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Analysis and pharmacokinetics of bulaquine and its major metabolite primaquine in rabbits using an LC-UV method — a pilot study☆

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Abstract

A precise and reproducible HPLC assay has been developed and validated for simultaneous determination of bulaquine (BQ) and its metabolite primaquine (PQ) in rabbit plasma. The method, applicable to 0.5 ml plasma, involves double extraction of samples with *n*-hexane: isopropanol (98:2, v/v) containing dimethyl octylamine (DMOA) (0.1%, v/v). Separations were accomplished by reversed-phase liquid chromatography using a Spheri-5 cyano column with a low pressure gradient with mobile phase consisting of ammonium acetate buffer (50 mM, pH 6.0) and acetonitrile with DMOA. The method was sensitive with a limit of quantitation of 20 ng ml^{-1} in rabbit plasma for both BQ and PQ and the recoveries were >85 and $>45\%$, respectively. Excellent linear relationships ($r > 0.99$) were obtained between the measured and added concentration ratios of the plasma concentrations over a range of 20–1000 ng ml^{-1} for both the analytes. Precision and accuracy were acceptable as indicated by relative standard deviations from 1.8 to 15.1%, bias values ranging from -14.2 to 15.7%. Moreover, BQ was stable in rabbit plasma for 15 days of storage at -60 °C and after being subjected to three freeze-thaw cycles. The method was applied to determine the levels and pharmacokinetics of BQ in rabbits following a single 2.5 mg kg^{-1} oral and intravenous dose. The BQ levels declined and the PQ levels increased with time. The PQ/BQ ratio after oral dose at 1 and 1.5 h were higher than that after intravenous dose. In the pilot preclinical pharmacokinetic study after a single 2.5 mg kg^{-1} oral dose, BQ levels were determined up to 6 h (post-prandial) and 8 h (fasting). The plasma concentration versus time data were best fitted to a two-compartment open model with first-order absorption and elimination processes without lag time. The $AUC_{0-\infty}$ and the elimination $t_{\frac{1}{2}}$ in fasted rabbit was higher than that in post-prandial rabbit indicating the effect of food on BQ pharmacokinetics.

Keywords: Reversed-phase chromatography; Bulaquine; Primaquine; Antimalarial; Plasma; Rabbit; Pharmacokinetics

1. Introduction

Bulaquine (**I**), 3-[1-[4-[(6-methoxy-8-quinolinyl) amino] pentyl amino] ethylidene] dihydro-2(3H) furanone, (Fig. 1), an analogue of primaquine (**II**),

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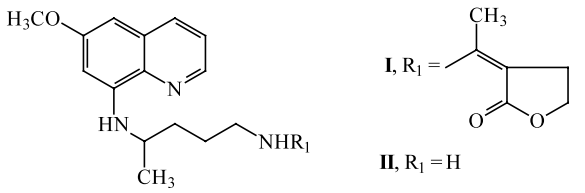

Fig. 1. Chemical structure of BQ (I) and PQ (II).

is a potent antimalarial agent developed at the Central Drug Research Institute, Lucknow, India. It is safer than primaquine (PQ) and causes only 1/3rd of methemoglobinemia [1–3]. It has shown curative and causal prophylactic activities against sporozoite-induced *Plasmodium cyanomolgi* infection in rhesus monkeys and is also safe in subacute toxicity studies in rats and rhesus monkeys with no teratogenic action [4,5].

Two HPLC methods have been reported for determination of Bulaquine (BQ) in biological fluids [6,7]. The first method coupled with UV detection determines BQ with a quantitation limit of 25 ng ml^{-1} [6]. The method could not be reproduced under present laboratory conditions, as the resolution of BQ, PQ and the rabbit plasma impurities were highly dependent on RP-18 column of different makes. The second method selectively determined only BQ by using non-aqueous mobile phase and fluorescence detection [7]. PQ was not determined due to its non-fluorescent nature. Moreover, an increase in the proportion of water or methanol in the mobile phase decreased the peak response of BQ by fluorescence quenching. Therefore, there was a need to develop and validate a robust method for simultaneous determination of BQ and PQ in biological fluid for the pharmacokinetic studies.

The present paper reports the development and validation of a method for the simultaneous determination of BQ and PQ in rabbit plasma with a quantitation limit of 20 ng ml^{-1} for both the analytes. The method was applied to determine the levels of BQ and PQ following a single 2.5 mg kg^{-1} intravenous and oral dose of BQ in rabbits. Moreover, a pilot preclinical pharmacokinetic study was carried out in overnight fasted and post-prandial rabbits after a single 2.5 mg kg^{-1} oral dose.

