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Liquid chromatography–tandem mass spectrometric assay with a novel method of quantitation for the simultaneous determination of bulaquine and its metabolite, primaquine, in monkey plasma

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Abstract

A sensitive and selective liquid chromatography–tandem mass spectrometry method (LC–MS–MS) for the simultaneous estimation of bulaquine and primaquine has been developed and validated in monkey plasma. The mobile phase consisted of acetonitrile/ammonium acetate buffer (20 m*M*, pH 6) (50:50 v/v) at a flow-rate of 1 ml/min. The chromatographic separations were achieved on two spheri cyano columns (5 μm, 30×4.6 mm I.D.) connected in series. The quantitation was carried out using a Micromass LC–MS–MS with an electrospray source in the multiple reaction monitoring (MRM) mode. The analytes were quantified from the summed total ion value of their two most intense molecular transitions. This is another novel method leading to increased sensitivity and precision. A simple liquid–liquid extraction with 2×1.0 ml *n*-hexane/ethyl acetate/dimethyloctyl amine (90:10:0.05, v/v) was utilized. The method was validated in terms of recovery, linearity, accuracy and precision (within- and between-assay variation). The recoveries from spiked control samples were ⩾90 and 50% for bulaquine and primaquine, respectively. Linearity in plasma was observed over a dynamic range of 1.56–400 and 3.91–1000 ng/ml for bulaquine and primaquine, respectively.

Keywords: Bulaquine; Primaquine

1. Introduction

Malaria is one of the major infectious diseases in the world today despite years of efforts to eradicate it and subsequently to reduce its impact on mortality and morbidity. Of the known 8-amino quinolines, only primaquine (PQ) (Fig. 1) is in clinical use as an anti-relapse drug against *Plasmodium vivax* [1]. It destroys pre erythrocytic stage of the parasite but suffers from the disadvantage of causing methemoglobinemia and oxidant induced hemolytic anemia, especially in individuals having G-6 phosphate dehydrogenase deficiency [2]. The demonstration by Schmidt and Genther in 1953 that a *P. cyanomolgi* infection in the rhesus monkey was the counterpart, both biologically and chemotherapeutically, of a *P.*

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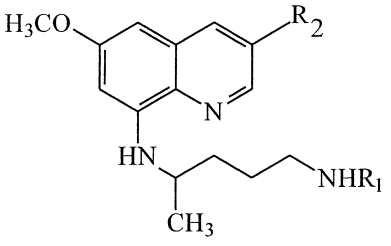


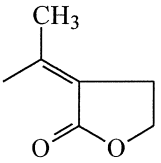
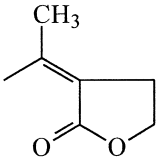
Compound	R_1	R_2
BQ		H
PQ	H	H
3-BBQ		Br
3-BPQ	H	Br

Fig. 1. Structures of BQ, PQ, 3-BBQ, and 3-BPQ.

vivax infection in humans had a profound impact on the development of the 8-amino quinolines. Bulaquine (BQ) 3-[1-[4-[(6-methoxy-8-quinolinyl) amino] pentylamino] ethylidene] dihydro-2 (3H) furanone (Fig. 1), an analogue of PQ, is a potent anti malarial discovered by the Central Drug Research Institute (CDRI). It is a weakly basic chemical entity, safer than PQ and causes only one-third of methemoglobinemia [3–5].

It was deemed necessary to develop an assay method for simultaneous quantitative estimation of BQ and PQ in biological fluids to generate pharmacokinetic data for BQ. Two analytical methods that have been published from this lab of which first utilizes high performance liquid chromatography–Ultraviolet (HPLC–UV) analysis and detects BQ and PQ with a quantitation limit of 25 and 10 ng/ml, respectively [6]. The second method involved HPLC–fluorimetry, which selectively determined only BQ by using non-aqueous mobile phase and fluorescence detection while PQ could not be determined due to its non-fluorescent nature [7]. Mass spectrometry (MS) is renowned for its sensitivity and selectivity, which presents several advantages in comparison to traditional HPLC [8]. The development of the atmospheric pressure ionization (API) source is an important breakthrough, and the use of liquid chromatography–tandem mass spectometry (LC–MS–MS) with an electrospray ionisation (ESI)/atmospheric pressure chemical ionization (APCI) interface is the technique that is currently considered the method of choice to perform pharmacokinetic studies [9–13].

This paper presents for the first time the development and validation of an assay method with a novel method of quantitation for the simultaneous estimation of BQ and PQ by LC–MS–MS using ESI in the positive ion mode.

