Ultrasensitive determination of pyrroloquinoline quinone in human plasma by HPLC with chemiluminescence detection using the redox cycle of quinone

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Abstract

A fast, accurate, and ultrasensitive high-performance liquid chromatography method with chemiluminescence detection (HPLC-CL) was optimized and validated for the determination of pyrroloquinoline quinone (PQQ) concentration in human plasma following solid-phase extraction (SPE). This method is based on the redox cycle of the reaction between PQQ and dithiothreitol, which generates reactive oxygen species that can be detected using luminol as a CL probe. The isocratic HPLC system comprised an ODS column and 4.0 mM tetra-*n*-butylammonium bromide in Tris-HNO_3 buffer (pH 8.8; 50 mM)-acetonitrile (7:3, v/v) as mobile phase. A novel, rapid, and simple SPE method was also developed providing excellent %recovery (≥95.2%) for PQQ from human plasma samples. The proposed method was linear over the range of 4.0–400 nmol/L plasma of PQQ with a lower detection limit (S/N = 3) of 1.08 nmol/L plasma (0.27 nM). The method was successfully implemented to determine PQQ concentration in the plasma of healthy individuals after administration of PQQ supplements.

Keywords: Pyrroloquinoline quinone; HPLC; chemiluminescence; redox cycle; human plasma.

1. Introduction

Pyrroloquinoline quinone (PQQ, 4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylic acid) is a cofactor for methanol dehydrogenase [1] and is categorized as a member of the vitamin B family [2]. PQQ plays important roles in mammals, including protection against liver [3], heart [4], and brain injuries [5], enhancement of the synthesis of DNA in human fibroblasts [6], and promotion of the production of the nerve growth factor [7]. In addition, it has a defensive effect against embryonic cataract induced by hydrocortisone [8] and acts as a radical scavenger [9, 10]. It also inhibits the formation of amyloid fibrils and protects from the toxicity of C-truncated alpha-synuclein variants, which are properties that make it an effective agent in the prevention of Parkinson's disease [11, 12]. Finally, it prevents cognitive deficiency in oxidative-stress-induced neurodegeneration [13], and its disodium salt is used as a dietary supplement. Considering the many physiological roles and activities of this compound, a highly sensitive analytical method for the determination of PQQ concentration in human plasma is required.

A number of methods have been reported for PQQ determination in different matrices. An HPLC-ESI-MS/MS-based method [14] and a GC-MS-based method (involving the preliminary derivatization of PQQ with phenyl trimethylammonium

hydroxide) [15] were utilized for the quantitative analysis of PQQ in various foods. Quantitative CE-UV [16] and HPLC-ECD [17], in addition to an HPLC-UV and cyclic voltammetry qualitative [18] approach, were implemented for the determination of PQQ standard. PQQ was also determined in different pig and bovine enzymes by HPLC-UV and mass spectrometry (MS) detection after PQQ conversion to a hydrazone derivative [19]. None of these methods [14–21] were applied to human plasma; in addition, many of them depended on the use of sophisticated instruments [14, 15, 17] and involved tedious and time-consuming derivatization procedures [15, 19]. Gas chromatography-MS (GC-MS [22]) has been the only bioanalytical method for PQQ concentration determination reported so far. However, the derivatization procedure with phenyltrimethylammonium hydroxide that this protocol involved required harsh conditions such as heating at 100°C for 15 min. The high cost and complexity of conducting MS experiments limit the widespread use of this method, which suffers from additional drawbacks such as a multi-step, lengthy, and complicated extraction procedure (Table 1). Therefore, developing a simple, sensitive, rapid, and convenient analytical method for the analysis of PQQ level in human plasma remains a very important research goal.

It has been reported that PQQ is present in human plasma at very low concentration [22, 23]. Hence, developing a reliable and efficient sample preparation approach and a subsequent sensitive analytical method to determine plasma PQQ level constitute an analytical challenge. Among the various detection methods, chemiluminescence (CL) detection provides high sensitivity that enables the determination of trace amounts of many species using a simple instrument. However, to date, no method relying on CL detection has been applied to the determination of PQQ concentration. Our research group reported a CL assay for the determination of quinone concentration that utilizes quinone characteristic redox reaction cycle [24–26]. In this approach, quinones are reduced by dithiothreitol (DTT) to yield the corresponding semiquinone radicals, which convert dissolved oxygen to the superoxide anion radical, which will, in turn, produce CL upon reaction with luminol (Fig. 1). The aim of the present study is to employ this redox cycle to develop an ultra-sensitive, simple, rapid, and convenient HPLC-CL detection method for the determination of PQQ concentration in human plasma.

Sample preparation is a very important step in the analysis of PQQ in plasma. Only one sample preparation procedure, which combines two liquid–liquid and one solid–phase extraction (SPE) steps, has been reported [22, 27] for the extraction of PQQ

from plasma. Due to the complication and time-consuming nature of this extraction method [22, 27], it was necessary to develop an alternative rapid, efficient, simple, and selective procedure for the extraction of PQQ from plasma. Hence, it was also one of our main targets to develop an efficient SPE procedure for extracting PQQ and then to couple it with the HPLC-CL detection for monitoring trace amounts of PQQ in plasma. The conditions and relevant parameters of the extraction of PQQ by SPE were carefully optimized, and the analytical performance of the method was evaluated.

