Increased bioavailability of primaquine using poly(ethylene oxide) matrix extended-release tablets administered to beagle dogs

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Primaquine (PQ) is used for the radical cure of *Plasmodium vivax* malaria and can cause serious side effects in some individuals. The development of an extended-release dosage with poly(ethylene oxide) as a hydrophilic polymer has been investigated to improve drug efficacy and tolerability. The aim of this study was to evaluate *in vivo* a new extended-release formulation of PQ (60 mg). The formulation was administered to beagle dogs and plasma PQ concentrations were compared to a conventional immediate-release formulation of PQ (60 mg). The evaluation was carried out using a validated high-performance liquid chromatography method using solid-phase extraction. Total PQ exposure in beagle dogs was 2.2 times higher (area under curve of 12 193 versus 5678 ng h/ml) and the elimination half-life of PQ was a 19-fold greater (12.95 hours versus 0.68 hours) with the extended-release tablets compared with the immediate-release tablets. These findings suggest that the extended-release formulation of PQ merits further evaluation for the treatment of *P. vivax* malaria and/or chemoprophylaxis.

INTRODUCTION

Malaria is a tropical disease, confined to underdeveloped regions of the globe, which has been traditionally neglected by the pharmaceutical industry and represents a serious world health problem (WHO, 2008; Vale *et al.*, 2009). Primaquine (PQ), 8-[(4-amino-1-methylbutyl) amino]-6-methoxyquinoline, is effective against the liver stages of *Plasmodium vivax* and *Plasmodium ovale* malaria and it is the only drug available for radical cure against these *Plasmodium* species. PQ also possesses causal prophylactic activity against both *P. falciparum* and *P. vivax* (Baird *et al.*, 2003; WHO, 2008). However, PQ has adverse side

effects and its toxicity is of concern, which limits its use and decreases patient adherence to treatment (White, 2008). The most important adverse side effects are acute intravascular haemolysis in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency, and gastrointestinal disturbances, such as abdominal pain, nausea, vomiting, methaemoglobinemia and leukocytosis (Hofsteenge *et al.*, 1986; Baird and Rieckmann, 2003). In the absence of definitive evidence of either G6PD-normal status or demonstrated tolerability to a given variant, PQ should not be administered. This restricts the use of PQ in the setting of interventions against epidemic or endemic *vivax* malaria (Baird and Rieckmann, 2003).

PQ is readily absorbed from the gastrointestinal tract and peak plasma concentrations are attained around 1–3 hours after oral administration, after which levels decline, with an elimination half-life of 3–6 hours. It is widely distributed in body

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tissues and is rapidly metabolized in the liver to its major inactive metabolite, carboxypri-maquine (Mihaly *et al.*, 1984). Due to the relatively short elimination half-life, the treatment of malaria with PQ requires frequent daily dosing for 14 days, thereby intensifying the adverse side effects (WHO, 2008; Vale *et al.*, 2009). To reduce the undesirable side effects, some researchers have suggested different routes of PQ administration, such as transdermal therapeutic systems (Mayorga *et al.*, 1996; Jeans and Heard, 1999), encapsulation in nanoparticles (Rodrigues, Jr *et al.*, 1995), liposomes (Stensrud *et al.*, 2000) and emulsification (Dierling and Cui, 2005). However, none of the above alternative routes have moved forward to clinical applications (Vale *et al.*, 2009). Swellable matrices have also been developed by direct compression and *in vitro* evaluation has been carried out (Cruz *et al.*, 2008a; Bertol *et al.*, 2010) in order to improve the treatment efficacy of PQ and to reduce the limitations of existing PQ therapies in G6PD-normal patients.

The development of controlled-release matrix formulations has been successfully applied in the pharmaceutical industry since these are considered reliable in terms of drug delivery, easy to formulate and manufacture (Colombo *et al.*, 2009). Hydrophilic matrices, such as poly(ethylene) oxides (PEOs), are among the most used controlled delivery systems (Maggi *et al.*, 2000; Kojima *et al.*, 2008) because they can provide an appropriate combination of swelling, dissolution or erosion mechanisms to control the drug release kinetics (Heller *et al.*, 2002; Lopes *et al.*, 2005).