2. Experimental

2.1. Materials

BQ (purity >99%) was provided by M/s Nicholas Piramal India Ltd, Mumbai and was used in the present study. Analytical grade ammonium acetate, ammonia solution and glacial acetic acid were procured from E. Merck (India), Mumbai. HPLC grade acetonitrile, *n*-hexane, and isopropanol were purchased from J.T. Baker, USA. Analytical grade propylene glycol and dipotassium hydrogen orthophosphate (K_2HPO_4) and orthophosphoric acid were purchased from Glaxo (India) Ltd, Mumbai. Dimethyl octylamine (DMOA) and primaquine diphosphate were bought from Aldrich (St. Louis). The internal standard (IS), 3-bromoprimaquine diphosphate, was a generous gift from Prof. James D. McChesney of the University of Mississippi, USA. Triple distilled water (TDW) obtained from an all Quartz glass distillation unit was used in the preparation of buffers and reagents. Heparin sodium for injection (Beparine®, 5000 IU ml^{-1}; Biological Evans Ltd, Hyderabad, India) was appropriately diluted with saline and was used to rinse the test tubes prior to blood collection for plasma.

Blood was collected from healthy male rabbits that received no drugs, in clean heparinized glass tubes and was centrifuged to separate plasma so as to generate a drug-free plasma pool.

2.2. Preparation of reagents and solutions

A 100 μg ml^{-1} stock solution of BQ was prepared by dissolving 5 mg of the compound in 50 ml acetonitrile containing DMOA (0.1%, v/v). Individual stock solutions containing 100 μg ml^{-1} of PQ and IS were prepared in TDW. Working dilutions of IS (20 and 50 μg ml^{-1}) were prepared in acetonitrile. The stock solutions of BQ and PQ were used to prepare mixed working standards (I–VII) containing 0.4, 0.8, 2, 4, 8, 20, and 40 μg ml^{-1} of both the analytes in acetonitrile. The analytical standards (50, 100, 250, 500, 1000, and 2500 ng ml^{-1}) were prepared by diluting 1.25 ml of each working standard (I–VI) and 500 μl of IS (50 μg ml^{-1}) with 10 ml of reconstitution solution

[50% acetonitrile in ammonium acetate buffer (50 mM, pH 7.0)]. These analytical standards were used to assess the HPLC system reproducibility and the recovery of BQ and PQ from spiked plasma samples.

Plasma calibration standards containing 20, 50, 100, 200, 500, and 1000 ng ml^{-1} of BQ and PQ were prepared individually by diluting 25 μl of the working standards (II–VII) to 1 ml with plasma. This method was used to prepare appropriate calibration standards in plasma in replicates. The calibration standards were prepared freshly on each day of analysis.

The solution formulation of BQ (5 mg ml^{-1}) was prepared by dissolving the weighed quantity of BQ in propylene glycol, ethyl alcohol and K_2HPO_4 buffer (20 mM, pH 7.5) in the ratio 35:30:35% (v/v), respectively. The formulation was checked for the stability of BQ (degradation, $<$ 1% within 4 h of preparation). To check the stability of BQ in the formulation, an aliquot after 0, 1, 2, and 4 h of preparation was appropriately diluted with reconstitution solution and analysed using the HPLC method described in Section 2.3. The concentration of BQ in these solutions was interpolated from the analytical standard curve.

2.3. Instrumentation

The HPLC system consisted of a pump (LC-10ATvp with SCL 10Avp system controller, Shimadzu, Japan) with flow control valve system (FCV-10ALvp) to pump the mobile phase [solvent A: 65% acetonitrile in ammonium acetate buffer (50 mM, pH 6.0 adjusted with glacial acetic acid) and solvent B: ammonium acetate buffer (50 mM, pH 6.0)] at a flow rate of 1 ml min^{-1}. A Model 7125i syringe loading injector (Rheodyne, USA) fitted with a fixed 50 μl loop was used to inject the samples. Separation was achieved on a Spheri-5 cyano column (5 μm, 220 × 4.6 mm, i.d.), coupled with a guard column packed with the same material (5 μm, 30 × 4.6 mm, i.d.) (Pierce Chemical Co., Rockford, IL). The optimum separation of BQ, PQ, and IS from the endogenous plasma component was achieved using the gradient elution. The gradient started with pump supplying solvents A and B in the ratio 55 and 45%, respectively. The concentration of the mobile phase component in solvent A increased linearly to 90% within 15 min followed by stabilization for 2 min and then decreased to 55% by 2 min. The eluants were monitored using a SPD-10Avp UV–Vis detector set at 261 nm and the chromatograms were integrated using Class-VP software (Shimadzu, Japan). The HPLC system was equilibrated for approximately 30 min at a flow rate of 1 ml min^{-1} before analysis commenced. A vortex-mixer (Cecon, India), ultrasonic bath (Bransonic, Shelto, CY), a Model SVC-220H speed vac concentrator (Savant, NY) and a Model C-30 centrifuge (Remi, India) were used for sample preparations.