Finally, this method was applied to the quantification of PQQ in human plasma. For this purpose, we collected plasma samples from healthy individuals who had taken PQQ supplements and studied how PQQ plasma concentration changed over time.

2. Experimental

2.1. Materials and reagents

All chemicals were of extra-pure analytical grade and solvents were of HPLC grade. PQQ disodium salt was sourced from Mitsubishi Gas Chemical Co. Inc. (Tokyo, Japan). Acetonitrile (ACN), methanol, and nitric acid were supplied by Kanto Chemical Co. (Tokyo, Japan). DTT and hydrochloric acid were sourced from Nacalai Tesque (Kyoto, Japan). Luminol and sodium dihydrogen phosphate were sourced from Wako

Pure Chemical Industries, Ltd. (Osaka, Japan). Tris (hydroxymethyl) aminomethane (Tris) was obtained from MP Biomedicals Co. (Santa Ana, USA). Tetra-*n*-butylammonium bromide (TBAB) was sourced from Tokyo Chemical Industries (Tokyo, Japan). Sodium hydroxide was purchased from Merck Co. (Darmstadt, Germany). Pyridine was sourced from Kishida Chemicals (Osaka, Japan). PQQ stock solutions (25 µM) were prepared in water and kept in a refrigerator at 4°C. Tris-HNO_3 buffer (50 mM) was prepared in water and its pH was adjusted to 8.8; subsequently, a 4.0 mM solution of TBAB was prepared in this buffer. DTT (0.15 mM) and luminol (1.5 mM) solutions were prepared fresh in ACN and NaOH*aq* (150 mM), respectively.

2.2. Instrumentation

The HPLC system (Fig. 2) consisted of three Shimadzu LC-20A pumps (Kyoto, Japan), a Rheodyne injector (Cotati, CA, USA) with a loop size of 20 µL, a CLD-10A CL detector (Shimadzu), and an EZ Chrom Elite chromatography data acquisition system (Scientific software, Pleasanton, CA, USA). PTFE tubing (7 m × 0.5 mm i.d., GL Sciences, Tokyo, Japan) was used as the reaction coil. The pH meter used was Horiba F22.

2.3. HPLC conditions

Chromatographic separation was performed on an InertSustain C18 (150 × 4.6 mm i.d., 3 μm particle size, GL Sciences, Tokyo) column. An InertSustain C18 cartridge guard column E (10 × 4.0 mm i.d., 3 μm, GL Sciences) was used. Isocratic elution was accomplished with a mixture of TBAB (4.0 mM) in Tris-HNO_3 buffer (pH 8.8; 50 mM)-ACN (7:3, v/v) at a flow rate of 1.0 mL/min. TBAB was utilized because it can form ion pair with PQQ and cause PQQ to be retained on a hydrophobic analytical column. The eluent from the column was mixed simultaneously with DTT (0.15 mM) and luminol (1.5 mM) solutions and then passed to the reaction coil. DTT and luminol solutions flow rates were set at 0.25 mL/min.

2.4. Calibration curve

Standard PQQ solutions were prepared in the 1–250 nM concentration range by diluting the PQQ stock solution (25 μM) with the mobile phase. A blank experiment was carried out simultaneously. The average peak areas were plotted versus the concentrations of PQQ (nM).

2.5. Plasma samples

Plasma samples were collected from six healthy individuals (two males and four females, mean age 25.8 ± 7.0 years). In order to investigate the variation of PQQ concentration in plasma after the oral administration of this chemical as a supplement, PQQ disodium salt supplement capsules (100 mg, BioPQQ, Mitsubishi Gas Chemical Co. Inc.) were administered to the healthy individuals once a day for 7 days. Each capsule was made of cellulose and contained Bio PQQ (10 mg), starch (150.5 mg), starch hydrolysate (82 mg), and calcium stearate (7.5 mg) [28]. Capsule stability was confirmed through a dissolution test performed according to the pharmacopoeia. On the 1^{st} day, whole-blood samples were collected before (0 h) administration of PQQ and then after 3 and 24 h. After daily administration of the supplement for 6 days, on the 7^{th} day, whole-blood samples were collected before (0 h) administration of the PQQ supplement and after 3 h. The blood samples were collected in blood-sampling tubes containing EDTA as an anticoagulant. After centrifuging at 3000 rpm for 10 min at 5°C, the supernatant was collected and stored at −80°C until analysis. This experiment was performed according to the guidelines of the Ethics Committee of the School of Pharmaceutical Sciences, Nagasaki University, after receiving approval from the said Committee.

