PQ and suggests a need for further clinical evaluation of the efficacy and safety of PQ enantiomers in humans.

The computed elimination half-lives of PQ, (−)-PQ and (+)-PQ were similar. Thus, in the event of future clinical evaluation of individual PQ enantiomers to determine the enantioselective therapeutically advantageous advantage of PQ, dosing frequency with enantiomers may not be different from current dose regimens evaluated for racemic PQ.

Comparative clinical pharmacokinetic profiles of (−)-PQ versus (+)-PQ and (−)-cPQ versus (+)-cPQ strongly support further clinical evaluation of individual enantiomers of PQ in terms of safety and other therapeutic advantages. Initial analyses of these results suggest a better therapeutic value of (−)-PQ compared with (−)-PQ. The lower clearance (CL/F) and higher exposure (AUC) for (−)-PQ compared with (−)-PQ suggest that a lower dose of (−)-PQ may be required for malaria patients. Further, any potential adverse effects of exposure to cPQ would be eliminated because (−)-PQ would generate very low levels of (−)-cPQ.

Additionally, it would be interesting to investigate the enantioselective profile of other PQ metabolites, which may be directly implicated in the relative efficacy and toxicity of PQ. The analytic methods for phenotyping the potentially reactive phenolic metabolites of PQ generated with human hepatocytes (Avula et al., 2013) and recombinant human CYP2D6 (Fasini et al., 2014) have been reported recently and may be applied to clinical studies with individual PQ enantiomers.

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Authorship Contributions

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