2.4. Sample preparation

A simple and efficient liquid–liquid extraction with 2 × 3.0 ml of extraction solvent [*n*-hexane: isopropanol, 98:02% (v/v) plus DMOA (0.1%, v/v)] was used to isolate the analytes from plasma. Aliquots of blank, spiked or test plasma (0.5 ml) in a 10-ml glass tubes was basified with 50 μl of potassium hydroxide (1 M). After adding 25 μl of IS (20 μg ml^{-1}), it was vortex-mixed with 3.0 ml of extraction solvent for 1 min and centrifuged at $1000 \times g$ for 10 min. The organic layer was transferred to another tube by snap freezing the aqueous layer in liquid nitrogen. The aqueous phase after thawing was again extracted with 3.0 ml extraction solvent and the combined organic phases (6 ml) were evaporated to dryness under reduced pressure in speed vac concentrator. The dry residue was reconstituted in 200 μl reconstitution solution, centrifuged and the clear supernatant ($\sim$150 μl) was injected onto the HPLC system.

2.5. Assay validation

A validation protocol was prepared and all the criteria commonly employed during the validation of the HPLC methods were assessed. The method was validated for 3 days in terms of HPLC system reproducibility, linearity, recovery, accuracy and precision, and stability studies in spiked plasma

samples stored at −60 °C and during freeze-thaw cycles.

The detection limit of the assay method (LOD) for BQ and PQ was defined as the drug quantity in the plasma after the sample clean up method that corresponds to three times the baseline noise (S/N > 3). The limit of quantification (LOQ) was defined as the concentration of the sample quantified with < 20% variation.

To determine the HPLC system reproducibility, pentaplet injections of each analytical standard were given and the coefficient of variation (CV) was determined from the following equation:

$$\%\mathrm{CV} = \frac{\mathrm{SD}}{\mathrm{Mean}} \times 100$$

Linearity for calibration standards ($n = 6$) in triplicates for 3 days was assessed by subjecting the spiked concentrations and the respective peak area ratios to least-square linear regression analysis with and without intercepts, and a weighted least-square regression ($1/x$ or $1/x^2$). A proper calibration model was chosen after examination of residuals and coefficient of correlation in each case. For calculation of the recoveries of BQ and PQ, spiked quality control (QC) samples were prepared at low (20 ng ml^{-1}), medium (200 ng ml^{-1}), and high (1000 ng ml^{-1}) concentrations. The samples were processed as mentioned above and the concentration of BQ and PQ were determined from the regression of the analytical standard curve. The recovery was calculated by comparing the observed concentration with the spiked concentrations.

For determination of the accuracy and precision, calibration standards and QC samples at low medium and high concentration were analysed in triplicate for 3 different days ($n = 3 \times 3 \times 3 = 27$). Intra- and inter-batch accuracy was determined by calculating the %bias from the theoretical concentration using the following equation:

$$\%\mathrm{Bias} = \frac{\text{Observed concentration} - \text{Nominal concentration}}{\text{Nominal concentration}} \times 100$$

Inter- and intra-batch precision in terms of relative standard deviation (%R.S.D.) was obtained by subjecting the data to one way analysis of variance.

To assess the freeze-thaw assay stability of BQ, the QC samples at low, medium, and high concentrations in triplicate for 4 different days were prepared. One set comprising of triplicate samples at each concentration level was assayed on the day of preparation (no freeze-thaw cycle). The remaining three sets were stored frozen at −60 °C and analysed after one, two, and three freeze-thaw cycles. Thawing was achieved by keeping the sample tubes unassisted at ambient temperature for 30 min. The results obtained after analysis on day of preparation were taken as standard (100%) for both the freeze-thaw and storage stability at −60 °C and the subsequent results were compared with the standard and expressed as percent deviation. For the stability of BQ in the spiked plasma samples stored at −60 °C, the QC samples at low, medium, and high concentrations in triplicate for 4 different days were prepared and stored at −60 °C. These set of samples were analysed after 1, 3, 7, and 15 days of storage and their concentrations read from the respective calibration standard curve on that day. Data was analysed as described for the freeze-thaw cycle stability and the results expressed as percent deviation.