2. Experimental

2.1. Chemicals and materials

Pure reference standard of BQ (99% pure) was obtained from the Pharmaceutics Division of CDRI, Lucknow, India. PQ diphosphate and dimethyl octylamine (DMOA) were procured from Aldrich chemicals (USA). 3-Bromo primaquine diphosphate (3-BPQ) was obtained as a generous gift from Prof. James D. McChesney of the University of Mississippi, USA and 3-bromo bulaquine (3-BBQ) was obtained from the Chemical Technology Division of CDRI. Hexane and ethyl acetate, HPLC grade, UltimAR™, were obtained from Mallinckrodt Baker Inc. Paris, KY, USA. Acetonitrile (ACN, HPLC grade), ammonia solution [25% (91% pure)] and ammonium acetate GR grade were obtained from E Merck Ltd, Mumbai, India. Analytical grade propylene glycol and di-potassium hydrogen orthophosphate (K_2HPO_4) were purchased from Glaxo Ltd., Mumbai, India. Iso propyl alcohol (IPA, HPLC grade) was procured from Thomas Baker (Chemicals) Limited, Mumbai, India. Purified water was obtained from $MilliQ_{PLUS}$ system. Drug-free heparinized plasma was obtained from young, healthy male rhesus monkeys housed in the Laboratory Animal Services Division of CDRI. All ethical guidelines for

maintenance and experimental studies with rhesus monkeys were followed.

2.2. Liquid chromatography

A Jasco PU980 intelligent pump was used to deliver a premixed mobile phase composed of ACN/20 m*M* ammonium acetate buffer, pH 6 (50:50 v/v), at a flow-rate of 1 ml/min. The mobile phase was degassed for 20 min in an ultrasonic bath (Bransonic Cleaning equipment Company, Shelton, USA) prior to the analysis. Chromatographic separations were achieved on two spheri cyano columns of 30 mm× 4.6 mm I.D., 5-μm particle size, connected in series using a stainless steel connector with zero dead volume. The samples were injected through a manual injector (Rheodyne model No. 7125 Cotati, USA) fitted with a 20-μl loop. Automated data acquisition was triggered using contact closure signals of the manual injector. The total effluent from the column was split such that one-tenth was injected onto the ESI.

2.3. Mass spectrometry analysis

The Micromass Quattro II (Micromass, Manchester, UK) Triple Quadrupole Mass Spectrometer was operated using a standard ESI source (Micromass, Manchester, UK). Data acquisition and analysis were performed using Mass Lynx version 3.3 software.

For optimization of MS parameters, approximately equimolar solutions of each analyte were prepared in the HPLC mobile phase. Nitrogen was used as both nebulizing gas (10 l/h) and as curtain gas (250 l/h). Cone voltages were optimized for each analyte by performing full scan acquisitions. The source temperature was set at 80 °C, and the ESI capillary at 3.5 KV.

LC–selective ion recording (SIR)-MS optimizations were performed by repetitive on column injections of the mixture of analytes using the isocratic HPLC method described above. The source temperature, cone voltages, capillary voltage, nebulizing and dry gas conditions were the same as described for the MS optimization. The dwell time and inter-channel delay were 0.8 and 0.02 s, respectively, with a span of 0.2 Da. SIR masses used for quantification were *m*/*z* 370, 260, 448 and 338 for BQ, PQ, 3-BBQ and 3-BPQ, respectively.

The optimized cone voltages for the respective analytes was set for the MS–MS experiments with argon as the collision gas at a pressure of 2.5×10^{-3} millibars. Collision energies (CE) for fragmentation of precursor to product ions were optimized by flow injection analysis, varying the CEs for each of the analytes to obtain the most intense precursor to product ion transitions. MS–MS acquisition was performed by setting the mass of the analytes with scan range of MS2 from 20 to 460.

For the optimization of LC–MRM-MS–MS, the source settings obtained above were utilized. The established operating conditions of MS in MRM mode are summarized in Table 1.

2.4. Standard and working solutions

Individual standard stock solutions of BQ (100 μg/ml) in ACN/DMOA (99.9:0.1, v/v) and its

Table 1
MRM conditions for BQ, PQ, 3-BPQ and 3-BBQ

Analyte	Retention time (min)	Function	Channel	Precursor ion (*m*/*z*) [M+H$^+$]	Product ion (*m*/*z*)	C.V. (V)	CE (eV)	Inter channel delay (s)	Dwell time (s)
BQ	1.7	MRM of 2 mass pairs	1	370	196	40			
			2	370	242				
PQ	4.5	MRM of 2 mass pairs	1	260	86	34			
			2	260	174		20	0.02	1.0
3-BPQ	6.1	MRM of 1 mass pair	1	338	86	32			
3-BBQ	2.0	MRM of 1 mass pair	1	448	196	30			

internal standard (I.S.), 3-BBQ (100 μg/ml in ACN), were prepared by accurately weighing the required amounts into separate volumetric flasks and dissolving in appropriate volumes of specified solvents. DMOA was found to be a necessary component in stock solution of BQ and in extraction solvent so as to prevent the conversion of BQ to PQ [6]. Stock solutions of PQ (100 μg/ml) and its I.S. 3-BPQ (100 μg/ml) were prepared in triple distilled water.