2.6. SPE procedure and determination of PQQ concentration in plasma

SPE was performed using a Varian Bond Elut C18 cartridge (500 mg/3 mL) supplied by Agilent Technologies (CA, USA). Aliquots of 200 µL of the plasma samples collected as described in section 2.5 or pooled plasma samples spiked with PQQ were pipetted into 1.5 mL tubes and acidified with 200 µL of 1 M NaH_2PO_4 /HCl (pH 2.0), and the tubes were subsequently vortexed for 1 min. The conditioning and equilibration of the C18 cartridge were carried out using 4 mL of methanol and 4 mL of purified water, respectively. A 300 µL aliquot of each acidified plasma sample was then loaded onto the cartridge. The cartridge was washed sequentially with 2 mL of purified water and 600 µL of 5% aqueous pyridine. Finally, the elution was carried out with 600 µL of 30% ACN, a 20 µL aliquot was injected into the HPLC system. The sample was diluted by SPE, and the final concentration of PQQ was 1/4 that of the original plasma sample.

2.7. Method validation

Method validation was performed according to FDA guidelines for the validation of bioanalytical [29] methods. The calibration curve (drawn on the basis of 8 points) was performed by measuring the PQQ concentration of plasma samples spiked with a PQQ standard covering the range of 4–400 nmol/L plasma. The limit of detection

(LOD) was determined at a signal-to-noise (S/N) ratio of 3.

The accuracy and precision of the method were determined by analysis of spiked human plasma at three concentration levels using five replicates for each concentration. The intra-day precision of the proposed method was assessed at low (4 nmol/L plasma), medium (40 nmol/L plasma), and high (200 nmol/L plasma) plasma concentration level using five determinations per sample on the same day. Inter-day precision was assessed by determining PQQ levels at the same three concentrations for five consecutive days. The values of %RSD for the five determinations express the precision of the method.

Furthermore, the stability of PQQ in plasma was studied at concentrations of 20 and 200 nmol/L plasma. Short- and long-term stabilities of PQQ in plasma were evaluated after letting samples stand at room temperature for 1, 4, and 24 h. In addition, the stability of PQQ in plasma with respect to freeze–thaw cycles was determined. The plasma samples spiked with PQQ were stored at −80°C and subsequently thawed to room temperature, and this process was repeated a total of three times. In addition, the plasma samples stored at −80°C for 7 days were also measured.

3. Results and Discussion

3.1. Optimization of the HPLC-CL conditions

To improve the sensitivity of the analytical approach, CL reaction conditions were optimized. The influences of different variables on the peak area and S/N ratio were investigated using a standard solution of PQQ. Previous studies [24–26] provided evidence that the CL generated increases steadily upon mixing quinone with DTT and luminol. Therefore, it is better to have the mixed solution pass through a reaction coil before measuring CL intensity. The effect of the length of the reaction coil was studied over the 6–8 m range in a coil with a constant internal diameter of 0.5 mm (Fig. 3a). The maximum peak area was obtained with a coil measuring 6 m, but the S/N ratio increased up to a coil length of 7 m and then decreased. Therefore, a reaction coil with a length of 7 m was selected as the optimum. The effects of the concentrations of the reagents were also examined. The influence of luminol was studied over a 0.5–3.0 mM range. The peak area and S/N ratio increased up to a 1.5 mM concentration and then decreased (Fig. 3b). Hence, 1.5 mM luminol was chosen as the optimum concentration. The effect of NaOH concentration was also studied over a 50–300 mM range. The highest peak area and S/N ratio were observed for a 150 mM solution of NaOH (Fig. 3c). The concentration of DTT was studied in the 0.05–0.4 mM range. The maximum peak area was observed at 0.1 mM concentration, whereas the maximum S/N ratio was observed at 0.15 mM concentration (Fig. 3d), so 0.15 mM DTT was selected as the

optimum concentration for conducting the experiment. Fig. 4a shows a typical chromatogram of a standard solution of 50 nM PQQ obtained by implementing the proposed method. The peak derived from PQQ was detected at 10.7 min.

3.2. Method linearity

The calibration curve of the method was constructed by plotting the average peak area of triplicate analyses versus the known PQQ concentration of standard solutions in the 1–250 nM range. The regression equation utilized was $Y = (13.0 \pm 1.0) \times 10^4 X - (13.3 \pm 11.9) \times 10^4$, where Y is the peak area and X is the concentration of PQQ (nM). The detection limit of PQQ was 0.23 nM (4.6 fmol/injection) at S/N = 3.

3.3. Optimization of the extraction method

The HPLC-CL method thus developed displayed enough sensitivity to be utilized in the determination of PQQ concentration in human plasma. We then investigated the performance of the method for the extraction of PQQ from plasma samples. Suzuki *et al.* [27] investigated the conditions for extracting PQQ from biological samples implementing an approach comprising of a liquid–liquid extraction followed by a solid-phase extraction. In this method, the PQQ present in a biological sample was

