Selective determination of ubiquinone in human plasma by HPLC with chemiluminescence reaction based on the redox cycle of quinone

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Abstract

Ubiquinone is an important biologically active compound in living body. The determination of ubiquinone in human plasma is useful for the investigation of bioavailability of ubiquinone and for early diagnosis of several diseases. Therefore, we developed an HPLC-chemiluminescence detection method for the analysis of ubiquinone in plasma samples. The method is based on luminol chemiluminescence detection of super oxide anion that generated by the redox cycle reaction between ubiquinone and dithiothreitol. The HPLC system involved an octyl column with a mobile phase of methanol. Ubiquinone eluted from the column was mixed with dithiothreitol and luminol solutions simultaneously and generated chemiluminescence was monitored by chemiluminescence detector. The calibration curve for standard ubiquinone solution was linear from 0.09 to 43.2 μg/mL (0.22-216 ng on column) with the correlation coefficient of 0.999, and the detection limit (S/N = 3) was 26 ng/mL (130 pg on column). Using the proposed HPLC method, the peak of ubiquinone in human plasma could be clearly detected on the chromatogram without any interferences from plasma components.

Keywords: Ubiquinone; Human Plasma; Chemiluminescence; Redox cycle; HPLC

1. Introduction

Ubiquinone (also called coenzyme Q_{10}) is an important biologically active compound which has several roles in the living body. For example, ubiquinone is an electron carrier in the mitochondrial electron transport chain and participates in aerobic cellular respiration and generating energy [1]. Besides, ubiquinone has antioxidant activity and protects circulating lipoproteins against oxidative damage, therefore the ubiquinone concentration in blood may reflect the redox status in human body [1,2]. Furthermore, it has been reported that the plasma levels of ubiquinone in patients with certain diseases including hyperthyroidism, melanoma, cystic fibrosis and phenylketonuria and mevalonic aciduria are significantly lower than those in healthy subjects [3-6]. Therefore, the determination of ubiquinone can be useful for diagnosis of these diseases. In addition, since ubiquinone is used as a therapeutic agent for heart diseases, monitoring of ubiquinone in human plasma is important to investigate its bioavailability and pharmacokinetic properties [7]. Considering these aspects, a sensitive and selective analytical method for ubiquinone is required in various situations.

Several analytical methods have been developed for the determination of ubiquinone in blood samples using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [8,9], electrochemical detection (ECD) [10,11] and

tandem mass spectrometry (MS/MS) [12]. However, HPLC-UV methods lack sensitivity and suffer from interferences due to co-existing substances. HPLC-ECD methods require a complicated system because ubiquinone should be converted to ubiquinol by chemical catalytic reduction with reducer column or electroreduction reaction with in-line electrode just before the detector. In spite of the high sensitivity and selectivity of HPLC-MS/MS method, the high cost of the mass spectrometer limits the widespread use. Among detection techniques, it is known that chemiluminescence (CL) detection provides high sensitivity with simple equipment. Therefore, HPLC with CL detection has been widely applied to the determination of biologically active compounds in blood samples [13,14]. However, until now, a method for the determination of ubiquinone in blood using CL detection has not been developed.

Recently, we have reported a novel CL assay for quinones including ubiquinone based on the their redox reaction cycle [15]. In this batch method, quinones were reduced to semiquinone radicals by dithiothreitol (DTT) as a reductant. Successively, semiquinone radicals convert dissolved oxygen to superoxide anion, which reacts with luminol to generate CL (Fig. 1). When the CL assay was used to determine the content of ubiquinone in pharmaceutical formulations, it was found that the assay was specific for ubiquinone even in the presence of pharmaceutical additives. In this study, we attempted to develop an HPLC-CL method based on this CL reaction

system for the determination of ubiquinone in biological samples. Finally, we successfully applied the developed HPLC method to determine ubiquinone in human plasma without any interferences from plasma components.