Mixed working stock (MWS) solutions of BQ and PQ, MWS 1 (BQ=5 μg/ml and PQ=10 μg/ml) and MWS 2 (BQ=16 μg/ml and PQ=40 μg/ml) were prepared by transferring appropriate volumes of stock solutions to a 10-ml volumetric flask and making up the volume with ACN. MWS 1 and MWS 2 were used in the preparation of analytical standards and calibration standards, respectively. Common working stock solutions of both I.S.s, CWIS 1 (3-BBQ=5 μg/ml and 3-BPQ=20 μg/ml) and CWIS 2 (3-BBQ=2 μg/ml and 3-BPQ=8 μg/ml) were prepared in ACN from their respective stock solutions by appropriate dilutions.

Analytical standards were prepared from MWS 1 by diluting it with reconstitution solution [ACN/ammonium acetate buffer, 20 m*M*, pH 7 (50:50 v/v)] to obtain a concentration range of 0.98–500 and 1.95–1000 ng/ml for BQ and PQ, respectively. CWIS 1 was spiked to each analytical standard to achieve a concentration of 250 ng/ml for 3-BBQ and 1000 ng/ml for 3-BPQ.

2.5. Calibration and quality control samples

Calibration standards were prepared from MWS 2 over a range of 1.56–400 and 3.91–1000 ng/ml for BQ and PQ, respectively, so that ACN content was ⩽2.5%. CWIS 2 was added to the plasma samples at time of extraction resulting in the same concentration of 3-BBQ and 3-BPQ as in analytical standards. Quality control (QC) samples at five different concentration levels [two low (L1, L2), two medium (M1, M2) and one high (H)] were prepared in triplicate once each day and were used to assess accuracy and precision of the assay method. All the calibration and QC samples were stored at −30 °C until analysis.

2.6. Sample preparation

Aliquots of 0.1 ml of plasma were placed in 5-ml tubes and basified with ammonia solution. CWIS 2 (12.5 μl) was added to these tubes and vortex mixed. Extraction solvent [1.0 ml; *n*-hexane/ethyl acetate/DMOA (90:10:0.05, v/v)] was added and each tube was vortex mixed for 1 min and then centrifuged at 1000 g for 5 min. The aqueous layer was frozen in liquid nitrogen and the organic layer was transferred to another tube. This was repeated and the combined organic layers were evaporated to dryness using Speed Vac Savant vacuum concentrator (Savant Instruments Inc., New York, USA). The residue was reconstituted in 150 μl of reconstitution solution and 20 μl was injected onto the LC–MS system. The calibration curve was obtained by weighted $1/x$ regression of the peak area ratios (BQ vs. 3-BBQ and PQ vs. 3-BPQ), versus nominal concentration using Microsoft Excel version 5.0.

2.7. Method validation

The validation of LC–MS–MS included within run and between run accuracy and precision determination on five different days and freeze–thaw effects. The accuracy and precision studies were carried out in triplicate. Both BQ and PQ gave two prominent product ion during MS–MS experiments (discussed in optimization of LC–MS–MS conditions). The sum of the responses obtained for the two intense transitions for both BQ and PQ were considered in method validation.

2.8. Specificity

The specificity was defined as non-interference in the regions of interest with the endogenous substances, in the determination of the concentration. Eight different lots of blank plasma were tested for interference or matrix effects.

2.9. Limit of detection (LOD) and lowest limit of quantitation (LLOQ)

The LOD of assay method for BQ and PQ was the drug quantity in plasma after sample clean-up corresponding to three times the baseline noise ($S/N>3$).

The LLOQ was defined as the concentration that was quantified with less than 20% variation in precision.

2.10. Accuracy and precision

The accuracy of each sample preparation was determined by injection of calibration samples and five QC samples in triplicate for five different days. The precision was determined by one-way ANOVA as within and between %RSD. The accuracy was expressed as %Bias:

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

2.11. Freeze thaw stability (f–t)

QC samples (0.1 ml) at low (Ll), medium (M2) and high (H) concentrations in duplicates were stored at −30 °C in glass tubes. One set of duplicate samples at each concentration was analyzed immediately after spiking, which served as the reference concentration. The other samples were analyzed after one, two and three f–t cycles. Thawing was achieved at ambient temperature for 30 min. The change in concentration during the f–t cycles was determined by comparing the concentrations after thawing with the reference concentration and was expressed as percent deviation from the reference concentration.

3. Results and discussion

3.1. Optimization of LC conditions

Liquid chromatography was performed on two-spheri cyano columns (30×4.6 mm, 5 μm) connected in series with the first one serving as a guard column for the next, hence providing longer lifespan for the stationary phase on repeated plasma injections. The final LC conditions were optimized to ACN/ammonium acetate buffer (20 m*M*, pH 6) (50:50 v/v) at 1.0 ml/min. Under these conditions a run time of 8 min with BQ, PQ and 3-BPQ eluting at 1.7, 4.5 and 6.1 min, respectively, could be achieved.