For the *in vivo* evaluation of the PQ/PEO matrix extended-release tablets, a bioanalytical method was developed and validated in the present study for the determination in beagle dogs, a common animal model used in pre-clinical pharmacokinetics studies. Analytical methods have been described for the measurement of PQ alone and its metabolites using different mobile-phases and extraction procedures (e.g. liquid-liquid

extraction, protein precipitation) (Baker *et al.*, 1982; Nora *et al.*, 1984; Parkhurst *et al.*, 1984; Ward *et al.*, 1984; Endoh *et al.*, 1992; Dean *et al.*, 1994; Dua *et al.*, 1996; Mayorga *et al.*, 1997; Lal *et al.*, 2003; Nitin *et al.*, 2003; Kim *et al.*, 2004; Cuong *et al.*, 2006; Singh and Vingkar, 2008). However, for these methods, a full validation was not demonstrated for PQ and several groups reported low extraction recoveries of the drug. PQ has an amphiphatic character with two pKa values (3.2 and 10.4) (Stensrud *et al.*, 2000) resulting in low recovery rates using conventional extraction methods, such as liquid-liquid extraction (Lal *et al.*, 2003; Nitin *et al.*, 2003). Considering the polar nature of the analyte, solid phase extraction (SPE) has been considered an effective and highly preferred technique (Pathak *et al.*, 2008). A high-performance liquid chromatography (HPLC) method with SPE has not been reported for the measurement of PQ in biological matrices.

The aim of this study was to develop and validate an HPLC method using SPE for the quantification of plasma PQ concentrations in beagle dogs and to compare the pharmacokinetics of PQ administered as immediate-release tablets (IRTs) and extended-release tablets (ERTs). Favourable findings with the ERT of PQ could represent a new option for malaria treatment and/or chemoprophylaxis with PQ.

MATERIALS AND METHODS

Reagents and Chemicals

The PQ reference standard was purchased from US Pharmacopeia (Rockville, MD, USA), raw material and IRT were kindly donated by Fundação Oswaldo Cruz/Far-Manguinhos (Rio de Janeiro, RJ, Brazil). Bromopride (internal standard, IS) was purchased from Sigma-Aldrich (St Louis, MO, USA). The excipients used were: PEO with molecular weight of 8×10^6 g/mol (Sigma-Aldrich), microcrystalline cellulose (Avicel® PH-102; Blanver, Cotia, SP, Brazil), colloidal

silicon dioxide (Aerosil®; Galena, Campinas, SP, Brazil) and talc (Charles B Chrystal Co., Inc., New York, NY, USA). HPLC-grade methanol and acetonitrile were obtained from J. T. Baker (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA). The ultrapure water used in analyses was obtained from a Milli-Q® system (Millipore, Bedford, MA, USA).

Preparation of Extended-Release Tablets

ERTs were prepared by direct compression of the physical mixtures of 52.6 mg of PQ phosphate (corresponding to 30 mg of PQ free base), 30% of hydrophilic polymer PEO and excipients, with a total mass of 175 mg (Cruz *et al.*, 2008a).

Instrumentation and Chromatographic Conditions

The HPLC analysis was performed on a Shimadzu LC-10A vp system (Kyoto, Japan). The UV detector was set at 254 nm and peak area was integrated automatically using a Shimadzu Class VP® V 6.14 software program. A reversed-phase Phenomenex Luna C18 column (250 × 4.6 mm, 5 µm) (Torrance, CA, USA) maintained at 35 ± 1°C was used. The mobile phase consisted of acetonitrile–methanol–water–acetic acid (18:3.5:78:0.5, v/v), pH 2.75, with a flow rate of 1.0 ml/min.

Preparation of Standard Solutions and Calibration Curves

The stock solution of PQ (400 µg/ml) was prepared in water and bromopride (IS) stock solution (400 µg/ml) was prepared in methanol–water (3:2, v/v). Calibration standard solutions were obtained from dilutions of PQ stock solution with water. Aliquots (25 µl) of the PQ standard solutions were added to 475 µl of blank dog plasma to prepare the calibration standards covering the concentration range of 10–3000 ng/ml. The quality control (QC) samples were prepared in pooled plasma, with final PQ concentrations

of 200 ng/ml (low), 1300 ng/ml (medium) and 2700 ng/ml (high), and stored at –20°C until analysis.

Plasma Extraction

A 500 µl aliquot of each standard, QC or blank plasma sample was transferred to a glass tube, followed by the addition of 37.5 µl of IS solution (200 µg/ml), 50 µl of ammonia hydroxide and 200 µl of water. All samples were mixed by vortex agitation for 30 seconds and 740 µl of samples were then loaded into SPE cartridges (Phenomenex Strata X, 30 mg/ml) preconditioned with methanol (1 ml) and water (1 ml). The extraction of PQ from cartridges was performed with two volumes (1 ml each) of a mixture containing methanol–acetonitrile–water–acetic acid (60:30:10:0.1, v/v). The eluate was evaporated to dryness at 60°C under reduced pressure in a speed-vac concentrator (Savant SPD 1010; Thermo Electron Corporation, Milford, MA, USA). The samples were reconstituted with 250 µl of mobile phase and mixed by vortex agitation for 1 minute and 20 µl was injected into the HPLC system.