2. Experimental

2.1. Chemicals

Ubiquinone was purchased from Kanto Chemical (Tokyo, Japan). DTT, ethanol and methanol were obtained from Nacalai Tesque (Kyoto, Japan). Luminol was purchased from Sigma (St. Louis, MO, USA) and sodium hydroxide (NaOH) was from Merck (Tokyo, Japan). Purified water was prepared by a Simpli Lab UV (Millipore, Bedford, MA, USA) water device. 2-Henicosyl-1,4-naphthoquinone as an internal standard (IS) was synthesized from 1,4-naphthoquinone and behenic acid in our laboratory according to the previously reported method [16]. Other chemicals were of extra pure grade. Stock solutions of ubiquinone were prepared in ethanol and diluted with ethanol to prepare the working solutions. Solutions of DTT and luminol were prepared in methanol and NaOH aqueous solution, respectively, just before analysis.

2.2. Instrument

The HPLC system (Fig. 2) consisted of three LC-10AS liquid chromatographic pumps (Shimadzu, Kyoto), a Rheodyne 7125 injector (Cotati, CA, USA) with a 5-μL sample loop, a CLD-10A chemiluminescence detector (Shimadzu), a UNI-1 noise filter (Union, Gunma, Japan) and an SIC chromatorecorder (Tokyo, Japan). PTFE tubing

(15 m x 0.5 mm i.d., GL Sciences, Tokyo) was used as reaction coil. A Cosmosil 5C8-MS (150 mm x 4.6 mm, i.d., 5 μm, Nacalai Tesque) and methanol was used as stationary and mobile phase, respectively. The eluent from the column was mixed with 1.5 mM of DTT in methanol and 1.0 mM of luminol in 150 mM NaOH aqueous solution, simultaneously. The flow rates of the mobile phase, DTT and luminol solution were set at 0.50, 0.30 and 0.25 mL/min, respectively. For the comparison study, a UV-8010 UV detector (TOSOH, Tokyo) was used at a wavelength of 275 nm.

2.3. Assay procedure for ubiquinone in human plasma

Fifty micro liters of plasma were spiked with 10 μL of 2.26 μg/mL IS (2-henicosyl-1,4-naphthoquinone) solution, and then 10 μL of 0.3% hydrogen peroxide was added to oxidize ubiquinol to ubiquinone. One-hundred micro liter of ethanol was added to denature protein and then vortexed for 1 min. To this solution, 600 μL of *n*-hexane was added to extract ubiquinone and IS. The organic layer was taken and evaporated under reduced pressure, and the residue was reconstituted with 50 μL of methanol and then 5 μL was injected into the HPLC system. The present experiments were approved by the Ethics Committee of the School of Pharmaceutical Sciences, Nagasaki University, and performed in accordance with established guidelines.

3. Results and discussion

3.1. Optimization of HPLC conditions

Considering the high lipophilic nature of ubiquinone, an octyl column and methanol were selected as stationary and mobile phase, respectively. Ubiquinone eluted from the column was mixed with DTT and luminol solutions in the reaction coil and the generated CL was monitored. Figure 3A shows a typical chromatogram obtained by the proposed HPLC system. The peaks of ubiquinone and IS were detected at 15 and 30 min, respectively. The effects of the conditions of the proposed HPLC-CL system on peak height and S/N ratio of ubiquinone were investigated to obtain the highest sensitivity. In our previous study [15], it was observed that the CL intensity of ubiquinone after mixing with DTT and luminol increased slowly. Therefore, the mixed solution was introduced to CL detector after passing through a PTFE tube as a reaction coil in order to obtain suitable CL at the detector. The effects of coil length ranging from 9 to 18 m on peak height and S/N ratio were examined (Fig. 4). The peak height increased with the increase of coil length until 16 m and then decreased. Although the maximum peak height was achieved at 16 m, the reaction coil length was selected at 15 m in consideration of suitable S/N ratio. The reaction

coil length corresponds to the reaction time of 100 s. The reagent concentrations were also optimized. The effects of DTT concentration on peak height and S/N ratio were examined. Since the peak height and S/N ratio increased with the increase of DTT concentration till 1.5 mM, 1.5 mM was selected. Also, the influence of luminol concentration was examined (Fig. 5). It was found that the peak height increased with the increase in luminol concentration; 1.0 mM of luminol, which gave the maximum S/N ratio, was selected. The effects of concentration of NaOH solution for dissolving luminol on peak height and S/N ratio were examined. The peak height reached the maximum when NaOH concentration was more than 140 mM, and the 150 mM of NaOH gave the best S/N ratio.