3.2. Optimization of LC–MS–MS conditions

ACN-ammonium acetate buffer that was found to be suitable during LC optimization was utilized to obtain electrospray response with 3-BPQ used as an I.S. for both BQ and PQ. In the positive ion mode, the protonated species $[M+H]^+$ at m/z 370, 260 and 338 were observed for BQ, PQ and 3-BPQ, respectively. The cone voltage optimization yielded best results at 40, 34 and 32 V for BQ, PQ and 3-BPQ, respectively. Initially, SIR mode was used but significant interference was observed in regions of interest in blank extracted plasma samples. Therefore, MRM was explored. Initially, product ions were generated through fragmentation of the molecular ions by collision activated dissociation (CAD), using argon as collision gas. CE and pressure of the collision gas were optimized at 20 eV and 2.5×10^{-3} millibars, respectively, to obtain the most intense product ions. The product ion spectra of $[M+H]^+$ ion of BQ, PQ and 3-BPQ showed intense fragments at 196, 86 and 86, respectively (Fig. 2). Initially, MRM conditions quantifying precursor to most intense fragment was used for calibration. The transitions monitored for BQ, PQ and 3-BPQ were 370>196, 260>86 and 338>86, respectively. However, the detection limits achieved could not detect sufficiently low concentrations. During MS–MS analysis a second product ion was prominent, but less intense compared to the product ions for BQ and PQ. The secondary product ions for BQ and PQ under the optimized conditions were m/z 242 and 174, respectively. Furthermore, the two transitions (BQ; 370>196 and 370>242, PQ; 260>174 and 260>242) were monitored in a single function and the sum of the responses (SUM) of the two individual transitions were considered for quantitation for both the analytes. This resulted in a twofold increase in sensitivity. The corresponding MRM conditions are summarized in Table 1.

Pilot calibration curves of BQ and PQ revealed that the peak area ratio of PQ with 3-BPQ varied linearly, while that of BQ gave a non-linear response with increasing number of injections. To overcome this effect I.S. with structure and fragmentation pattern closer to BQ were tested. 3-BBQ was found to exhibit a similar ionisation and fragmentation pattern as BQ and was therefore chosen as its I.S.

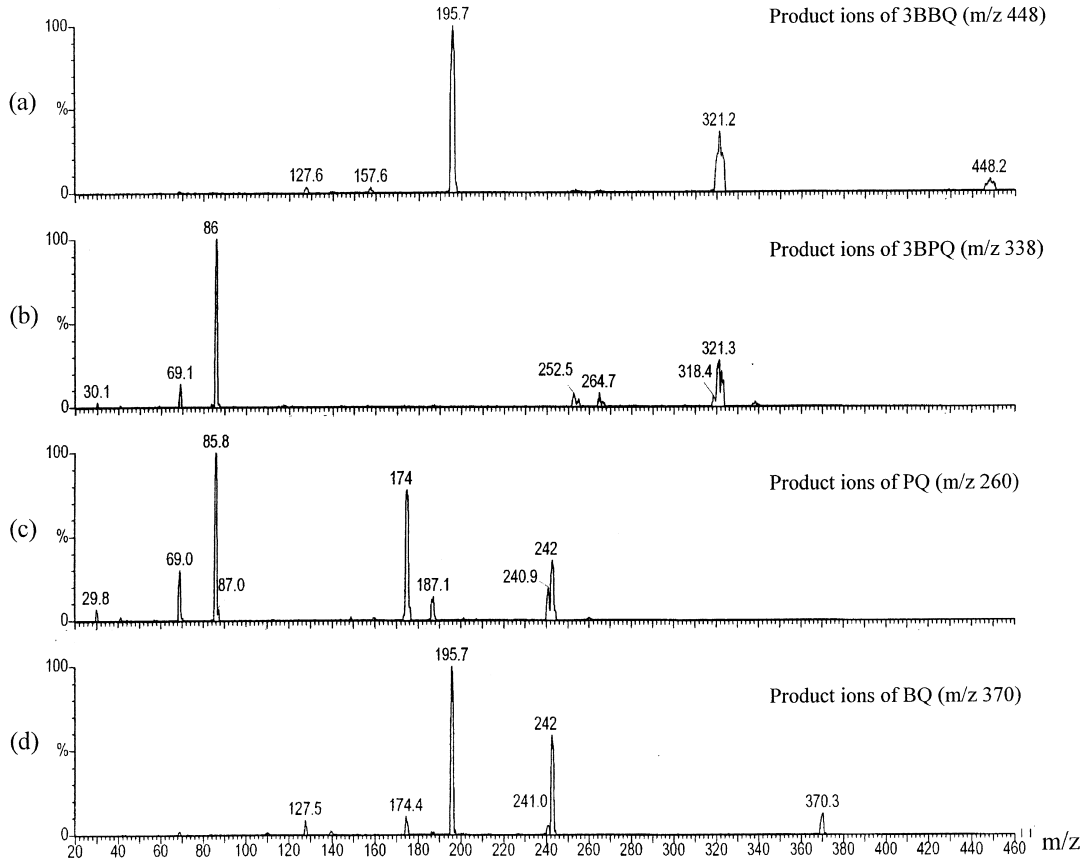


Fig. 2. MS–MS spectra of (a) 3-BBQ, (b) 3-BPQ, (c) PQ, (d) BQ, showing prominent precursor to product ion transitions.

