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Determination Method for Pyrroloquinoline Quinone in Food Products by HPLC-UV Detection Using a Redox-Based Colorimetric Reaction

Mizuho Fukuda,¹ Naoya Kishikawa,¹,* Taketo Samemoto,¹ Kaoru Ohta,¹ Kaname Ohyama,¹ Mahmoud Hamed El-Maghrabey,¹,b Kazuto Ikemoto,¹ and Naotaka Kuroda*¹

¹Course of Pharmaceutical Sciences, Graduate School of Biomedical Sciences, Nagasaki University; 1–14 Bunkyo-machi, Nagasaki 852–8521, Japan; bc Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Mansoura University; Mansoura 35516, Egypt; and c Mitsubishi Gas Chemical Company, Inc., Niigata Research Lab.; Niigata 950–3112, Japan.

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We have developed an HPLC-UV method for the determination of pyrroloquinoline quinone (PQQ), which utilizes a redox-based colorimetric reaction. In the proposed colorimetric reaction, the redox reaction between PQQ and dithiothreitol generates superoxide anion radicals that can convert 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) to formazan dye. After PQQ separation on an octadecyl silica column, it was mixed online with dithiothreitol and INT, and the formed formazan dye was monitored by absorbance at 490 nm. The detection limit (S/N = 3) of the proposed method was 7.6 nM (152 fmol/injection). The proposed method could selectively detect PQQ in food products without any clean-up procedures.

Key words pyrroloquinoline quinone; food product; HPLC; colorimetric reagent; redox cycle

Introduction

Pyrroloquinoline quinone (PQQ) is one of the quinones that has been attracting attention in recent years due to its high functionality. PQQ is a water-soluble quinone that was discovered from methanol-assimilating bacteria in 1979,¹ and it has been reported to act as a cofactor for alcohol dehydrogenase and methanol dehydrogenase. It was reported that foods such as green pepper and broccoli sprout contain high levels of PQQ.² As a specific device and is not widely distributed.

We confirmed that quinones could be determined based on the redox cycle of quinone. For this purpose, a redox colorimetric reagent, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT), was employed as a post-column detection reagent instead of luminol. Although the HPLC-CL method allows sensitive detection of PQQ, there is a limitation that the CL detector is a specific device and is not widely distributed.

The most common detection mode is UV detection, and till now, PQQ was determined once by HPLC with UV detection after derivatization with acetone. However, the sensitivity was not good, and the method used a short wavelength for detection at 254 nm, which resulted in crowded chromatograms and decreased the method selectivity.¹³ In this study, we attempted to develop a sensitive and selective HPLC method for the determination of PQQ with a UV/Vis detector at a long detection wavelength based on the redox cycle of quinone. For this purpose, a redox colorimetric reagent, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT), was employed as a post-column detection reagent instead of luminol. We confirmed that quinones could be determined based on the absorbance measurement of formazan dye formed from the reaction between INT, quinone, and reductant, including DTT.²²,²³ In this study, we attempted to develop a sensitive and selective HPLC method for the determination of PQQ with a UV/Vis detector at a long detection wavelength based on the redox cycle of quinone. For this purpose, a redox colorimetric reagent, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT), was employed as a post-column detection reagent instead of luminol. We confirmed that quinones could be determined based on the absorbance measurement of formazan dye formed from the reaction between INT, quinone, and reductant, including DTT.²²,²³ (Fig. 1). After establishing the method by the optimization of HPLC conditions, the proposed HPLC-UV method was applied to determine PQQ content in food products.

Experimental

Materials and Reagents PQQ disodium salt was sourced from Mitsubishi Gas Chemical Co. Inc. (Tokyo, Japan).

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Sodium hydroxide was purchased from Merck Co. (Darmstadt, Germany). Sodium carbonate, DTT, tetra-n-butylammonium bromide (TBAB), sodium dihydrogen phosphate dihydrate, and disodium hydrogen phosphate were obtained from Nacalai Tesque (Kyoto, Japan). Acetonitrile and INT were from Kanto Chemical Co., Inc. (Tokyo, Japan) and Dojindo Laboratories (Kumamoto, Japan), respectively. Water was distilled and passed through a Yamato Pure Line WL21P system (Tokyo, Japan).

PQQ was used after being dissolved in water and appropriately diluted. INT was dissolved in 50 mM carbonate buffer (pH 10.2) and adjusted to 120 $\mu$M, and DTT was dissolved in acetonitrile and adjusted to 50 $\mu$M. The INT solution was stored in an amber bottle and used within 12 h of preparation.

**HPLC Instruments and Conditions** The HPLC system (Fig. 2) consisted of three Shimadzu LC-10AS pumps (Shimadzu, Kyoto, Japan), a Rheodyne 7125 injector (Cotati, CA, U.S.A.) with 20 $\mu$L loop, a HITACHI L-7300 column oven (Hitachi, Tokyo, Japan), a reaction coil made of polytetrafluoroethylene tubing (0.5 mm i.d. $\times$ 10 m), a JASCO 875-UV Intelligent UV/VIS Detector (JASCO, Tokyo, Japan) and a recorder.
Shimadzu CR-8A recorder. Cosmosil 5C18-AR-II (4.6 mm i.d. ×250 mm, 5 μm, Nacalai Tesque) was used for the separation of PQQ, and the column temperature was maintained at 35°C. The mobile phase was a mixture of 5 mM phosphate buffer (pH 7.4) containing 4 mM TBAB and acetonitrile (7:3, v/v) and flowed at 0.5 mL/min. The eluate from the column was mixed with 50 μM DTT in acetonitrile and 120 μM INT in carbonate buffer (pH 10.2) simultaneously at 0.25 mL/min each. The mixed solution was introduced into the UV detector after passing through the reaction coil, and the absorbance at λ = 490 nm was measured. The injection volume into the HPLC system was 20 μL.