3.2. Calibration curve and detection limit

A calibration curve for standard ubiquinone was constructed by plotting the peak height ratio to IS against the concentration of ubiquinone. The calibration curve of the proposed HPLC method was linear from 0.09 to 43.2 μg/mL (0.22-216 ng on column) with the correlation coefficient of 0.999, and the detection limit (S/N = 3) was 26 ng/mL (130 pg on column). The regression equation (mean ± standard error, n = 3) was 2.23 ± 0.02 + 0.15 ± 0.01. The sensitivity of the proposed HPLC-CL method was

approximately 60 times and 10 times higher than those of HPLC-UV [8] and HPLC with post-column colorimetric reaction method [9], respectively. Additionally, the sensitivity was almost comparable to those of HPLC-ECD with catalytic reduction [10], HPLC-ECD with electroreduction [11], HPLC-MS/MS [12] and ultra-performance liquid chromatography with mass spectrometry (UPLC-MS) [17] in spite of the complexity of the proposed detection system was lower compared to those of these methods.

3.3. Determination of UQ in human plasma

The developed HPLC-CL system was applied to determine the concentration of ubiquinone in human plasma. The preparation procedure for human plasma was modified according to the previous report [10]. Since it has been reported that the most of ubiquinone molecules are presented as reduced form (ubiquinol) in human plasma [18], oxidation by hydrogen peroxide was employed for the conversion of ubiquinol to ubiquinone prior to the extraction. A typical chromatogram of plasma extract from healthy subject by the proposed HPLC-CL method is shown in Fig. 3B. The peaks of ubiquinone and IS in plasma could be clearly detected on the chromatogram without interferences from other plasma components. To demonstrate the sensitivity and selectivity of the proposed method, the plasma extract was also

injected to HPLC-UV system using the same separation conditions as the HPLC-CL system. Figure 3C shows a chromatogram of plasma extract obtained by UV detection. In contrast with the result obtained by CL methods, several large peaks were observed and the determination of ubiquinone was difficult due to extremely weak peak response of ubiquinone. The calibration curve for recovered ubiquinone from human plasma was linear over the range from 0.09 to 8.63 μg/mL with a correlation coefficient of 0.999. The recovery and reproducibility of the proposed method were evaluated by the analysis of the plasma samples spiked with a known amount of ubiquinone. As shown in Table 1, the RSDs for within-day and between-day reproducibility ranged from 0.8 to 4.5% and from 2.7 to 3.9%, respectively, and the excellent recoveries were achieved more than 98.2%. The stability of ubiquinone in plasma samples was evaluated by comparing the results of stored samples under different conditions with freshly prepared samples. The short- and long-term stabilities were evaluated during storage at room temperature for 4 h and at −80 °C for 2 weeks, respectively. The freeze–thaw stability was evaluated after three freeze–thaw cycles from −80 °C to room temperature. As shown in Table 2, the results suggested the sufficient stability of ubiquinone during the storage period at room temperature for 4 h, at −80 ºC for 2 weeks and after three freeze-thaw cycles. In addition, the matrix effects were evaluated by comparing the peak heights obtained from the standard solution of ubiquinone with the post-extraction

plasma samples spiked with ubiquinone at three concentration levels. The matrix effects (mean ± SD, n = 3) for ubiquinone at concentrations of 0.43, 1.73 and 8.63 ng/mL were 95.6 ± 4.0%, 99.8 ± 5.3% and 95.3 ± 1.8%, respectively. The results revealed that there were no significant matrix effects from plasma extracts. Therefore, the proposed method allows highly accurate determination of ubiquinone in human plasma. The determined value of ubiquinone in human plasma (0.46 ± 0.18 μg/mL, n = 3) was well in accordance with the published data [11,19].

4. Conclusion

The present study is the first attempt to measure ubiquinone concentration in biological fluids using CL detection. We developed a new HPLC method for the analysis of ubiquinone based on the CL generated during the reaction of ubiquinone with DTT and luminol. The sensitivity of the method was sufficient to determine the concentration of ubiquinone in human plasma. The peak of ubiquinone in plasma was clearly detected without interferences when the proposed method was applied to human plasma sample. Therefore, the proposed method should be useful in a variety fields such as biological and pharmaceutical analyses.