Drug Administration, Blood Sampling and Pharmacokinetic Analysis

An oral dose of PQ (60 mg) formulated as either ERT or IRT was administered to six female beagle dogs (body weight range: 12.10–17.45 kg), with 200 ml of water after an overnight fast. The beagle dogs originated from 'Serveis de Support a la Recerca Estabulari-UB'. Principles in good laboratory animal care were followed and animal experimentation was in compliance with the 'Ethical Committee of Animal Experimentation' of the Federal University of Santa Catarina. After the wash-out period, corresponding to seven elimination half-lives of PQ (i.e. 92 hours), the first group received the IRT and the second group received the ERT. Animals had access to water and food 4 hours after drug administration. Blood samples (3 ml) were collected in heparinized tubes before tablet administration and at different

time points after dosing: 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5 and 6 hours for IRT and 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 17, 19, 21, 30 and 48 hours for ERT. The blood was immediately centrifuged at 3000 rev min⁻¹ for 15 minutes, and the plasma separated and stored at -20°C until analysis.

PQ plasma concentration-time profiles were plotted and the pharmacokinetic parameters estimated using non-compartmental analysis (Pharmkit; Johnston and Woolard, 1983). The maximum concentration ($C_{\max}$) of PQ and the time at which it was reached ($T_{\max}$) were obtained directly from the plasma concentration-time profiles of the drug. The area under the curve (AUC) for the PQ concentration-time profile from 0 to 48 hours [$AUC_{(0-48)}$] and to infinity [$AUC_{(0-\infty)}$] were calculated for each animal using the log trapezoidal rule. The $AUC_{(0-\infty)}$ was calculated by extrapolating the terminal slope of the curve to infinity as the ratio between the last measured concentration and the drug elimination constant (K_e). K_e was estimated by calculating the linear regression (least squares) method using the concentrations (log transformed multiplied by -2.303) that constitute the terminal decay phase. The elimination half-life ($T_{1/2}$) was derived from K_e , where $T_{1/2} = \ln 2 / K_e$. The relative bioavailability (f_{rel}) values of the two PQ formulations were calculated as the ratio $AUC_{(0-\infty)}^{\text{ERT}} / AUC_{(0-\infty)}^{\text{IRT}} \times 100\%$ for comparison. The pharmacokinetic data of the two formulations were compared statistically by ANOVA ($P < 0.05$).

RESULTS

Method Validation

The HPLC method was validated by the determination of the following parameters: specificity, lower limit of quantification (LLOQ), linearity, recovery, accuracy, precision and stability (FDA, 2001). Method specificity was assessed using six blank dog plasma samples, including hemolysed and

lipemic plasma. The comparison between the blank and spiked dog plasma (75 ng/ml) chromatograms indicated that no significant interfering peaks were detected from endogenous substances (Fig. 1). The retention times for IS and PQ were 4.0 and 9.6 minutes, respectively. At the LLOQ (10 ng/ml) the precision (expressed as relative standard deviation, RSD) of the method was 14.7%, with an inaccuracy of 14.6%. The limit of detection of PQ was calculated and found to be 2 ng/ml. Validated HPLC methods aim for good specificity and low sensitivity. The LLOQ (10 ng/ml) and limit of detection (2 ng/ml) achieved with this method could be improved further if necessary. After SPE, the samples can be concentrated by reconstituting them with a lower volume of mobile phase (<250 μ l) and the injection volume (20 μ l) can be increased (i.e. 50 μ l). For the calibration curve (10–3000 ng/ml), the typical coefficient of determination and equation of the curve were $r^2 = 0.999$, $y = 2.67 \cdot 10^{-5} \times + 0.0001$, respectively, indicating excellent linearity of the method.

To evaluate the inter-day precision and accuracy of the method, QC samples were analysed daily with an independent calibration curve of standards for 3 days, while intra-day precision and accuracy were evaluated by measuring QC samples ($n = 6$) on the same day. The evaluation of precision and accuracy was based on the criterion (FDA, 2001) that the RSD of each concentration should be within $\pm 15\%$ of the nominal concentration, except for the LLOQ, which had an acceptance level of $\pm 20\%$. As shown in Table 1, the intra- and inter-day precision and accuracy data for the method were within the acceptable range.

