Automated Solid-Phase Extraction Method for the Determination of Piperaquine in Plasma by Peak Compression Liquid Chromatography

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Abstract

A validated bioanalytical method for the determination of piperaquine (PQ) in plasma by solid-phase extraction (SPE) and liquid chromatography (LC) using peak compression is presented. Protein is precipitated from plasma with acetonitrile–1% aqueous acetic acid (85:15, v/v). An internal standard (IS) is added to the samples before they are loaded onto a strong cation exchanger (Isolute PRS) SPE column. PQ and the IS are analyzed by LC on a Zorbax SB-CN column (250 × 4.0 mm) with the mobile phase acetonitrile–phosphate buffer [I = 0.1, pH 2.5 (12:88, v/v)] and UV detection at 345 nm. Trichloroacetic acid (TCA) is added to the samples prior to injection into the chromatography system. PQ elutes in a gradient of TCA, which enables peak compression of PQ and significantly higher peak efficiency as a result. The intraassay precision for plasma is determined to be 5.4% at 3.00µM and 5.8% at 0.050µM. The interassay precision for plasma is 1.3% at 3.00µM and 10.0% at 0.050µM. The lower limit of quantitation and the limit of detection are 0.025 and 0.005µM, respectively.

Introduction

Piperaquine (PQ), 1,3-bis-[4-(7-chloroquinolyl-4)-piperazinyl-1]-propane, is a drug closely related to chloroquine (CQ) and other 4-aminoquinolines. PQ is an antimalarial drug that was synthesized and developed by Chinese scientists approximately 30 years ago. It has been used as an affordable alternative to CQ in CQ-resistant areas, but has not received much attention outside of China.

Because CQ is, today, largely ineffective because of resistance development, the wider use of a PQ base or PQ phosphate (PQP) in combination with other antimalarial drugs is under consideration within organizations such as the World Health Organization (WHO). Such combinations are already registered and deployed in P.R. China and Vietnam [e.g., Artekin (dihydroartemisinin, PQP, and trimethoprim) and CV8 (dihydroartemisinin, PQP, trimethoprim, and primaquine phosphate). A few field studies in China have been performed indicating that PQ would be an effective and safe antimalarial drug (1–4). Besides PQ, the analogues hydroxypiperaquine and hydroxypiperaquine phosphate have also been tested in field studies in China with good results (5,6). To the best of our knowledge, there are no published articles regarding the pharmacokinetics of PQ or any methods to quantitate PQ in biological fluids. Half-lives for PQ of approximately 9 days and t_{max} values of approximately 3 h have been reported in mice (7).

A bioanalytical method for the determination of PQ in plasma by solid-phase extraction (SPE) and liquid chromatography (LC) using peak compression, suitable for pharmacokinetic studies, has been developed and validated.

The assay has been validated with respect to accuracy, precision, linearity, selectivity, stability, and recovery according to published guidelines (8).

Experimental

Chemicals and materials

PQ was obtained from Guangzhou University of Traditional Chinese Medicine (Guangzhou, China). The internal standard (IS), 3-methyl-4-(3-hydroxy-4-diethylaminopropyl)-7-chloro-

Figure 1. Structures of PQ and the IS.
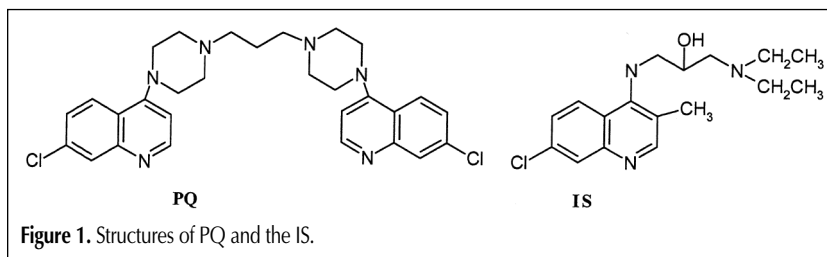
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quinoline, was obtained from Glaxo Wellcome (Hertfordshire, U.K.). The structures are shown in Figure 1. Acetonitrile (Chromasolv) and methanol (Chromasolv) were obtained from Riedel-de Haën (Seelze, Germany). Trichloroacetic acid (TCA) (pro analysi), triethylamine (for synthesis), ortho-phosphoric acid (pro analysi), sodium hydroxide (pro analysi), and 37% hydrochloric acid (pro analysi) were obtained from VWR International (Darmstadt, Germany).