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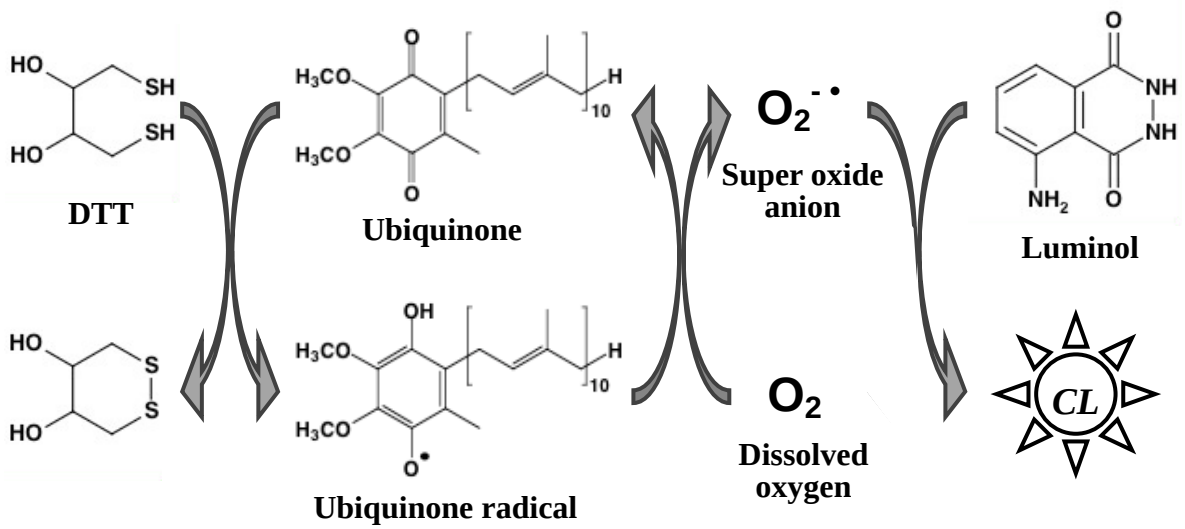