The extraction recovery was evaluated by dividing the mean for the extracted QC samples by the mean for the unextracted samples (spiked with the extracted blank plasma residues) at the same concentration. The mean ($\pm$ RSD%) extraction recoveries for the three QC concentrations ($n = 6$) were $88.6 \pm 13.2\%$ for the low QC, $110.8 \pm 10.3\%$

FIG. 1. Chromatograms obtained for specificity test. (a) Blank dog plasma; (b) lipemic dog plasma; (c) hemolysed dog plasma; (d) PQ (75 ng/ml) and IS (bromopride 15 µg/ml) dog plasma.

for the medium QC, $95.7 \pm 10.9\%$ for the high QC and $99.0 \pm 11.2\%$ for IS.

The stability of PQ in dog plasma was evaluated for various storage periods and related to the initial concentration as zero cycle (i.e. samples that were freshly prepared and processed immediately). The samples were considered stable when the deviation (expressed as percentage bias) from the zero cycle was within $\pm 15\%$. The freeze-thaw stability of PQ in the QC samples ($n=3$) was determined over three

freeze-thaw cycles within 3 days. In each cycle, the frozen plasma samples were thawed at room temperature for 2 hours and refrozen for 24 hours. The short-term stability of PQ was evaluated using three aliquots of each unprocessed QC sample kept at room temperature ($25 \pm 5^\circ\text{C}$) for 7 hours and then analysed. For the processed sample stability, three aliquots (low, medium and high QC samples) were processed and placed at room temperature and analysed after 48 hours. For the long-term

TABLE 1. Intra-day and inter-day precision and accuracy data for determination of PQ in plasma

Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	Precision (RSD%)	Accuracy (%)
Intra-day ^a			
200	208	9.8	104.1
1300	1319	6.8	101.5
2700	2798	8.6	103.7
Inter-day ^b			
200	214 ^c	6.8	107.2
1300	1345 ^c	7.9	103.5
2700	2791 ^c	9.2	103.4

^aMean of six replicates.^bMean of five replicates.^cMean of 3 days.

stability of PQ, three aliquots of each QC sample were frozen at -20°C for 60 days and then analysed. The results for the PQ stability are shown in Table 2 and indicate that PQ is stable in neat plasma for up to 7 hours at room temperature (short-term) and also after three freeze–thaw cycles. Plasma samples of PQ were stable for at least 60 days at -20°C (long-term). The results demonstrated that samples could be analysed up to 48 hours after extraction with acceptable precision and accuracy.

TABLE 2. PQ stability in plasma

Stability	Zero cycle concentration (ng/ml) ^a	Concentration found after storage (ng/ml) ^a	RSD (%)	Bias (%) ^b
Long term (60 days)	203	212	10.2	4.3
	1288	1310	5.7	1.7
	2829	2652	4.8	−6.3
Short term (7 hours)	203	231	6.3	13.8
	1288	1417	4.1	10.0
	2829	3132	8.2	10.7
Processed samples (after 48 hours)	203	198	9.9	−2.4
	1288	1169	6.0	−9.2
	2829	2587	7.6	−8.6
Three freeze–thaw cycles	203	223	9.6	9.9
	1288	1408	4.5	9.3
	2829	2894	8.6	2.3

^aMean of three replicates.^bBias=(measured concentration−nominal concentration/nominal concentration) × 100%.

Pharmacokinetic Studies of the Two Formulations of PQ

The mean plasma PQ concentration–time profiles of the beagle dogs following IRT and ERT administration are shown in Fig. 2, with the derived pharmacokinetic parameters of PQ summarized in Table 3. The pharmacokinetics of PQ were statistically different ($P<0.05$) for the two formulations. The $C_{\max}$ of PQ was 2.2 times higher after IRT compared with ERT administration (2419 versus 1083 ng/ml). A longer time was required to reach the $C_{\max}$ of PQ after ERT compared with IRT (4.4 hours versus 1.6 hours). The total PQ exposure was 2.2 times higher after ERT than IRT [$\text{AUC}_{(0-\infty)}$: 12 193 ng h/ml versus 5678 ng h/ml] and the $T_{1/2}$ of PQ was markedly longer after ERT than IRT (12.95 hours versus 0.68 hours).