extracted into an organic layer then re-extracted into an aqueous layer. After the second extraction, HCl was added to the aqueous layer to acidify the sample, and PQQ was extracted using a C18 cartridge; the recovery rate at this point was 50–105%. This procedure has been implemented in the method for the determination of PQQ concentration in biological samples by GC-MS reported by Kumazawa *et al* [22]. This extraction procedure is time-consuming because it involves two extraction modes to be implemented consecutively. Therefore, we set out to develop a fast and convenient extraction method to trace the amount of PQQ in human plasma. We studied the deproteinization method using an organic solvent and a liquid–liquid extraction method using ethyl acetate, but in neither case were we able to achieve a sufficient recovery rate. Therefore, the SPE method using the C18 cartridge, which is the most commonly used solid phase relying on hydrophobic interactions, was examined. The solvent to be used in SPE was determined on the basis of a report by Suzuki *et al* [27]. We tried many washing solvents, including water, ACN, aqueous ACN (10–50%), and aqueous pyridine (2–10%) alone or in combination with each other, with the washing step being followed by elution with different mobile phases: 30% aqueous ACN, water, or 5% aqueous pyridine. We obtained the best results using water followed by 5% aqueous pyridine for the washing step and 30% aqueous ACN as eluting solvent, as mentioned in

section 2.6. The SPE procedure developed required a smaller sample volume than that needed in the reported method, and it involves a simple operation. Additionally, 30% aqueous ACN was used as eluent instead of 5% aqueous pyridine, which allowed direct injection of the eluate into the HPLC-CL system without the need for evaporation and reconstitution steps. Furthermore, this method showed high selectivity for PQQ, with no evidence of interference by plasma components in the detection of PQQ. In fact, in our method, PQQ concentration was measured using the reactive oxygen species generated by the redox cycle of quinone, so endogenous quinones, including vitamin K homologues and coenzyme Q10, could theoretically interfere with the measurement of PQQ level. As anticipated, however, such endogenous biological components were removed by the SPE method we hereby propose.

3.4. Method validation with plasma

The method was applied to the determination of PQQ concentration in human plasma. PQQ was selectively extracted by SPE from plasma samples spiked with a PQQ standard solution and the analyte was subsequently detected. The plasma components did not affect the detection of PQQ (Figs. 4b and 4c). The calibration curve for the analysis was drawn using human pooled plasma that had been spiked with PQQ over

the range of 4–400 nmol/L plasma. The regression equation of the calibration curve was $Y = (2.7 \pm 0.2) \times 10^4 X + (4.3 \pm 2.9) \times 10^4$, where Y is the peak area and X is the concentration of PQQ (nM) and the LOD of was 0.27 nM (5.4 fmol/injection). The LOQ was found to be 1.0 nM (4.0 nmol/L plasma) with good accuracy (%found > 95%) and acceptable precision (%RSD $\leq$ 5.6%) that are in good agreement with the FDA validation guidelines [29]. The small increase in background luminescence was the result of the presence of reactive oxygen species or other components in plasma. However, the LOD of standard and human plasma sample calculated by S/N ratio showed only a limited change. The intra- and inter-day accuracy and precision of the method were determined by the analysis of plasma samples spiked with PQQ at three concentrations (4, 40, and 200 nmol/L plasma). For all samples, the accuracy was in the 95.2–100.3% range and the %RSD for intra- and inter-day precision did not exceed 6.1% (Table 2). These results indicated the high reproducibility of this method.

The stability of PQQ in plasma was examined using plasma samples spiked with PQQ at concentrations of 20 and 200 nmol/L plasma and determining (i) short-term stability at room temperature for 1 h and 4 h, (ii) long-term stability for 24 h at room temperature, (iii) stability after three freeze–thaw cycles, and (iv) stability of a frozen sample kept at −80°C for 7 days. In particular, the stability of the PQQ in the

plasma samples treated as mentioned above was determined by comparing with freshly prepared one. The recovery rate of PQQ in all samples was ≧81.8% under the studied conditions, and the stability of PQQ in plasma was confirmed (Table 3).

3.5. Variations of PQQ concentration in plasma after administration of PQQ supplements

The developed HPLC-CL method was applied to the determination of PQQ concentration in human plasma after continuous administration of PQQ supplements. Typical chromatograms of human plasma at (a) 0 h (before administration), (b) 3 h, and (c) 24 h after administration of PQQ on the 1st day of the experiment are reported in Figs. 5a, 5b, and 5c, respectively. PQQ concentrations in plasma are also depicted in Fig. 6a. As shown in these figures, PQQ was not detected in plasma before administration of PQQ supplements. On the other hand, PQQ was detected in the plasma of all individuals in the range of 16.4–53.8 nmol/L plasma after 3 h of administration. Although PQQ was also detected in plasma after 24 h of administration, the concentrations of PQQ were greatly reduced at this stage compared to those of the samples collected after 3 h of administration. After continuous administration for 6 days, significant concentrations of PQQ were detected in the plasma samples even before the administration of supplements. In addition, PQQ concentrations after 3 h of

administration on the 7th day of the experiment were higher (Fig. 6b) than their counterparts determined on the 1st day. These results suggested that the continuous administration of PQQ supplements could increase the basal blood concentration of PQQ. The values of PQQ concentration in plasma were summarized in Table S1 (supporting file). Also, it is noteworthy that there is a large variance in PQQ concentration among volunteers (%RSD reached 62.1%) which is also reported by Harris *et al.* [23] with %RSD of about 70.3%. This could be due to individual variation in metabolism of PQQ.