A concentrated stock solution (0.61M) of TCA was prepared in deionized water.

Deionized water was obtained from a Milli-Q deionized water system (Millipore, Bedford, MA). The phosphate buffer solutions (I = 0.05 and 0.1) were prepared by mixing different amounts of sodium hydroxide and ortho-phosphoric acid with Milli-Q deionized water.

Instrumentation and chromatographic conditions

The LC system used in the assay consisted of an LKB 2150 LC pump (Pharmacia Biotech, Uppsala, Sweden) and a WISP 710B autoinjector (Waters, Milford, MA). The detector was a Spectroflow 757 absorbance detector (Kratos, Ramsey, NJ) set at 345 nm. Data acquisition was performed using Chromatography Station for Windows 1.7 (DataApex Ltd., Prague, The Czech Republic) on an AT&T Pentium 90-MHz personal computer (Dell Computer Corporation, Bracknell, Berkshire, U.K.). The mobile phase was acetonitrile–phosphate buffer [I = 0.1, pH 2.5 (12:88, v/v)] with a flow rate of 1.0 mL/min through a 5-µm, 250- × 4.6-mm i.d. SB-CN column (Zorbax, Wilmington, NC) protected by a 5-µm SecurityGuard C18 short guard column (Phenomenex, Torrance, CA).

SPE

Extraction was carried out on an automated ASPEC XL SPE system (Gilson, Middleton, WI) using PRS extraction columns containing propylsulfonic acid as support (1 mL, 100 mg) (IST Ltd., Hengoed, Glamorgan, U.K.). The ASPEC system used a positive air pressure instead of vacuum to get the liquids through the columns.

Preparation of calibration standards and samples for determination of precision

Concentrated stock solutions of PQ and the IS (500µM) were prepared in methanol–hydrochloric acid (99:1, v/v). The solutions were stored at approximately +4ºC and protected from light. Appropriate amounts of the stock solution of PQ were added to blank plasma to yield spiked calibration standards ranging from 0.025 to 5µM. The calibration standards were prepared in batches of 10 mL and stored in 0.5-mL aliquots at approximately –17ºC. Calibration curves were prepared using 8 different concentrations of PQ. The peak–height ratio of PQ to IS against concentrations of PQ with nonweighted linear regression was used for quantitation.

For the determination of precision in plasma, samples were prepared at three concentrations (0.050, 0.750, and 3.00µM) in batches of 20 mL

and stored in 0.5-mL aliquots at approximately –17ºC.

Samples for freeze–thaw stability were prepared at two concentrations (0.050 and 3.00µM) in batches of 10 mL and stored in 0.5-mL aliquots at approximately –17ºC.

The lower limit of quantitation (LOQ) was selected at the concentration at which the assay precision was within 20% and the signal-to-noise ratio exceeded 10:1. The limit of detection (LOD) was determined at the concentration at which the signal-to-noise ratio exceeded 3:1.

Sample preparation

To 500 µL plasma, 1000 µL acetonitrile–1% acetic acid (85:15, v/v) was added and the tube contents were mixed for 10 s. A volume of 500 µL of the IS [2µM in a phosphate buffer (pH 4, I = 0.05)] was added and the tube contents were mixed again for 10 s. The tubes were then centrifuged at 23,900 g for 10 min. The liquid phase was decanted into new tubes and then loaded onto an SPE column. The extraction procedure for plasma on the ASPEC XL is shown in Table I. The eluates were evaporated to dryness at 70ºC under a gentle stream of air and reconstituted in 250 µL acetonitrile–deionized water–TCA (5:93:2, v/v). A volume of 100 µL was injected into the LC system.

Influence of the pH on the absorbance

Some compounds have very pH-dependent absorbance. In order to achieve high sensitivity in the detector, it is important that both the pH of the mobile phase and the pH of the samples are chosen in which the compound has the highest absorbance. Spectrophotometric scans between 450 and 250 nm were made for PQ solutions (50µM) in the pH range of 2 to 12 in order to evaluate the pH effect on the absorbance.