Fig. 1. Mechanism of the CL reaction for ubiquinone based on the redox cycle

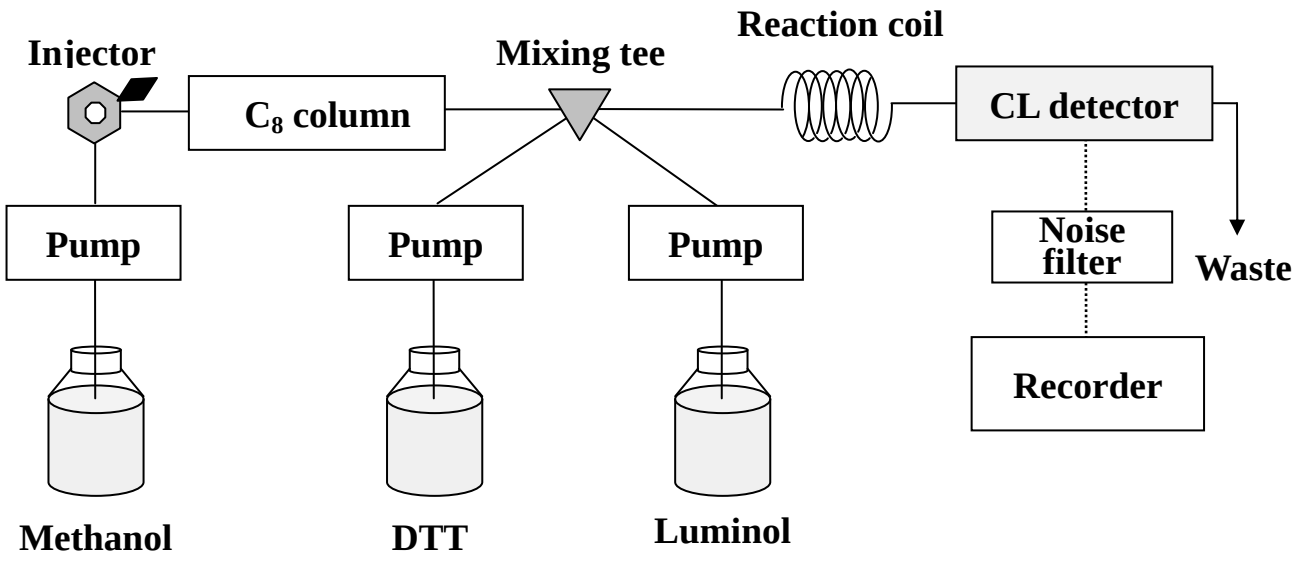

Fig. 2. Schematic diagram of the HPLC-CL system.