4. Conclusion

This study is the first report on the use of an HPLC-CL system for the determination of PQQ concentration in plasma. The developed method relies on CL detection of 3-aminophthalate produced by the reaction of PQQ with DTT and luminol. A novel, rapid, and simple SPE procedure was also developed and coupled to the proposed HPLC-CL method for ultrasensitive determination of PQQ concentration in human plasma samples without any matrix interference. In addition, it was possible to detect PQQ in plasma samples from individuals who had taken PQQ supplements. Hence, the developed method is suitable for the determination of plasma concentration

of PQQ to study the pharmacokinetics of this compound. In addition, the method can be utilized to elucidate in detail the biodistribution of PQQ by its application to other biological samples.

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Table 1 Critical comparison of the performance of the proposed and reported methods for the determination of PQQ concentration.

Method	Reagent, temperature / reaction time	% Recovery	Sample	LOD, nM (fmol/injection)	Remarks	Ref.
HPLC-ESI-MS/MS	No reaction	30-40	Foods	(1.5)	- High LOQ (150 fmol/injection) - Sophisticated and expensive instruments	[14]
GC-MS	Phenyl trimethylammonium hydroxide, 100 °C / 15 min	>50	Foods / biological tissues and fluids	1.5	- Tedious extraction - Sophisticated and expensive instruments - Harsh reaction condition	[15] [22]
CE-UV	No reaction	N/A*	Mixture of PQQ derivatives	7000	Poor sensitivity	[16]
HPLC-ECD	No reaction	N/A*	Standard solution	(10000)	Sophisticated instrument	[17]
HPLC-UV, Cyclic voltammetry	No reaction	N/A*	Standard solution	N/A*	Qualitative study	[18]
HPLC-UV and MS	Phenylhydrazine or 2.4-Dinitrophenyl-hydrazine	5–33	Enzymes of pig and bovine	N/A*	Qualitative study	[19]
HPLC-UV	Neurotransmitter amino acids, 37 °C / 24 h	N/A*	Amino acids incubations	50	Not suitable for biological samples	[20]
CZE-UV	Amino acids, room temperature / 24 h	N/A*	Growth medium of bacteria and amino acids incubations	100	Low sensitivity	[21]
HPLC-CL	Luminol and DTT, room temperature	95–100	Human plasma	0.27 (5.4)	- Automated online reaction - High sensitivity	

*N/A: data is not available.

Table 2 Accuracy and precision of the proposed method for the determination of PQQ concentration in PQQ-spiked human plasma samples.

Spiked conc. (nmol/L plasma)	Found conc. (nmol/L plasma)	Accuracy (%)	Precision (%RSD*)
Intra-day (n = 5)			
4	4.01	100.3	5.5
40	38.9	97.3	1.6
200	193.6	96.8	6.1
Inter-day (n = 5)			
4	3.81	95.2	5.6
40	39.0	97.5	1.9
200	194.8	97.3	3.3

*RSD: relative standard deviation.

Table 3 Stability of PQQ in human plasma.

Condition	%Recovery	
	20 nmol/L plasma	200 nmol/L plasma
Room temperature (1 h)	104.4	100.2
Room temperature (4 h)	95.7	99.5
Room temperature (24 h)	81.8	98.5
Three freeze–thaw cycles (–80 ℃)	98.0	87.7
Frozen at (–80 ℃) for 7 days	108.9	84.8

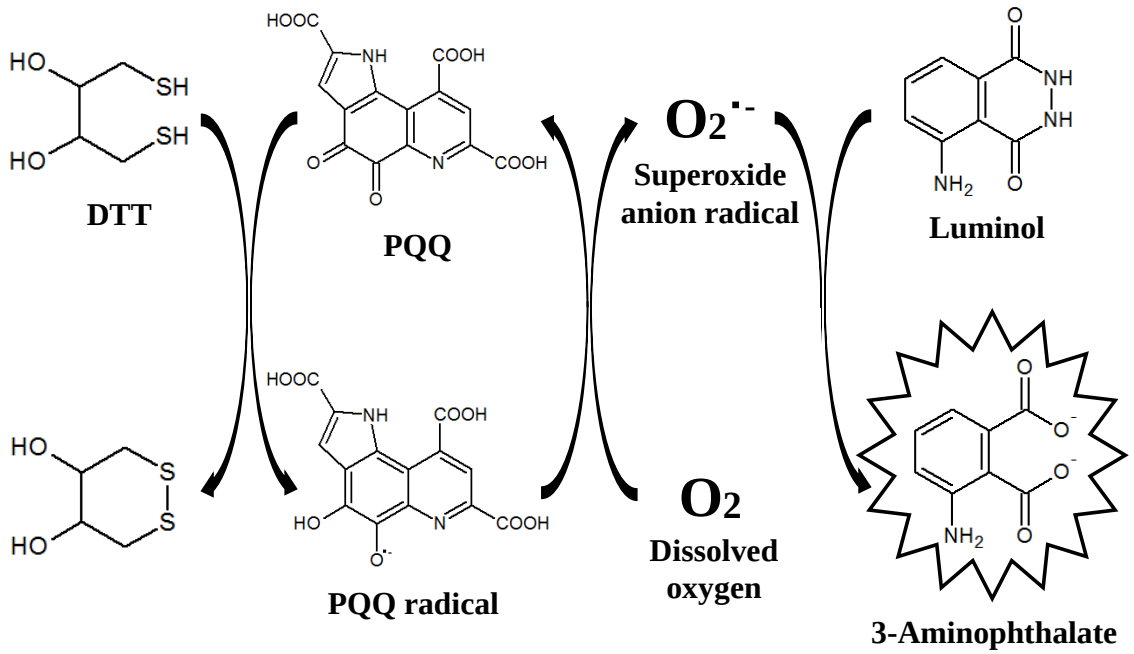

Fig. 1 Mechanism of the CL-producing reactivity of PQQ based on the quinone redox cycle.