Peak shape and efficiency improvements

The PQ peak exhibited a significant chromatographic tailing both on CN and C18 LC columns with bad peak efficiency as a consequence. Several attempts to improve the peak shape and

Table I. ASPEC SPE Procedure for the Extraction of Plasma

SPE step	Liquid dispensed	Dispensing volume (µL)	Dispensing flow rate (mL/min)	Pressuring air volume (µL)
Conditioning	Methanol	2000	3	0
	Acetonitrile–deionized water–1% aqueous acetic acid (42.5:50:7.5, v/v)	2000	3	200
Sample Loading	Plasma	1800*	0.5	500
Washing	Methanol–buffer†	2000	3	1000
Elution	Methanol–triethylamine (98:2, v/v)	650	0.5	0
	Methanol–triethylamine (98:2, v/v)	650	0.5	700

* The volume after protein precipitation is less than this volume. This is the volume that is in the program for ASPEC XL. This means that the whole liquid phase is loaded onto the SPE column.
† I = 0.1, pH 2 (80:20, v/v).

efficiency were made. Triethylamine was added to the mobile phase by competitive binding to the silanol groups in the LC column to see if it could reduce the tailing for the basic PQ. TCA, octanesulfonic acid, and pentanesulfonic acid were added to the mobile phase and samples prior to injection into the LC system to see if this could improve peak shapes (i.e., peak compression). Peak compression is a general term for different methods to concentrate an analyte band during a chromatographic run. Fornstedt et al. (9,10) and Nilsson (11,12) have reported compressed peaks after the coelution of the analyte peak and a system peak. The system peak had no absorption at the analysis wavelength. Nilsson (11) used the organic modifier *N,N*-dimethyldecylamine both in the mobile phase and the samples and achieved a six- to sevenfold improvement of the LOQ for a phenolic tertiary amine. Other experiments (9,10,12) have used a cation modifier in the mobile phase and injected the analyte in a solution containing an organic anion, which gave rise to a large system peak. The analyte was eluted in the fallback of those negative system peaks, which consisted of a steep gradient of increasing cation concentration resulting in very high efficiencies for the analyte peak. Hooijschuur et al. (13) have recently reported on peak compression effects after the addition of a modifier to the sample alone rather than to the mobile phase. The addition of a cocktail of different alcohols (1-butanol, 3-pentanol, and 2-methyl-3-pentanol) to the samples improved the efficiencies for several peaks in the chromatogram.

Accuracy, intra-, and interassay precision

The accuracy and precision of the method was estimated by the analysis of spiked plasma at three concentrations (0.050, 0.750, and 3.00μM) in triplicate for five days. Concentrations were determined using a calibration graph prepared on the day of analysis. Intra- and interassay precisions were calculated.

Linearity

Calibration curves were constructed using eight standards and obtained by calculating the peak–height ratios of PQ to the IS against the corresponding concentration. Linear calibration curves were generated by nonweighted linear regression analysis. It was necessary to divide the calibration range into two calibration curves because the highest calibration point was 200 times that of the lowest calibration point. One calibration curve was made for the standards in the range of 0.025 to 0.200μM and another one for all the standards in the range of 0.025 to 5.00μM.

Extraction recovery

The extraction recovery was determined from the plasma samples used for the precision study. Three concentrations (0.050, 0.750, and 3.00μM) in triplicate were analyzed for five days. The peak heights of the extracted samples were compared with a dilution in the same way in the reconstitution solvent as the actual dilution during the extraction. The nonextracted samples that dissolved in the reconstitution solvent were injected directly into the LC system. Peak heights of the SPE samples from plasma were compared with the peak heights of the nonextracted samples.

Stability

Not much is known about the stability of PQ. Bench-top stability was checked by the injection of a low (0.150μM) and a high (1.50μM) concentration of PQ in the reconstitution solvent every hour for 2 days.

The freeze/thaw stability was evaluated by the analysis of spiked plasma at two concentrations (0.050 and 3.00μM) in duplicate for 5 freeze/thaw cycles.

The stability in the elution solvent (methanol–triethylamine, 98:2, v/v) was investigated during 24 h. A 10-mL elution solvent at a PQ concentration of 1.00μM was portioned into 20 aliquots. Three samples were evaporated and reconstituted at 0, 3, 5, 21, and 24 h after preparation. The samples were kept at approximately 20°C until evaporation. All samples were analyzed together.

Spiked plasma calibrators were kept at approximately –17°C for two months. A set of calibrators was analyzed after 1 day, 2 weeks, 1 month, and 2 months. Calibration curves were constructed using the peak height of PQ against the corresponding concentration. The slopes and regression coefficients were calculated and compared for the different time points.