2.6. Determination of levels of bulaquine and primaquine in rabbits

A preclinical study was carried out in male rabbits ($n = 3$) in a crossover design after oral and intravenous administration. The rabbits (2.5–3.25 kg) were obtained from the Laboratory Animal division of the institute. They were acclimatized in the animal room of the division at least a day prior to the commencement of the study and were maintained on standard food provided by the

Laboratory Animal division. All the experiments were carried out according to the guidelines specified by the local ethical committee for animal experiments. The freshly prepared formulation of BQ was administered to the rabbits and food and water was provided after 2 h of oral dosing. For oral dosing, each rabbit was placed in a rabbit restrained cage and the solution formulation was administered (0.5 ml kg^{-1}) orally using a catheter (40 cm) followed by flushing the catheter with 3.0 ml vehicle. Blood samples (1–2 ml) were withdrawn from the marginal ear vein at 0 (predose), 0.50, 1, and 1.5 h using a 21G needle in a clean and heparinized test tube. For intravenous administration, each rabbit was placed in a rabbit restrained cage, the marginal ear vein was dilated with xylene and the solution formulation was administered (0.5 ml kg^{-1}) using a 26G needle. Heparinized blood samples (1–2 ml) were withdrawn from the marginal ear vein at 0, 0.50, 1, and 1.5 h, plasma was harvested and stored at −60 °C pending analysis. The plasma samples were assayed within 15 days of collection.

2.7. Pharmacokinetic study of bulaquine in rabbits

Healthy male rabbits were administered a single 2.5 mg kg^{-1} oral dose after an overnight fast and post-prandially ($n = 1$ each). Heparinized blood samples were collected at 0, 0.08, 0.25, 0.50, 0.75, 1, 1.5, 2, 3, 4, 6, 8, and 24 h post dose. The plasma was harvested and stored at −60 °C pending analysis. The concentration-time data were subjected to both non-compartmental and compartmental approaches using WIN-NONLIN computer software [8]. The terminal half-life ($t_{\frac{1}{2}}$) was calculated from the ratio 0.693/β, where β is the rate constant for terminal elimination phase. The clearance (Cl/F) and the volume of distribution (Vd/F) were calculated from Cl/F = Dose/AUC and Vd/F = Cl/β, respectively, where F is the fraction of the administered dose absorbed. The area under the concentration-time curve (AUC) was calculated by the trapezoidal method, with extrapolation from the last observed concentration using the relationship C(last)/β [9].

3. Results

3.1. Chromatography

Symmetrical and well resolved peaks were obtained for BQ, PQ and IS. Typical chromatograms of an analytical standard containing BQ and PQ (each 250 ng ml^{-1}) and IS (2500 ng ml^{-1}), drug-free rabbit plasma, rabbit plasma containing BQ and PQ (each 500 ng ml^{-1}) and IS (1000 ng ml^{-1}), rabbit plasma taken just prior to dosing (0 h) and 0.5 h after 2.5 mg kg^{-1} oral dose are depicted in Fig. 2. The extraction procedure and the chromatographic conditions yielded a clean chromatogram for the analytes. The endogenous impurities did not interfere with the elution zone of any of the analytes. The retention times for BQ, PQ, and IS were 8.0±1.0, 11.0±0.75, and 13.5±0.50 min, respectively. The overall chromatographic run time was 24 min.