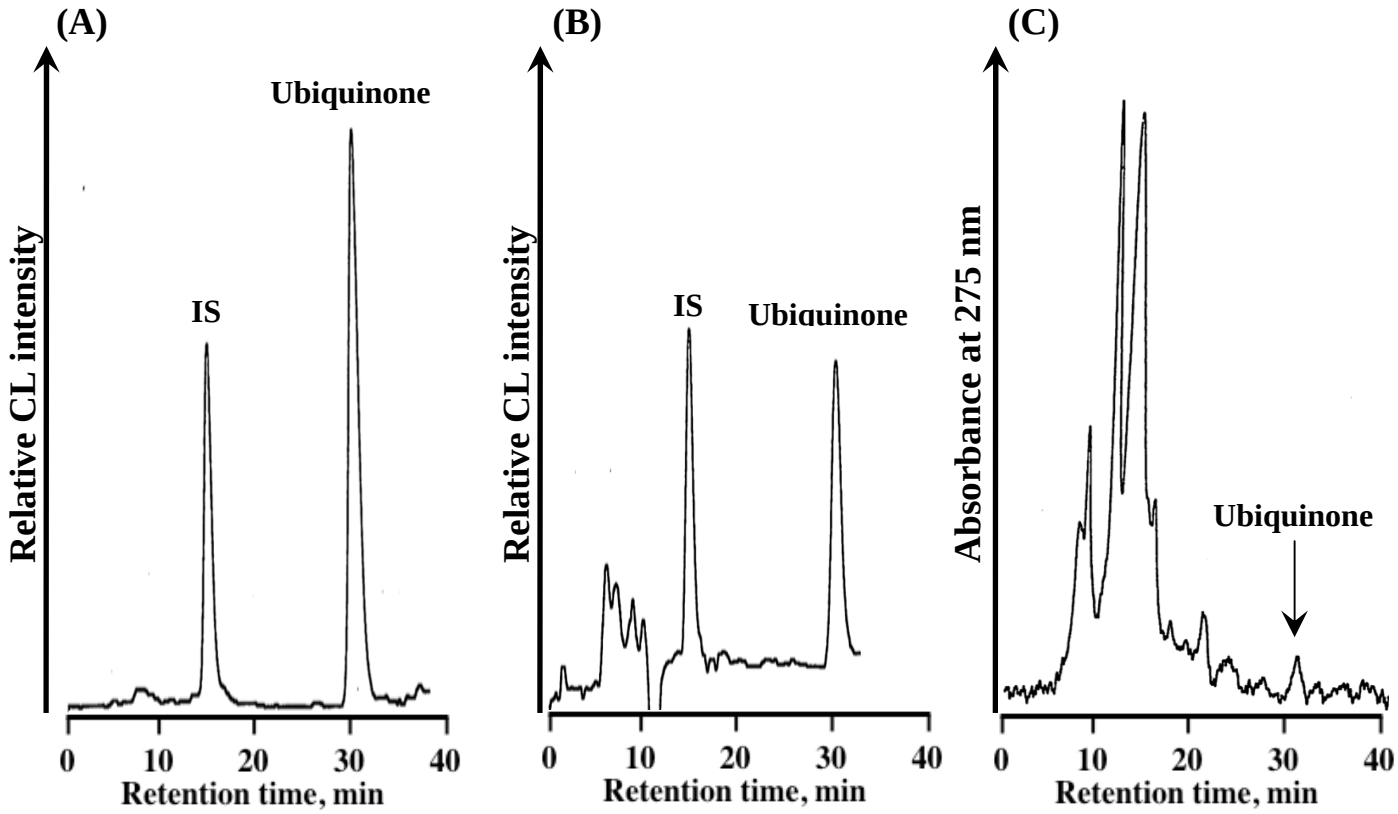

Fig. 3. **Chromatograms for (A) standard solution of 0.86 µg/mL ubiquinone and 0.45 µg/mL IS and (B) human plasma containing 0.40 µg/mL ubiquinone and 0.45 µg/mL IS obtained with the proposed CL detection and (C) human plasma containing 0.40 µg/mL ubiquinone obtained with UV detection.**

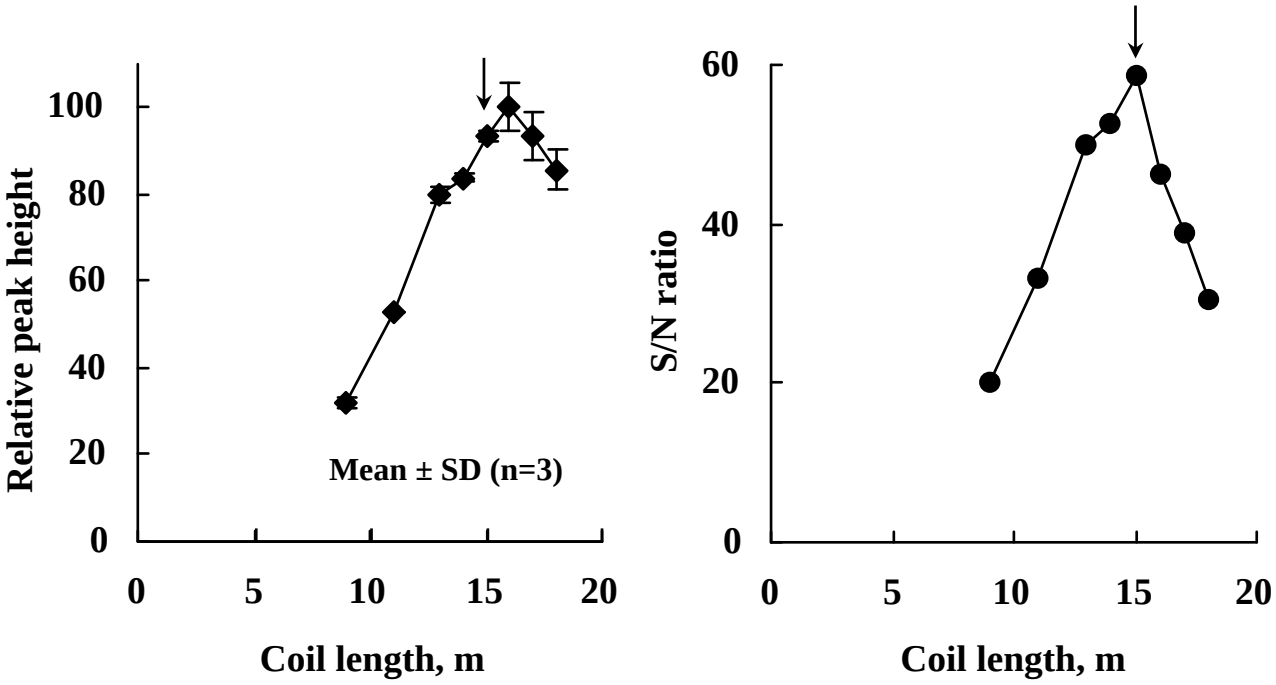

Fig. 4. Effects of reaction coil length on peak height and S/N ratio. The concentrations of ubiquinone, DTT, luminol and NaOH are 0.43 μg/mL, 1.5 mM, 1.0 mM and 150 mM, respectively.