Effect of heat treatment

When handling biological samples, there is always a risk of exposure to human immunodeficiency virus (HIV). HIV is thermolabile and is inactivated by heating at 56°C for 30 min (14,15). Spiked plasma samples (3.00μM) were incubated for 30 and 60 min at 60°C. Five aliquots (500 μL) of each sample were analyzed together with five aliquots (500 μL) of a spiked plasma sample (3.00μM) as a reference.

Selectivity

Blank plasma from six different healthy volunteers were analyzed, and their chromatograms were examined for endogenous compounds that could interfere with the quantitation of PQ. Several common antimalarials and some of their metabolites [e.g., amodiaquine, monodesethyl–amodiaquine, atovaquone, CQ, desethyl–CQ, mefloquine, mefloquine metabolite, proguanil, 4-chlorophenylbiguanid (proguanil metabolite), cykloguanil (proguanil metabolite), primaquine, pyrimethamine, pyronaridine, sulfadoxine, and trimethoprim] were injected into the LC system to see if they interfered with the peaks of PQ and the IS.

Results and Discussion

Not much is known about the physicochemical properties of PQ. Our experience is that PQ has poor solubility in pure methanol. The solubility is greatly increased when the methanol is acidified with hydrochloric acid. PQ is practically insoluble in water and PQP is soluble in hot water.

PQ has to be considered as a weak base even though it has four pKa values. The pKa values were calculated with a commercial pKa program (16) (pKa_1 = 8.6, pKa_2 = 8.6, pKa_3 = 5.8, and pKa_4 = 5.8). Studies on mice have suggested that PQ is not metabolized because only the parent drug has been found in the urine (7).

Influence of the pH on the absorbance

The absorbance of PQ seemed to be highly dependent on the pH. The absorbance was significantly higher at low pH (e.g., below pH 3) than at high pH (e.g., above pH 7) (Figure 2). The pH of the mobile phase was chosen to be pH 2.5 in order to achieve as sensitive a detection as possible without damaging the column.

Peak shape and efficiency improvements

The addition of triethylamine to the mobile phase did not affect the efficiency for the PQ peak nor did the addition of any of the different acids. The addition of TCA to the samples gave a significant increase of the efficiency for the PQ peak (from 4000 to 15000 theoretical plates). It also increased the retention time for PQ from 4.3 to 4.9 min. The efficiency and retention time for the IS peak increased marginally. The addition of TCA gave a small distortion in the baseline in the form of a small plateau around the PQ peak. The plateau started earlier with an increasing concentration of TCA in the samples. It was necessary to add at least 2% of the TCA stock solution (0.61M) to the samples to separate the plateau start from the PQ peak (Figure 3). The reason for the increase in efficiency is that the PQ peak was eluted in a steep gradient of TCA, which has no absorbance at the analysis wavelength. This effect can be seen in Figure 4 in which a blank injection solution was injected at 230 nm and a PQ–IS solution was injected at the analysis wavelength of 345 nm. TCA has a slightly higher capacity factor than PQ but lower than the IS, thus the IS peak remains almost unaffected. No further attempts were made to affect the IS peak because the asymmetry factor was already low and the sensitivity not a problem. The pH in the samples was 2.0 after the addition of TCA.

Accuracy, intra-, and interassay precision

The precision and accuracy of the assay is summarized in Table II. At an LOQ of 0.025µM, the signal-to-noise ratio was greater than 10:1 and intra- and interassay precisions were less than 20%. At an LOD of 0.005µM, the signal-to-noise ratio was greater than 3:1. These limits will hopefully be low enough to permit pharmacokinetic studies. To the best of our knowledge, there is no published information about drug levels or even estimated drug levels in humans.

Linearity

Linear calibration curves were obtained with correlation coefficients greater than 0.99 for all five days.

Recovery

The recovery was above 70% for all three concentrations and can be found in Table III.

Stability

The bench-top stability was good for both 0.150 and 1.50µM. The relative standard deviation (RSD) values were 3.3 and 1.6% for 0.150 and 1.50µM, respectively ($n = 21$).

PQ was stable during the 5 freeze/thaw cycles. The RSD values 5.1 and 3.6% for 50 and 3.00µM,

respectively, were both within the precision of the assay at the respective levels.