3.2. Validation

Initially, the HPLC system reproducibility was checked with pentaplet injections of each analytical standard in a single run. The variations in the peak area ratios of each standard with IS was maximal (BQ, 4.3%; PQ, 3.7%) at 0.05 μg ml^{-1} and was <2.6% at other concentration levels indicating that the system yields a reproducible

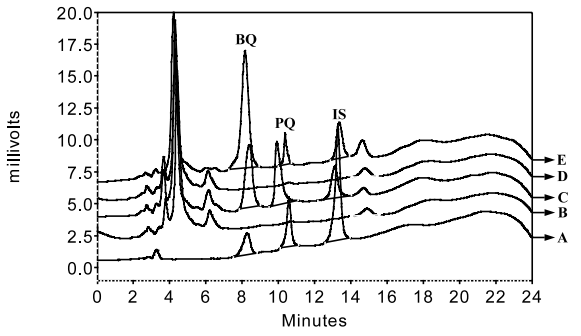

Fig. 2. Typical chromatograms of (A) an analytical standard containing 250 ng ml^{-1} BQ, PQ and 2500 ng ml^{-1} IS; (B) an extract of the drug-free rabbit plasma; (C) rabbit plasma containing 500 ng ml^{-1} BQ, PQ and 1000 ng ml^{-1} IS; (D) an extract of the rabbit plasma taken just before dosing (0 h); and (E) rabbit plasma sample taken 0.5 h after 2.5 mg kg^{-1} oral dose.

data. Moreover, the peak area ratios were linear with the concentrations. The total assay was validated (linearity, precision, and accuracy) by assaying 6 calibration standards and QC samples at low (20 ng ml^{-1}), medium (200 ng ml^{-1}), and high (1000 ng ml^{-1}) concentrations in triplicate on 3 different days. LOD for BQ and PQ were 10 ng ml^{-1} while LOQ for both the compounds were 20 ng ml^{-1}. Linearity of the method was confirmed over the concentration range of 20–1000 ng ml^{-1} using 0.5 ml plasma samples. A typical calibration curve for BQ and PQ had the regression equation of $Y = 0.001X - 0.007$ ($r = 0.9982$) and $Y = 0.003X + 0.015$ ($r = 0.9987$).

Absolute recoveries of BQ and PQ at the 3 concentrations in triplicate for 3 different days ranged from 88.2 to 95.8 and 47.5 to 55.4%, respectively (Table 1). The recovery of IS at the concentration used in the experimental procedure from extracted plasma was found to be 60.0 ± 5.6% ($n = 18$).

The intra- and inter-batch accuracy and precision are presented in Table 2. An acceptance limit of ±20% was employed for the lowest concentration (20 ng ml^{-1}) and ±15% was applied for medium and high samples (200 and 1000 ng ml^{-1}) [10]. The observed intra- and inter-assay biases (13.0 and 15.7%, respectively) were maximal at 20 ng ml^{-1} and were < 12% at other concentrations. The R.S.D. for BQ and PQ were within ±16% at the three concentration levels. After three freeze-thaw cycles, the %deviation from the concentration observed on the day of spiking were −6.3, 4.2, and 6.5% for the low, medium and high concentrations, respectively (Table 3). At LQC, %deviation in calculated concentrations of BQ from plasma frozen for 15 days at −60 °C compared with the concentration observed on the day of spiking was 13.8% (Table 3).

3.3. Preclinical pharmacokinetics in rabbits

Mean ± SD levels of BQ and PQ and the PQ/BQ ratios are summarized in Table 4. Following oral dose, the BQ levels after 1 and 1.5 h were lower than that after intravenous dose. The PQ levels at these time-points after oral dose were higher than that after intravenous dose (Table 4).

The plasma concentration-time data of BQ and PQ are presented in Figs. 3 and 4. The 5-min sample showed highest BQ and PQ concentration. The various pharmacokinetic parameters for BQ and PQ are listed in Table 5. The extent of absorption ($AUC_{0-\infty}$, 2315 ng h ml^{-1}) and elimination ($t_{\frac{1}{2}}$, 1.90 h) in fasted rabbit was higher than the values ($AUC_{0-\infty}$, 658 ng h ml^{-1} and $t_{\frac{1}{2}}$, 1.08 h) in post-prandial rabbit.

4. Discussion

BQ is unstable in commonly used organic solvents and acidic conditions. Under these conditions, BQ is converted into PQ. The addition of DMOA to the solvents results in lesser conversion [6]. Therefore, the solutions of BQ were prepared in solvents containing DMOA (0.1%, v/v). Moreover, the analytes (BQ and PQ) were found to be stable after reconstitution for at least 8.0 h at 4 °C as the %CVs at all the concentration levels were less than 5%.