MS–MS spectra of 3-BBQ are shown in Fig. 2 and the MRM conditions are summarized in Table 1.

3.3. Extraction and matrix suppression

MS, though very selective and sensitive may be adversely affected by ionization suppression due to other components present in the biomatrix. Initially, hexane with 0.05% DMOA was employed as extraction solvent. Although the recovery of BQ and 3-BBQ were above 90%, the recovery of PQ and 3-BPQ was only 15–20%. The extraction solvent was changed from pure hexane with 0.05% DMOA to hexane/IPA/DMOA (98:2:0.05, v/v). Recovery of PQ and 3-BPQ increased to 50–55% but there was significant suppression of ionization response of both BQ and 3-BBQ probably due to the impurities extracted by IPA. Ethyl acetate was tried and it was seen that hexane/ethyl acetate/DMOA (90:10:0.05, v/v) gave the best results. The recoveries of BQ and PQ were above 90 and 50%, respectively (Table 2). Matrix suppression of the analytes was minimized but loss in sensitivity was noticed after 15–20 plasma injections. To circumvent this effect it was decided to reconstitute the dry residue after extraction into 150 μl instead of 100 μl. This approach,

Table 2
Mean recoveries of BQ and PQ in spiked plasma

Conc. (ng/ml)		Absolute recovery (mean±SD, $n=5$) (%)	
BQ	PQ	BQ	PQ
1.56	3.91	100±15	60.8±5.5
3.13	7.81	103±11	57.0±4.0
12.5	31.3	100±5.4	58.6±6.2
50	125	101±4.5	56.5±4.0
400	1000	103±9.0	55.7±7.9

along with change in extraction solvent to ethyl acetate completely eliminated the matrix suppression problem.

3.4. Linearity and calibration standard range

The peak area ratios of BQ to 3-BBQ and PQ to 3-BPQ in plasma varied linearly with the concentration over the range 1.56–400 and 3.91–1000 ng/ml, respectively. The calibration model was selected based on the analysis of the data by linear regression with and without intercepts ($y=mx+c$ and $y=mx$) and weighting factors ($1/x$, $1/x^2$ and $1/\log x$). The best fit for the calibration curve could be achieved by a linear equation of $y=mx+c$ and a $1/x$ weighting factor.

3.5. Specificity

Although the extraction procedure was relatively simple, high specificity was achieved using tandem MS by monitoring the dissociation of the precursor ions to their respective product ions in individual channels of different functions. LC–MS–MS analysis of the eight lots of blank plasma samples showed no endogenous peaks interference with BQ, PQ and their respective I.S.s. Representative chromatograms of extracted blank plasma fortified with BQ, PQ, 3-BBQ, 3-BPQ overlaid with extracted blank plasma are shown in Figs. 3 and 4. Levels of BQ and PQ were detected following a single oral dose of 10 mg/kg in male rhesus monkeys. An oral liquid formulation of BQ was prepared in propylene glycol/ethanol/K_2HPO_4 buffer (20 m*M*, pH 7): 35:30:35, v/v. Typical chromatograms showing BQ and PQ in test samples at 15 min, 2 h and 6 h with their I.S. are shown in Fig. 5. This proves that the method was specific for spiked as well as test samples.

3.6. Sensitivity

When the method was validated using the SUM of both the transitions for BQ and PQ, the LOD determination demonstrated that all the analytes gave a signal-to-noise ratio of 3:1 and above for 0.5 ng/ml for BQ and 2 ng/ml for PQ. The LLOQ of 1.56 and 3.91 ng/ml for BQ and PQ, respectively, was established using 100 μl of plasma. On the other hand, when only the single transitions of BQ (370 > 196) and PQ (260 > 86) were monitored, the LLOQ was 3.13 and 7.81 ng/ml for BQ and PQ, respectively. Monitoring of only secondary transitions for BQ (370 > 242) and PQ (260 > 174) could quantify BQ and PQ with LLOQ of 6.25 and 15.62 ng/ml, respectively. Thus, there was an increase in the sensitivity for both BQ and PQ with summation of the two transitions employed for quantitation of the analytes.

3.7. Assay validation

3.7.1. Recovery

The average recoveries from monkey plasma for BQ over the calibrated range were 99–101% and for PQ were 55–60%. Mean recoveries of BQ and PQ at five QC levels are shown in Table 2.

3.7.2. Freeze–thaw (f–t) stability in monkey plasma

The deviation observed after one, two and three f–t cycles was within the acceptable limits of ±20% at LOQ and within ±15% at the other two concentration levels for BQ and PQ (Table 3) [14].