PQ was stable in the elution solvent (methanol–triethylamine, 98:2, v/v) for at least 24 h. The RSD value for all the samples
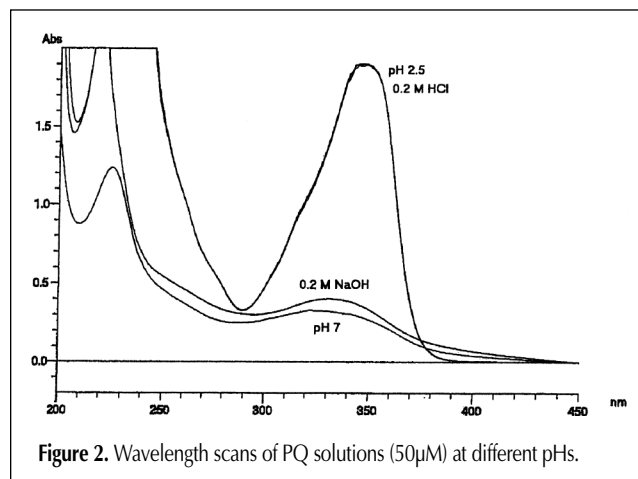

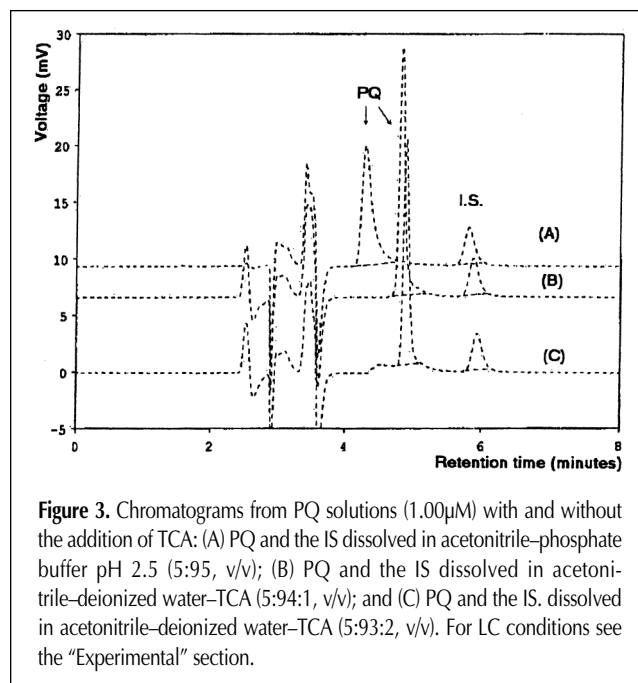**Figure 2.** Wavelength scans of PQ solutions (50µM) at different pHs.

Figure 3. Chromatograms from PQ solutions (1.00µM) with and without the addition of TCA: (A) PQ and the IS dissolved in acetonitrile–phosphate buffer pH 2.5 (5:95, v/v); (B) PQ and the IS dissolved in acetonitrile–deionized water–TCA (5:94:1, v/v); and (C) PQ and the I.S. dissolved in acetonitrile–deionized water–TCA (5:93:2, v/v). For LC conditions see the "Experimental" section.

Table II. Accuracy, Intra-, and Interassay Precision for the Determination of PQ in Plasma

	Added (µM)	Found (µM) mean ± standard deviation	%RSD	%Deviation (found versus added)
Intra-assay (n = 15)	0.050	0.048 × 0.003	5.8	–4.2
	0.750	0.738 × 0.051	6.9	–1.6
	3.00	2.91 × 0.16	5.4	–3.0
Interassay (n = 5)	0.050		10.0	
	0.750		4.6	
	3.00		1.3	

(n = 15) was 2.3% at a concentration of 1.00µM. This correlated well with the bench-top stability variation.