DISCUSSION

Based on a previous study of the *in vitro* analysis of PQ/PEO matrix tablets (Cruz *et al.*, 2008b), the chromatographic conditions for the HPLC method applied herein were selected and optimized in order to quantify PQ accurately without interference

FIG. 2. Mean log PQ plasma concentrations ($\pm$ standard deviation) after a single 60 mg oral dose of extended-release tablets (ERTs) or immediate-release tablets (IRTs) of PQ to six beagle dogs.

from endogenous beagle dog plasma components. A mobile phase consisting of acetonitrile/methanol/water/acetic acid and a C18 column gave the best signal-to-noise

TABLE 3. Pharmacokinetic parameters of PQ following oral administration of 60 mg of PQ in ERT or IRT to beagle dogs ($n=6$, mean $\pm$ SD)

Parameter	IRT (reference)	ERT (test)
$AUC_{(0-48)}$ (ng h/ml) ^a	5629 $\pm$ 205	10 765 $\pm$ 151
$AUC_{(0-\infty)}$ (ng h/ml) ^b	5677 $\pm$ 202	12 193 $\pm$ 141
C_{max} (ng/ml) ^c	2419 $\pm$ 152	1083 $\pm$ 90.1
T_{max} (h) ^d		4.4 $\pm$ 0.70
	1.6 $\pm$ 0.67	
K_e (h ⁻¹) ^e	1.07 $\pm$ 0.2	0.06 $\pm$ 0.01
$T_{1/2}$ (h) ^f	0.68 $\pm$ 0.15	12.95 $\pm$ 2.1
f_{rel} ^g	NA ^h	227.7%

^a $AUC_{(0-48)}$: area under the concentration-time curve from time 0 to 48 hours.

^b $AUC_{(0-\infty)}$: area under the concentration-time curve from time 0 to infinity.

^c C_{max} : maximum blood concentration.

^d T_{max} : time-to-peak concentration.

^e K_e : elimination constant.

^f $T_{1/2}$: half-life of elimination.

^g f_{rel} : relative bioavailability.

ratio for PQ measurement. An SPE procedure was developed that efficiently removed proteinacious material from PQ and concentrated the drug. The method validation demonstrated very good linearity, specificity, precision, accuracy and stability of PQ over the calibration range studied. The extraction recovery of PQ from dog plasma was close to 100%. The method was successfully applied to the determination of plasma PQ concentrations in the dogs following administration of the two formulations of PQ: IRT and ERT.

The new formulation (ERT) containing 60 mg of PQ produced significantly lower C_{max} and higher T_{max} values of PQ in the dogs compared with the commercialized IRT. These findings indicate a much slower rate of PQ absorption after ERT dosing. ERT administration also resulted in a substantial increase in the total PQ exposure and a considerably longer $T_{1/2}$ value compared with IRT. At 20 hours after ERT administration, the mean plasma PQ concentration had decreased to 80 ng/ml, and

after 48 hours only traces of PQ were detected. In contrast to ERT, the mean plasma PQ concentration had decreased to 40 ng/ml at 5 hours after IRT dosing. Compared with IRT, the bioavailability of PQ was 2.2 times higher with ERT, which demonstrates an enhancement in the extent of PQ absorption with the extended-release formulation.

Currently, the recommended regimen for the treatment of *P. vivax* malaria is 30 mg daily for 14 days, particularly in geographical areas where PQ-tolerant *P. vivax* is present (WHO, 2010). Compliance with a 14-day regimen can be problematic for individuals because of side effects and/or unwillingness to complete the course. In an effort to enhance compliance, shorter regimens of higher doses of PQ (60 mg daily for 7 days) have been recently evaluated, with good tolerability and efficacy outcomes (Krudsood *et al.*, 2008; Pukrittayakamee *et al.*, 2010). It is well recognized that drug release at a constant rate is highly desirable for maintaining blood drug concentrations in the therapeutic range, thereby avoiding the peak and valley profile characteristics of conventional dosage forms in a multidose regime (Sun *et al.*, 2003; Hoffman, 2008). The lower maximum plasma concentration, greater exposure and longer elimination half-life of PQ using ERT may be highly advantageous given the potential to reduce the duration of the treatment while maintaining the efficacy.

Some of the advantages of the ERT formulation of PQ demonstrated in the present study can be compared to the PQ analog tafenoquine® (GlaxoSmithKline), which is currently undergoing phase III clinical evaluations. In humans, tafenoquine has a much longer elimination half-life (around 14 days) than PQ and thus it requires a shorter administration regimen for the radical cure of vivax malaria (Walsh *et al.*, 2004) and weekly dosing for chemoprophylaxis (Shanks *et al.*, 2001; Nasveld *et al.* 2010). Although tafenoquine is a promising new antimalarial drug, it is

still contraindicated in individuals with G6PD deficiency.

In conclusion, the results of this study demonstrate that ERT containing PQ does provide advantages over IRT, with higher bioavailability and a longer elimination half-life. The favourable pharmacokinetics of PQ could lead to improve compliance and efficacy. Further studies with the ERT formulation are warranted to determine whether the improved pharmacokinetic properties of PQ result in improved efficacy and reduced toxicity.

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