3.7.3. Accuracy and precision

The overall %Bias and RSD at the five concentrations is presented in Table 4. Utilizing SUM for quantification of BQ and PQ, the result shows that the bioanalytical method is accurate (±20% difference from nominal concentration at the LLOQ) and the precision is within the acceptance limits of ⩽20% at LLOQ and ⩽15% at all other concentration levels studied [14].

3.8. Bench top and dry residue stability

There was no significant difference between the responses of standards at time zero and after 12 h in terms of % C.V. (⩽5%) for both BQ and PQ, indicating the stability of BQ and PQ at room temperature for 12 h. Moreover, the analytes were found to be stable after reconstitution for at least 12 h

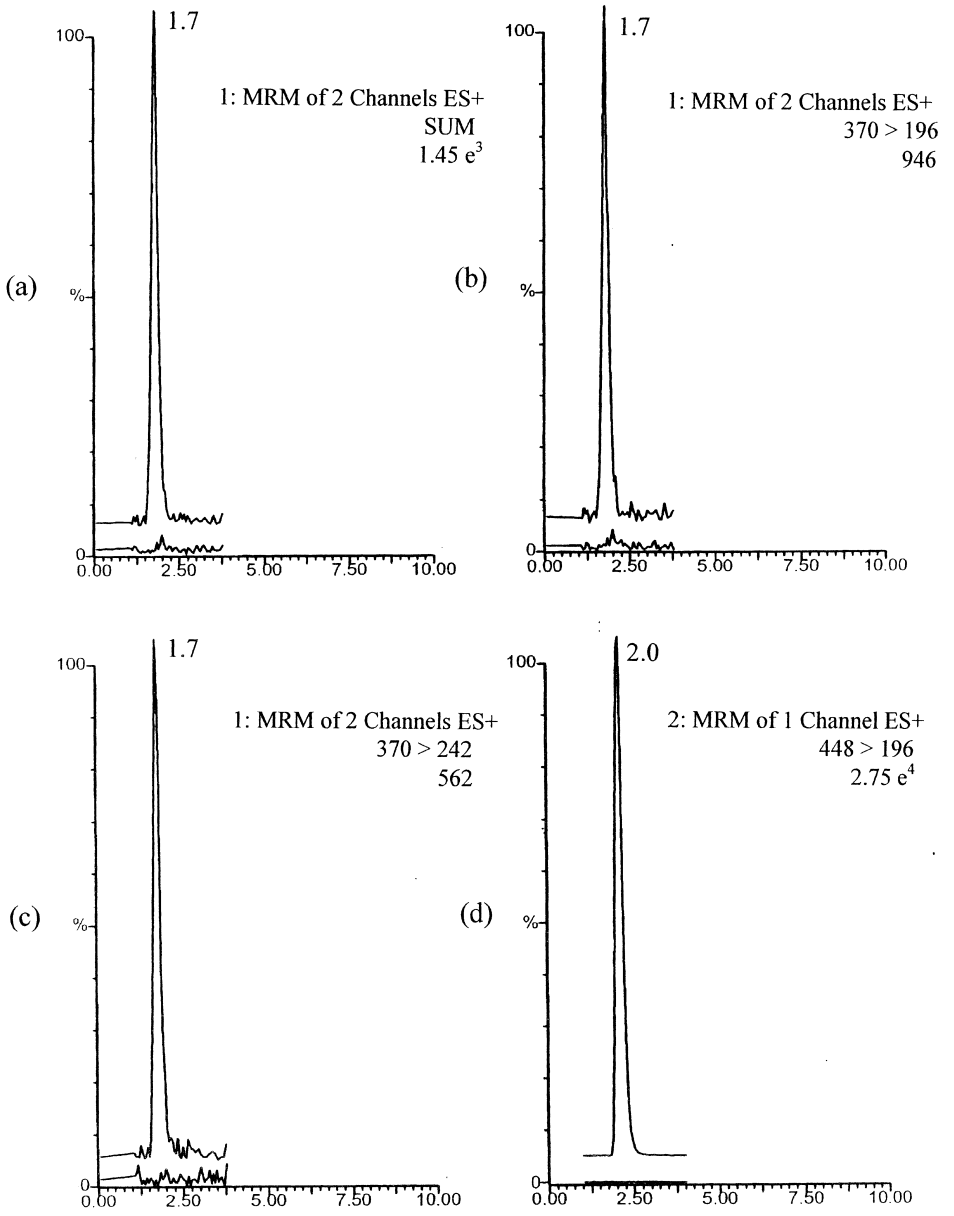


Fig. 3. Representative chromatograms of BQ (1.56 ng/ml) and 3-BBQ (250 ng/ml) in fortified blank plasma overlaid with extracted blank plasma. (a) SUM of both the transitions of BQ along with blank extracted plasma; (b) 370 to 196 transition along with blank extracted plasma; (c) 370 to 242 transition along with blank extracted plasma; (d) 448 to 196 transition for 3-BBQ along with blank extracted plasma.

at 4 °C as the % C.V. at all the five concentration levels was less than 5%. The dry residue was reconstituted just prior to injection on to LC–MS–MS. The dry residue stored at −30 °C after extraction was found to be stable for 7 days with %C.V.≤5% at all concentration levels.