Development of the method was initiated with RP-18 column (100 × 4.6 mm, i.d.) and isocratic elution using a mobile phase containing phosphate

Table 1
Recovery of BQ and PQ

Theoretical concentration (ng ml^{-1})	BQ		PQ	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)
20	91.5	5.4	52.6	9.3
200	93.4	2.2	51.1	4.1
1000	91.9	3.5	52.4	4.6

Table 2
Accuracy and precision of BQ and PQ

Theoretical concentration (ng ml^{-1})	Accuracy (% bias)		Precision (% R.S.D.)	
	Intra-batch	Inter-batch	Intra-batch	Inter-batch
(I) BQ				
20	13.0	15.7	3.4	11.0
200	10.5	8.6	1.8	10.5
1000	2.3	5.4	3.0	11.6
(II) PQ				
20	−14.2	−13.6	7.6	15.1
200	5.6	7.1	3.1	11.5
1000	8.8	8.6	3.2	12.4

buffer. BQ eluted as a broad peak (t_R, >15 min) even with 80% acetonitrile in mobile phase and PQ and IS did not resolve. Therefore, phosphate buffer was replaced with ammonium acetate buffer which marginally improved the peak response of BQ while PQ and IS were still unresolved. This followed the use of RP-18 columns of different length and make (Pierce, Phenomenex and Discovery). For better resolution and sensitivity, varying molarities of buffer, acetonitrile composition and gradient conditions with RP-18 columns were tried which did not yielded any improvement. This prompted the change of column from RP-18 to cyano to see the workability of the method. The cyano columns of different makes (Pierce and Phenomenex) and isocratic elution with mobile phase containing ammonium acetate buffer showed good peak response for BQ but PQ and IS were still not resolved. Moreover, rabbit plasma endogenous components interfered with the elution of PQ and IS. Among the various modifications tried, the low pressure binary gradient described above and the use of a Spheri-5 cyano column (220 × 4.6 mm, 5 μm) resulted in optimal resolution and good peak responses with no endogenous interference in the elution zone of the analytes. Slight changes in mobile phase conditions (solvent A 65 ± 3% ACN), pH of the ammonium acetate buffer (6.0 ± 0.2), molarity (50 ± 5 mM) did not significantly affect the peak response, resolution and retention times of the analytes signifying the robustness of the current HPLC method. The method was found to be reproducible when tested on different HPLC systems and also by different users in our laboratory. The HPLC analysis showed that the extraction procedure and the chromatographic conditions yield clean chromatogram for the analytes in rabbit plasma. The extraction of the analytes was initially tried with diethyl ether (with 0.1% DMOA), which yielded endogenous impurities in the elution zone of analytes. As an alternative, hexane with 0.1% DMOA was employed as the extraction solvent. With this extraction solvent, the recovery of BQ was $\geqslant 90\%$ whereas the recoveries of PQ and IS was $\leqslant 15$–20%. There-

Table 3
Stability of BQ in spiked plasma samples

Theoretical concentration (ng ml^{-1})	Freeze-thaw (f-t) stability (% deviation)			Storage stability at −60 °C (% deviation) on day			
	f–t 1	f–t 2	f–t 3	1	3	7	15
20	−8.1	−9.5	−6.3	−8.1	6.6	8.2	13.8
200	−1.0	−0.6	4.2	−1.0	11.0	10.1	2.4
1000	−1.0	2.8	6.5	−1.0	10.8	8.4	1.0

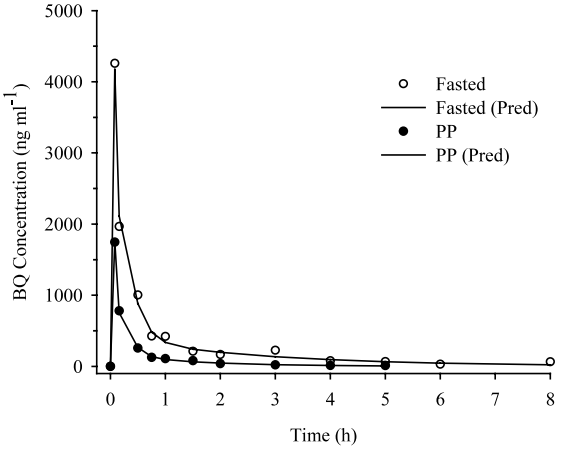

Fig. 3. Concentration-time profile of BQ after a single 2.5 mg kg^{-1} oral dose to an overnight fasted (○) and post-prandial (PP, ●) rabbit.