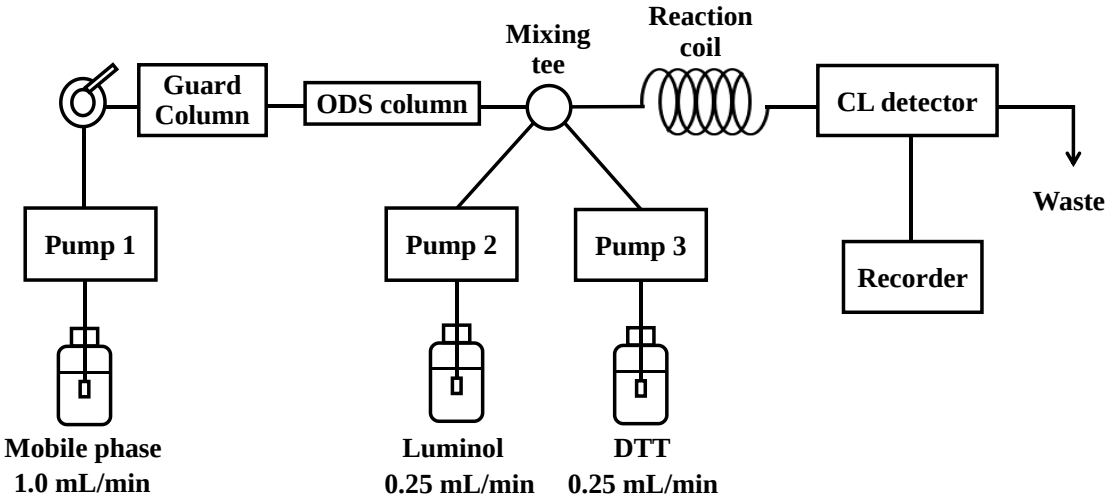

Fig. 2 Schematic diagram of the HPLC-CL system.