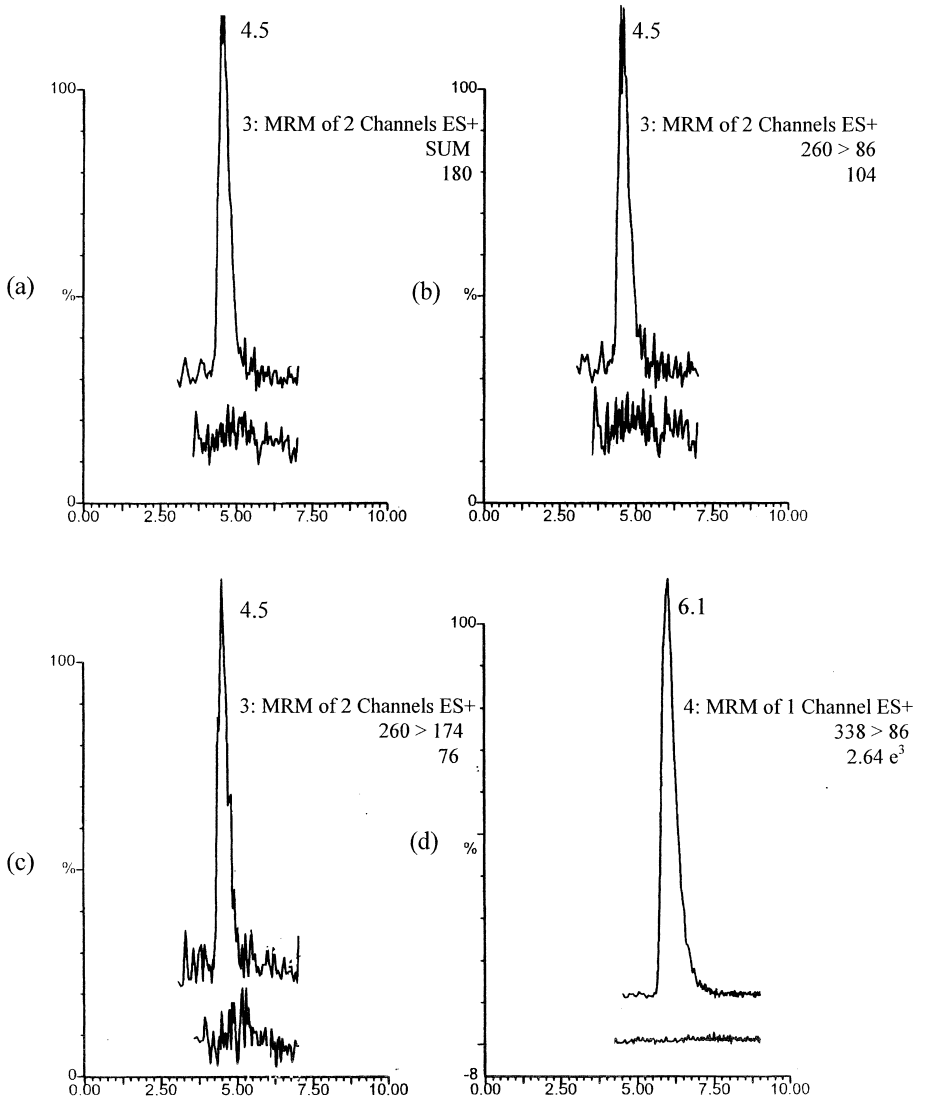


Fig. 4. Representative chromatograms of PQ (3.91 ng/ml) and 3-BPQ (1000 ng/ml) in fortified blank plasma overlaid with extracted blank plasma. (a) SUM of both the transitions of PQ along with blank extracted plasma; (b) 260 to 86 transition along with blank extracted plasma; (c) 260 to 174 transition along with blank extracted plasma; (d) 338 to 86 transition for 3-BPQ along with blank extracted plasma.

4. Conclusion

An LC–MS–MS bioanalytical method has been developed and validated in monkey plasma over a concentration range of 1.56–400 and 3.91–1000 ng/ml for BQ and PQ, respectively. Separate structural analogues were used as internal standards for each analyte to account for the variations due to matrix effect, extraction variability and instrument performance. The liquid–liquid extraction method gave good and consistent recoveries for BQ, PQ and their respective I.S.s from monkey plasma, with no de-