### UV-Vis Spectral Measurement

In the absorption cell, 20 μL of 25 μM PQQ on water and 1500 μL of 120 μM INT in 50 mM carbonate buffer (pH 10.2) solution were mixed, and then 1500 μL of 50 μM DTT in acetonitrile was added. The absorption spectrum was measured immediately with an UV-visible absorption spectrophotometer (Shimadzu UV-1800).

### Application to Food Products

Supplementary capsules containing PQQ (Quality Supplement and Vitamins, Inc., Ft. Lauderdale, FL, U.S.A.) were opened, and 510 mg of the content was dissolved in 1 L of water. After 50 times dilution with water, 20 μL of the diluted solution was injected into the HPLC system after filtration through a 0.45 μm membrane filter. Besides, the method was applied for the determination of PQQ in juice samples. The commercially available vegetable juice (Yasaiseikatsu100, KAGOME Co., Ltd., Tokyo, Japan) was injected into the HPLC system after filtration without any clean-up procedure.

### Results and Discussion

#### Absorbance Change of INT after Reaction with PQQ and DTT

We confirmed whether PQQ could be detected using a colorimetric reagent based on the redox cycle of quinone although the absorbance of PQQ itself was negligible. The mixed solution of only INT and DTT was colorless (Fig. 3a), while the reaction solution consisting of PQQ, INT, and DTT immediately turned orange (Fig. 3b). From this result, it was considered that the superoxide anion radicals generated by the reaction of PQQ and DTT converted the coexisting INT into a formazan dye that has an absorption maximum at 490 nm.

#### Optimization of HPLC Conditions

Since PQQ is a highly water-soluble compound, TBAB was added to the mobile phase to retain PQQ on the octadecyl silica column. Under the separation conditions described above, PQQ was detected at 28.5 min on the chromatogram (Fig. 4).

To obtain higher sensitivity, the colorimetric reaction conditions were optimized using the standard solution of PQQ. The INT concentration was examined in the range of 60–200 μM. The peak height increased as the INT concentration increased, and the maximum peak height was obtained at 120 μM. At a higher concentration, the peak height was slightly decreased, so 120 μM was selected as the optimum INT concentration (Fig. 5a). The DTT concentration was examined in the range of 20–60 μM. The peak height increased as the DTT concentration increased, and the maximum peak height was obtained at 40 μM or higher. Therefore, 50 μM was selected as the optimum DTT concentration, which gave the most stable peak intensity (Fig. 5b). In addition to DTT, sodium borohydride was used as a reductant, but no absorbance peak of PQQ was detected. The reaction coil length was examined in the range of 4.5–13 m. The peak height increased with the extension of the coil length, and the maximum peak height was obtained at
Therefore, we selected 10 m as the reaction coil (Fig. 5c).

**Calibration Curve and Reproducibility** A calibration curve was constructed by plotting the peak height versus the concentration of PQQ. An excellent linear relationship ($r = 0.998$) was obtained in the concentration range of 20–2500 nM, and the detection limit ($S/N = 3$) was 7.6 nM (152 fmol/injection). The proposed method was 1.3, 13, 17, and 66 times more sensitive compared with HPLC with voltammetric detection, UV detection, fluorescence detection, and amperometric detection, respectively. On the other hand, the proposed method was less sensitive compared with GC-MS and LC-MS/MS, respectively. However, the proposed method does not require pre-column derivatization or complicated and expensive equipment.

The reproducibility of the proposed method was investigated at low, medium, and high concentrations (100, 500, 2500 nM) within the calibration curve range. The relative standard deviations (RSD) for within-day ($n = 5$) analyses were 2.3, 0.9, and 1.2%, respectively, and between-day ($n = 4$) investigations were 8.8, 9.2, and 9.2%, respectively.

**Application to Food Products** The amount of PQQ contained in the supplement was measured by the proposed method. The PQQ peak was detected at the same retention time as the standard solution (Fig. 6), and no peaks derived from contaminants were detected. The recovery calculated by spiking PQQ at 500 nM to the supplement sample was 96.9 ± 5.7% ($n = 4$). The PQQ content per capsule determined by the proposed method was 9.41 ± 0.18 mg ($n = 6$), which was almost consistent with the indicated value of 10 mg. Moreover, the proposed method was applied to detect PQQ in the vegetable juice sample. Figures 7a and 7b show typical chromatograms of the vegetable juice and the vegetable juice spiked with standard PQQ. Although several peaks derived from components in the vegetable juice were detected, the PQQ peak could be detected without any interferences. The content of PQQ in vegetable juice was determined to be 300 nM.

**Conclusion** In the present study, we developed an HPLC-UV method for the determination of PQQ using a redox-based colorimetric reaction. The proposed method utilizes a quinone-specific property, which is the generation of superoxide anion radicals through the redox reaction cycle. The superoxide anion radicals generated from PQQ and DTT converted colorimetric reagent, INT, into a formazan dye having UV absorption at 490 nm. The proposed method is the first one to utilize a redox-based colorimetric reaction for the determination of quinone. The proposed method was able to measure PQQ with the detection limit ($S/N = 3$) of 7.6 nM using common HPLC equipment. The method was applied successfully for the determination of PQQ in supplemental capsules, and also we were able to detect PQQ in juice samples. The proposed method would be a useful analytical tool in the field of food chemistry and following PQQ levels in different edible products and supplements.

**Conflict of Interest** This study was funded by Mitsubishi Gas Chemical Company, Inc.

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