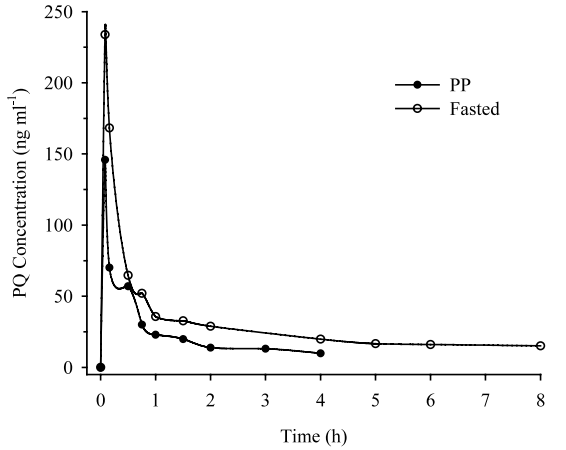

Fig. 4. Concentration-time profile of PQ after a single 2.5 mg kg^{-1} oral dose of BQ to an overnight fasted (○) and post-prandial (PP, ●) rabbit.

fore, it was decided to increase the polarity of this extraction solvent to enhance the recoveries of PQ and IS. Hence, extraction using various combinations of hexane: isopropanol and hexane: ethylacetate with DMOA were tried. The extraction with hexane: ethylacetate resulted in interference of endogenous impurities with the elution zone of the analytes. Thus, various combinations of hexane: isopropanol with 0.1% DMOA were tried of which 2% isopropanol in hexane with 0.1% DMOA gave best results. Single extraction with 3.0–5.0 ml of extraction solvent resulted in 70 and 30% recoveries for BQ and PQ, respectively. Therefore, double extraction with the same solvent system was carried out which gave enriched and consistent recoveries of the analytes. Moreover, the endogenous components of the rabbit plasma did not interfere with the elution of any of the analytes indicating that the method was selective (Fig. 2).

Table 4
Mean ± SD levels of BQ and PQ in rabbits after a single 2.5 mg kg^{-1} oral and intravenous dose in rabbits ($n = 3$)

Time (h)	Concentration (ng ml^{-1})		PQ/BQ (%)
	BQ	PQ	
(I) Oral			
0.5	1004.0 ± 85.9	92.1 ± 12.7	9.2 ± 0.6
1.0	421.2 ± 122.6	57.7 ± 11.6	14.0 ± 2.2
1.5	212.4 ± 33.5	51.4 ± 17.0	23.8 ± 5.2
(II) Intravenous			
0.5	981.1 ± 152.5	77.9 ± 59.4	8.8 ± 8.1
1.0	540.8 ± 119.5	52.0 ± 27.6	10.8 ± 8.4
1.5	407.6 ± 134.0	44.1 ± 16.1	12.7 ± 9.0

The linearity of the method was confirmed over the concentration range 20–1000 ng ml^{-1} with correlation coefficients exceeding 0.99, in addition to the visual examination of the calibration curves. Absolute recovery of BQ was $>85\%$ whereas that of PQ was $\sim 50\%$, therefore, the inclusion of an IS was deemed necessary.

The variations in the observed analyte concentrations for intra- and inter-assay accuracy (%bias) and precision (%R.S.D.) were within the acceptable limits of ±20% at lowest concentration and ±15% at all other concentrations (Table 2) [10].

During the stability study of BQ on storage at −60 °C, the day-to-day variation (%CV) was $\leqslant 9.44\%$ and changes (%deviation) from the nominal concentrations were within the acceptable limit ($\leqslant 15\%$) demonstrating that BQ was stable in plasma for at least 15 days (Table 3). After three freeze-thaw cycles, the %deviation from the concentration observed on day of spiking were within the acceptable limit of ±20% at low and ±15% at other concentrations indicating that the com-

Table 5
Pharmacokinetic parameters of BQ and PQ after a single 2.5 mg kg^{-1} oral administration of BQ to an overnight fasted (F) and post-prandial (PP) rabbit (each $n = 1$)

Parameters	BQ		PQ	
	Overnight fasted rabbit	Post-prandial rabbit	Overnight fasted rabbit	Post-prandial rabbit
K_{01} (h^{-1})	0.07	0.07	–	–
$AUC_{0-\infty}$ (ng h ml^{-1})	2315	658	334	138
MRT (h)	1.40	1.02	2.48	1.19
Elim. $t_{\frac{1}{2}}$ (h)	1.90	1.08	4.07	2.58
Vd/F (l kg^{-1})	0.43	0.93	–	–
Cl/F (l h^{-1} kg^{-1})	1.20	3.60	–	–
AUC_{PP}/AUC_F	0.28		0.41	
PQ/BQ Ratio			0.14	0.21

pound was stable in plasma through three freeze-thaw cycles. The variations in the stability studies represent both stability parameter and inherent intra-/inter-batch variations. The variations in the observed concentrations of BQ were comparable to intra-/inter-batch precision and within the acceptable limit indicating insignificant degradation of BQ. Moreover, no trend was observed in the read concentrations either on 15th day of storage at −60 °C or three freeze-thaw-assay cycles.