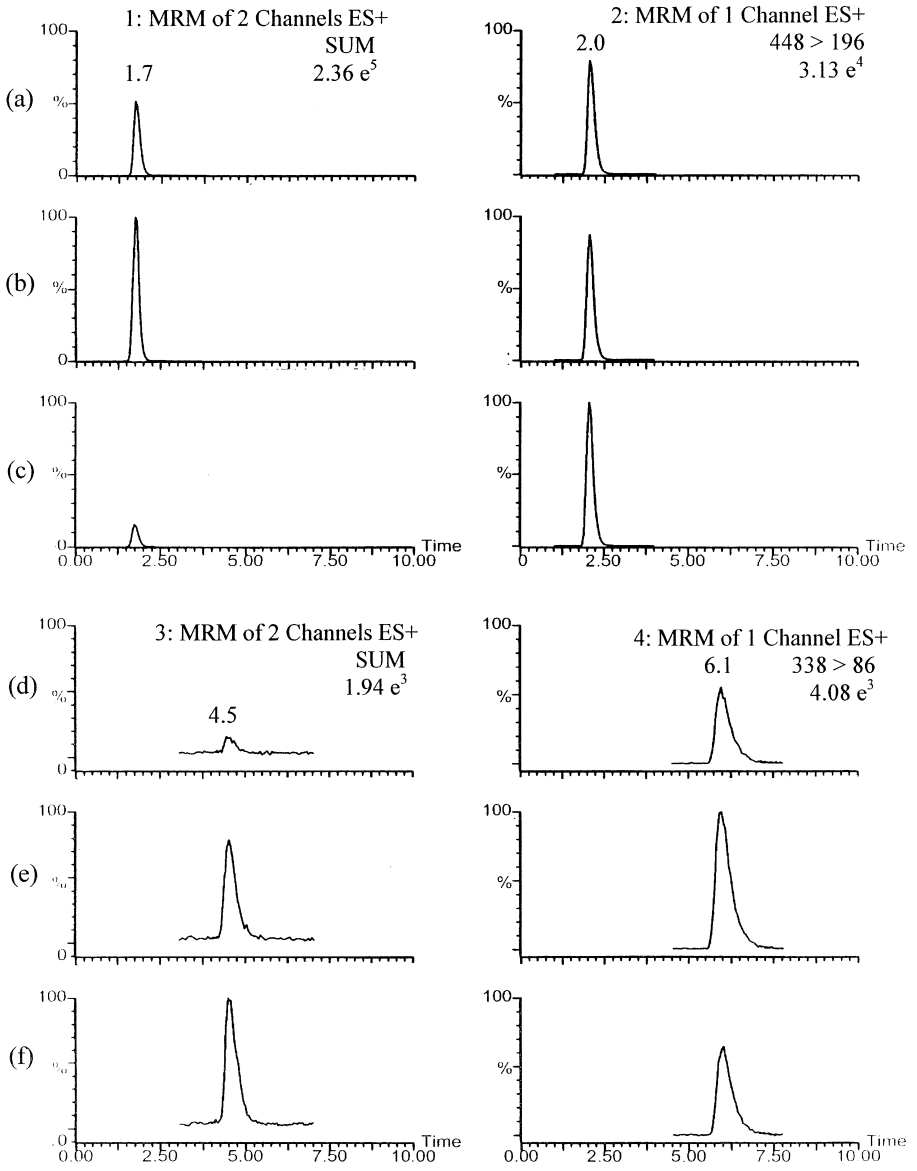


Fig. 5. Typical chromatograms showing BQ and PQ in test samples. (a–f) represent BQ and PQ in test samples after 0.25, 2.0 and 6.0 h, respectively. The concentrations of BQ in a, b and c are 154, 273 and 39.3 ng/ml while that for PQ in d, e and f are 16.5, 63.3 and 114 ng/ml, respectively.

tected interference. There was an increase in sensitivity by a factor of 16 and 2.5 for BQ and PQ, respectively, with an overall decrease in sample processing volumes by fivefold leading to higher sensitivity and throughput from the earlier HPLC–UV method [6]. Hence, the method is suitable for carrying out preclinical pharmacokinetic studies of BQ. It is also proposed that the novel method of

Table 3
Freeze–thaw (f–t) stability data for BQ and PQ

Nominal conc. (ng/ml)	f–t1	f–t2	f–t3
BQ			
1.56	−4.0	6.4	7.4
50	9.1	−1.8	7.9
400	1.5	1.0	0.1
PQ			
3.91	−19.9	18.6	14.8
125	−1.5	−14.0	−7.9
1000	1.3	−5.7	−5.8

quantitation utilized in this assay may lead to even more sensitivity in the case of compounds forming multiple product ions with similar intensities.

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Table 4
Accuracy (%Bias) and precision (%RSD) of BQ and PQ in plasma when SUM of the transitions for both the analytes were monitored

Analyte	Conc. (ng/ml)	% Bias		% RSD	
		Intra batch	Inter batch	Intra batch	Inter Batch
BQ	1.56	12.8	3.2	10.2	18.1
	3.13	7.8	4.8	3.0	14.0
	12.5	−1.1	−2.3	4.2	10.2
	50.0	−2.9	−0.5	2.7	12.8
	400	−1.5	−3.0	5.2	6.8
PQ	3.91	−0.2	2.0	2.8	7.5
	7.81	−3.3	−1.6	2.8	5.2
	31.3	−1.6	−3.2	3.9	7.5
	125	−2.9	1.2	4.4	14.4
	1000	−5.7	−7.7	4.6	12.4