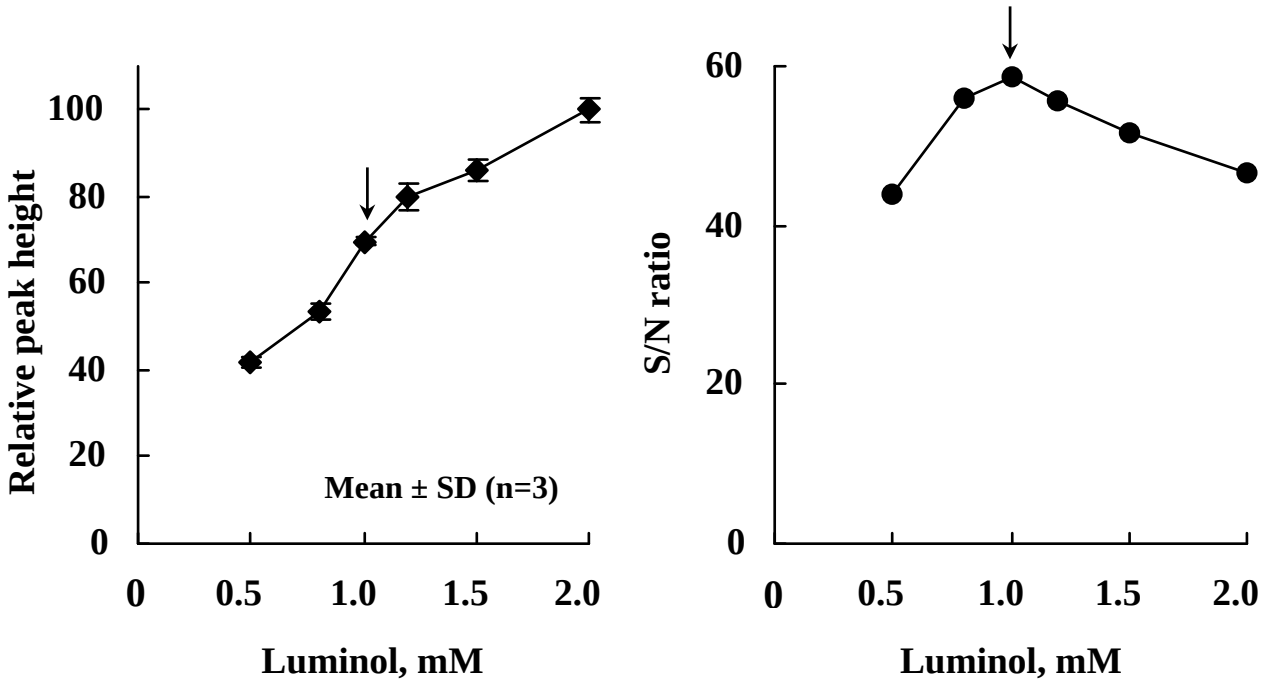

Fig. 5. Effects of luminol concentration on peak height and S/N ratio. The concentrations of ubiquinone, DTT and NaOH are 0.43 μg/mL, 1.5 mM and 150 mM, respectively. The reaction coil length is 15 m.

Table 1 Recovery of ubiquinone from spiked plasma samples

Within-day (n=5)			
Spiked concentration (μg/mL)	**Found ± SD (μg/mL)**	**RSD (%)**	**Recovery (%)**
0	**0.40 ± 0.02**	**4.5**	**-**
0.43	**0.84 ± 0.02**	**2.2**	**101**
1.73	**2.10 ± 0.06**	**2.7**	**98**
8.63	**9.07 ± 0.08**	**0.8**	**100**
Between-day (n=5)			
Spiked concentration (μg/mL)	**Found ± SD (μg/mL)**	**RSD (%)**	**Recovery (%)**
0.43	**0.85 ± 0.03**	**3.4**	**103**
1.73	**2.10 ± 0.06**	**2.7**	**99**
8.63	**9.01 ± 0.35**	**3.9**	**100**

Table 2 The stability of ubiquinone in human plasma samples

	Accuracy (%) (mean ± SD, n = 3)		
	Spiked concentration (μg/mL)		
	0.43	1.73	8.63
Room temperature for 4 h	90.5 ± 8.6	94.9 ± 4.1	95.5 ± 6.5
Freezer at -80 °C for 2 weeks	92.1 ± 7.3	93.0 ± 8.4	93.2 ± 4.8
Three-times freeze–thaw (-80 °C)	93.8 ± 3.1	94.8 ± 4.7	96.6 ± 6.5