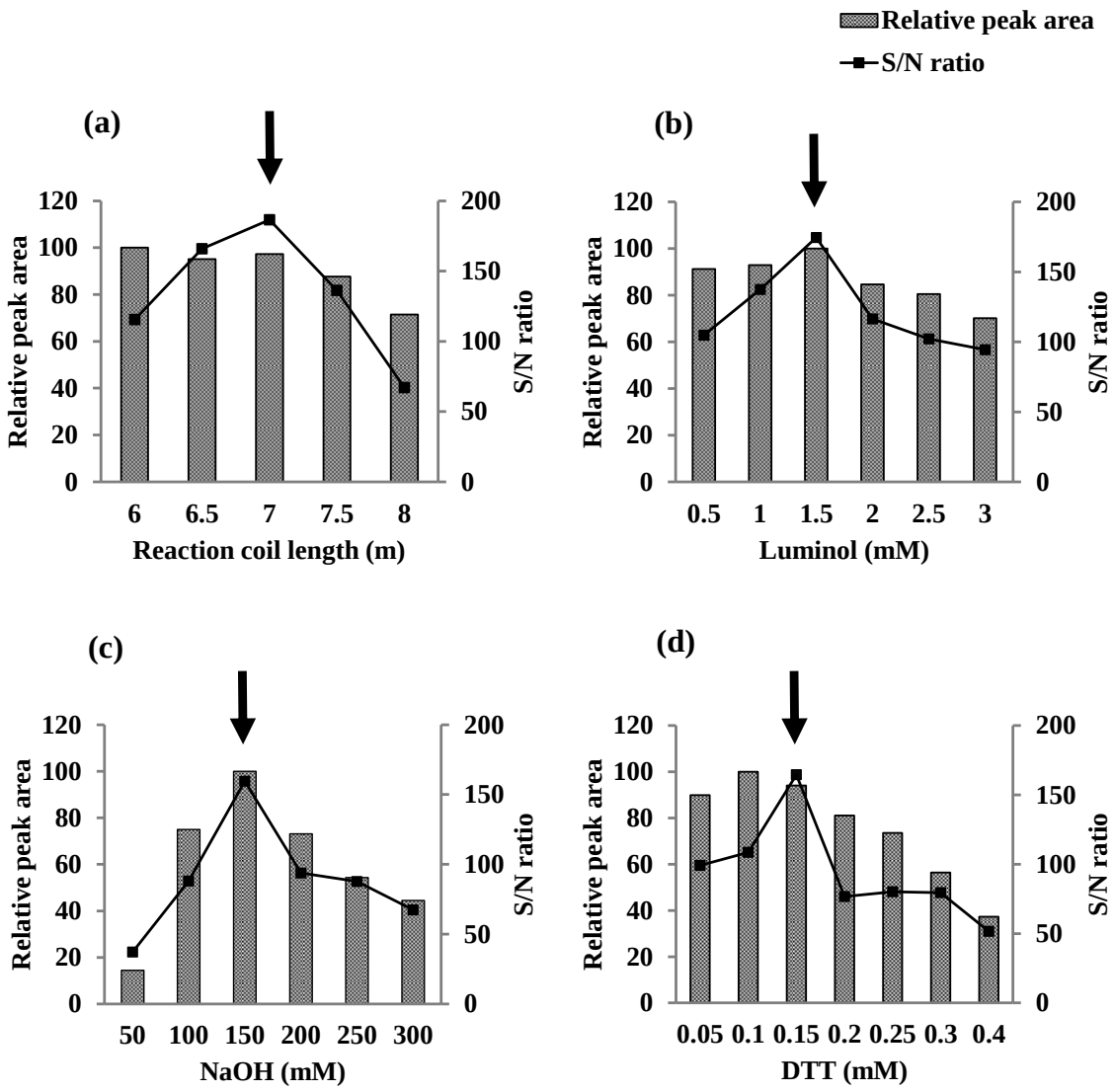

Fig. 3 Effects of reaction coil length and different reagent concentrations on the relative peak area and S/N ratio in CL measurements of samples containing PQQ at 50 nM concentration. Effect on peak area and S/N ratio of (a) reaction coil length, (b) luminol concentration, (c) NaOH concentration, and (d) DTT concentration.

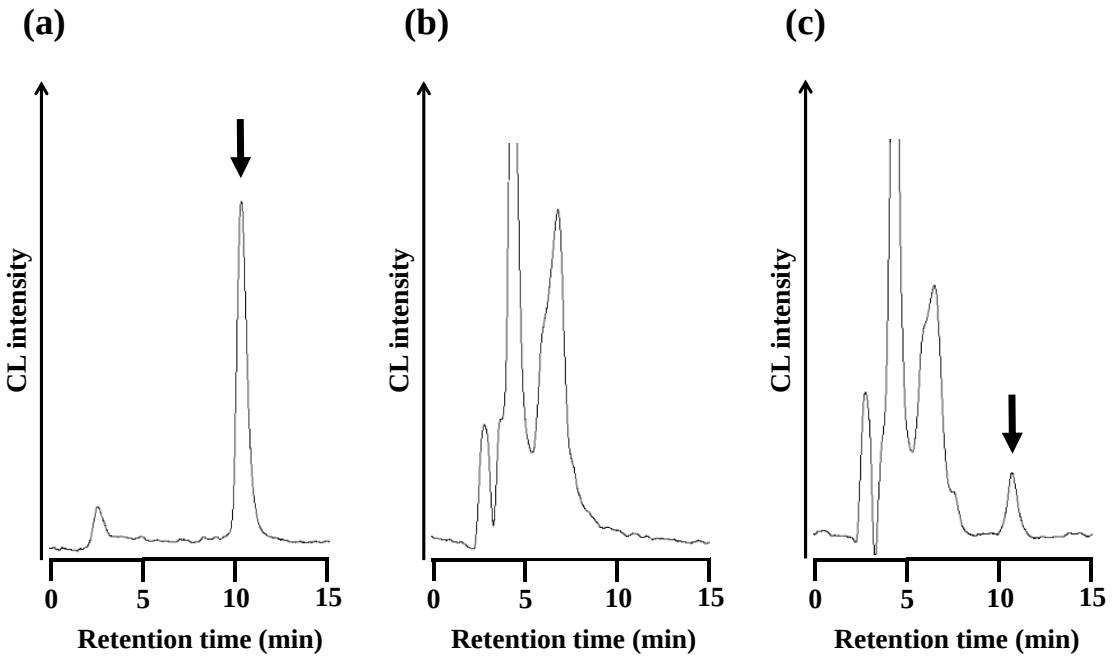

Fig. 4 Chromatograms of (a) standard 50 nM solution of PQQ, (b) blank human plasma, and (c) human plasma spiked with a standard solution of PQQ (the final concentration is 10 nM).