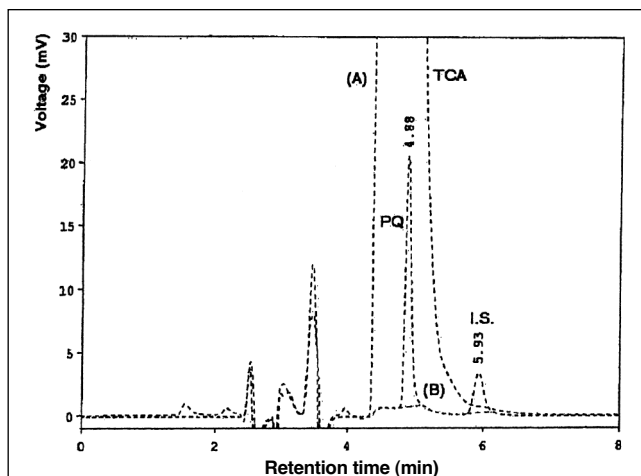Plasma samples were stable for at least 2 months at approxi-

Figure 4. Chromatograms from reconstitution solvent: (A) acetonitrile–deionized water–TCA (5:93:2, *v/v*) at 230 nm and (B) PQ and the IS (1.00µM) dissolved in acetonitrile–deionized water–TCA (5:93:2, v/v) at 345 nm. For LC conditions see the "Experimental" section.
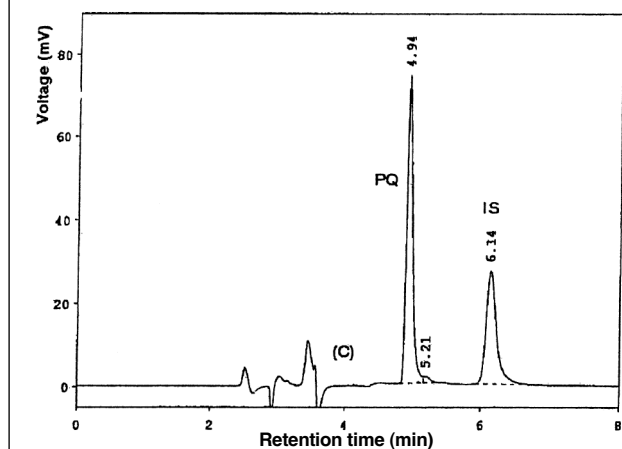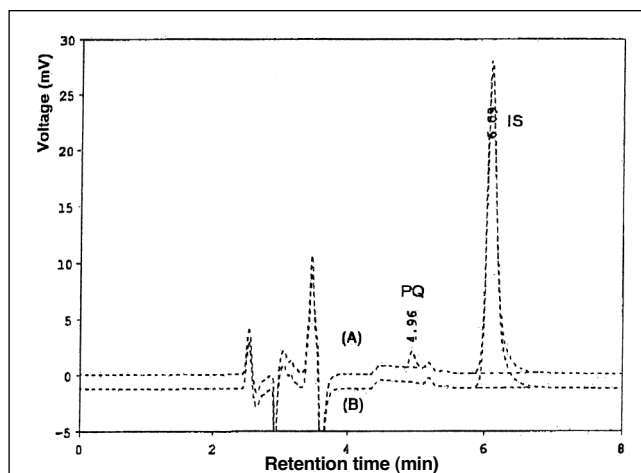

Figure 5. Chromatograms from spiked plasma: (A) plasma spiked with PQ (0.050µM); (B) blank plasma; and (C) plasma spiked with PQ (3.00µM). For SPE and LC conditions see the "Experimental" section.

mately –17°C. There were no significant differences in the slope, intercept, or regression coefficients for the calibration curves from the different time points.

Effect of heat treatment

The concentration of PQ was not altered, within the precision of the assay, by heating at 60°C for 60 min. The effect of heating spiked plasma samples is shown in Table IV.

Selectivity

None of the commonly used antimalarial drugs analyzed interfered with the retention times of the analytes. No endogenous interference from plasma was observed. Typical LC chromatograms from blank plasma, spiked plasma at 0.050µM, and spiked plasma at 3.00µM are presented in Figure 5.

Conclusion

A sensitive, selective, and accurate method using automated SPE and LC has been developed and validated. It is the first available method that permits the determination of the novel antimalarial PQ in plasma. It is suitable for pharmacokinetic studies in humans worldwide.

Acknowledgments

Table III. Recovery of PQ from Plasma*

	Concentration (µM)	Mean ± standard deviation (%)
PQ	0.050	74.9 × 8.2
	0.750	70.0 × 9.5
	3.00	79.6 × 8.6

* n = 15.

Table IV. Effect of Heat Treatment of 3.00µM Spiked Plasma Samples at 60°C

	Found (µM) (mean ± standard deviation)	%RSD
0 min (n = 5)	2.98 × 0.07	2.3
30 min (n = 5)	3.04 × 0.04	1.1
60 min (n = 5)	3.05 × 0.06	1.9

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