Using the present method, plasma levels of BQ and its metabolite PQ following intravenous and oral administration were determined. Following both oral and intravenous administration, the levels of BQ decreased and the levels of PQ increased with time (Table 4). The PQ-to-BQ plasma ratio (PQ/BQ) increased with time. The PQ/BQ ratio after the oral dose was higher than that after intravenous administration indicating higher metabolism of BQ to PQ after oral dosing.

Adequate levels of BQ after oral dose prompted us to conduct a pilot pharmacokinetic study in rabbit. Following oral administration in fasted and post-prandial rabbits, maximum plasma levels of both the analytes were observed at 5 min indicating rapid absorption of BQ and simultaneously its conversion into PQ (Figs. 3 and 4). The BQ plasma levels were determined up to 8 h in fasted rabbit. Whereas, BQ levels were determined up to only 6 h in post-prandial rabbits. However, PQ levels were determined up to 8 and 5 h in fasted and post-prandial rabbits, respectively (Fig. 4). Plasma BQ concentration-time data was best fitted to a two-compartment open model with first-order absorption and elimination processes without lag time. The elimination half-life in fasted rabbit (1.90 h) was higher than that observed in post-prandial rabbit (1.08 h) but were comparable to those observed after 10 mg kg^{-1} intravenous dose in rabbits [7]. AUC in post-prandial rabbit was only 28% to that observed in fasted rabbit clearly indicating that the BQ absorption is effected in presence of food (Table 5). The Vd/F was larger than the blood volume of rabbits, indicating rapid uptake of the compound by quickly perfused organs such as the liver and kidney. Clearance was also high in both the cases (hepatic blood flow for rabbit, 0.02–0.06 l min^{-1} kg^{-1}) [11] indicating high extraction ratio across the eliminating organs. The preclinical pharmacokinetic study was completed in only one rabbit in each group and clearly indicates the effect of food on the BQ pharmaco-

kinetics. Therefore, further studies are needed to establish the same in larger number of animals.

5. Conclusions

An HPLC assay was developed and validated for the simultaneous determination of BQ and its metabolite PQ in rabbit plasma. The extraction procedure is easy. The method was shown to be accurate and reliable over a concentration range of 20–1000 ng ml^{-1} with an acceptable R.S.D. and bias. The method was employed to study the pharmacokinetics of BQ in rabbits after oral dosing. BQ showed elimination half-life >1.5 h and showed effect of food on its pharmacokinetics. Further studies are needed to confirm the findings.

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References

[1] B. Bhat, M. Seth, A.P. Bhaduri, Prog. Drug Res. 28 (1984) 197–231.
[2] B. Bhat, A.P. Bhaduri, Indian J. Chem. 24B (1985) 419–423.
[3] S.K. Puri, R. Srivastava, V.C. Pandey, N. Sethi, G.P. Gupta, Am. J. Trop. Med. Hyg. 41 (1989) 638–642.
[4] G.P. Dutta, S.K. Puri, A.P. Bhaduri, M. Seth, Am. J. Trop. Med. Hyg. 41 (1989) 635–637.
[5] N. Sethi, S. Srivastava, R.K. Singh, S.K. Puri, Indian J. Parasitol. 17 (1998) 15–28.
[6] J.K. Paliwal, R.C. Gupta, P.K. Grover, J. Chromatogr. B. 616 (1993) 156–160.
[7] J.K. Paliwal, R.C. Gupta, J. Pharm. Biomed. Anal. 17 (1998) 775–783.
[8] WINNONLIN (version 1.5). SCI Software, Lexington, KY, USA.
[9] M. Gibaldi, D. Perrier, Pharmacokinetics, second ed., Marcel Dekker, New York, 1982, pp. 409–417.
[10] R. DeAngelis, J.J. Hubbel, L. Kanics, et al., Burroughs Wellcome, Research Triangle Park, NC (NCIC/91/001) (1992) 1–49.
[11] B. Davis, T. Moris, Pharm. Res. 10 (1993) 1093–1095.