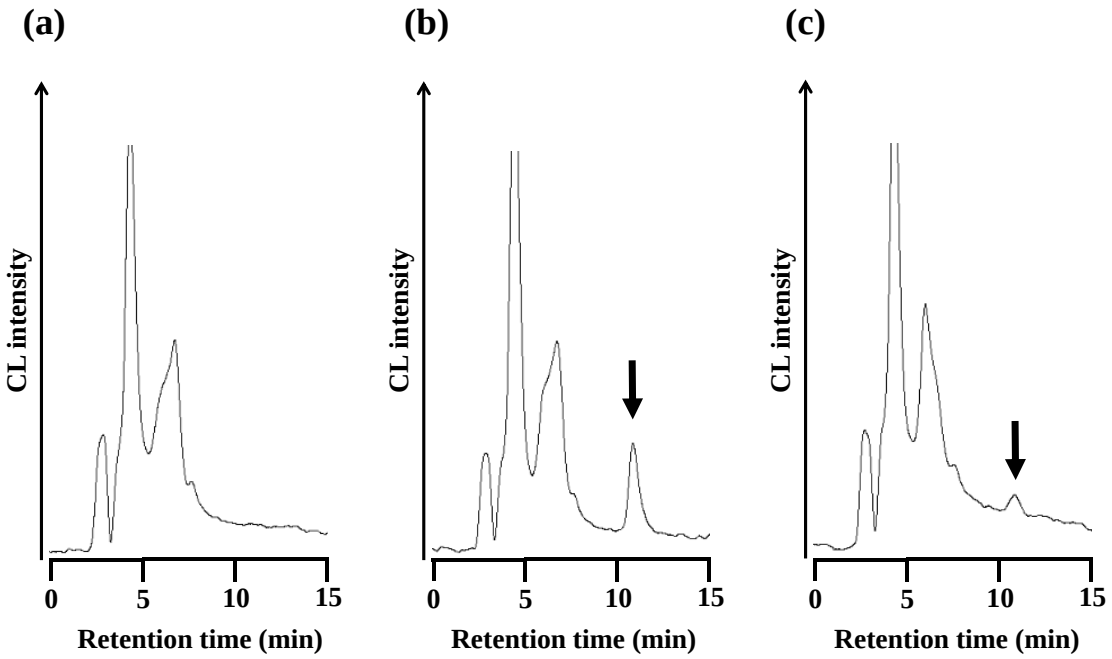

Fig. 5 Chromatograms of human plasma samples collected (a) before, (b) after 3 h, and (c) after 24 h of the administration of PQQ supplements on the 1st day of the experiment.

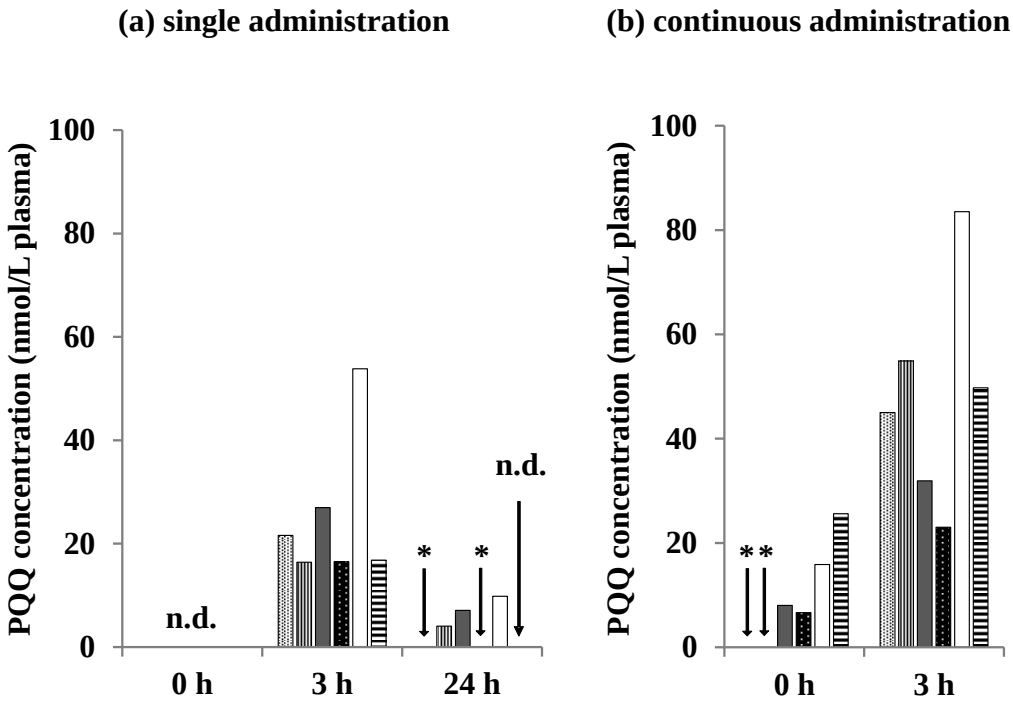

Fig. 6 PQQ concentrations in plasma samples collected from six individuals on (a) the 1st and (b) 7th day of the experiment. Each bar shows the PQQ concentration in individuals' plasma at each blood sampling and n.d. means not detected. * Indicates that the measured concentration lies between the LOD and the LOQ.

Table S1. PQQ concentrations in plasma samples collected from six individuals on the 1st and 7th day of the experiment.

Experiment day	Time	Range (nmol/L plasma, n = 6)	Mean ± SEM*** (nmol/L plasma, n = 6)
1st day	0 h	n.d.*	n.d.*
	3 h	16.4–53.8	25.4 ± 5.9
	24 h	n.d.*–9.8	7.0 ± 1.7
7th day	0 h	Trace**–25.6	14.0 ± 4.4
	3 h	23.0–83.5	48.0 ± 8.6

* n.d. means not detected

** Trace means that the value lies between LOD and LOQ

*** SEM is the standard error of mean.