J Fluoresc (2011) 21:2093–2100
DOI 10.1007/s10895-011-0908-1

ORIGINAL PAPER

Determination of Ubiquinone in Blood by High-Performance Liquid Chromatography with Post-Column Fluorescence Derivatization Using 2-Cyanoacetamide

Yukio Nohara · Junko Suzuki · Hiroaki Kubo

Received: 11 March 2011 / Accepted: 27 May 2011 / Published online: 7 June 2011

Abstract It was shown that ubiquinone (CoQ_{10}) and ubiquinol ($CoQ_{10}H_2$) produce fluorescence products under alkaline conditions when reacted with 2-cyanoacetamide. The reaction mixture from CoQ_{10} gave fluorescence with excitation and emission maximum wavelengths at 442 nm and 549 nm, respectively. This reaction was considered to proceed via Craven's reaction. Moreover, 2-cyanoacetamide was shown to be a useful reagent for high-performance liquid chromatography (HPLC) with post-column fluorescence derivatization of CoQ_{10} and $CoQ_{10}H_2$ in blood. CoQ_{10} showed a linear response in the range of 0.32–1276 ng, and the detection limit (S/N=3) was 0.16 ng. Moreover, the sample pretreatment by deproteinization and extraction of CoQ_{10} and $CoQ_{10}H_2$ from plasma using 1-propanol with potassium formate was effective for excellent separation of CoQ_{10} and $CoQ_{10}H_2$ from other fluorescent substances in the blood. This simple and rapid pretreatment was considered to minimize the oxidation of $CoQ_{10}H_2$. On the other hand, CoQ_{10} and $CoQ_{10}H_2$ in plasma samples obtained by finger prick were detected, as in venous blood obtained by venipuncture. Our method involving the simple and rapid collection of plasma by finger prick and sample pretreatment is thought to be applicable for the determination of $CoQ_{10}H_2$/total CoQ_{10} ratio as a biomarker of oxidative stress.

Keywords Ubiquinone · Ubiquinol · Finger prick · 2-Cyanoacetamide · Fluorescence · HPLC

Y. Nohara (✉) · H. Kubo
Faculty of Pharmacy, Iwaki Meisei University,
Iwaki, Fukushima 970–8551, Japan
e-mail: ynohara@iwakimu.ac.jp

J. Suzuki
School of Pharmacy, Kitasato University,
Minato-ku, Tokyo 108–8641, Japan

Introduction

It is known that ubiquinone (coenzyme Q10, CoQ_{10}) is an essential component of the mitochondrial respiratory chain responsible for oxidative phosphorylation, and may prevent oxidative damage in the human body as an antioxidant [1, 2]. CoQ_{10} is an endogenous enzyme cofactor produced in all living cells in humans; however, its cellular concentrations decrease both with age and in certain pathological conditions, which include myocardial disease [3, 4], phenylketonuria [5], mitochondrial disease [6], mevalonate kinase disease [7]. Because the administration of CoQ_{10} to humans has been considered as an effective way of maintaining the blood level of CoQ_{10}, it has been used as a dietary and health supplement for the prevention of lifestyle-related disease induced by free radical damage and aging [8–10]. CoQ_{10}, which is similar in chemical structure to vitamins K and E, is characterized by a benzoquinone ring attached to 10 repeating series of side chain isoprene units. Its reduced biologically active form of CoQ_{10}, ubiquinol ($CoQ_{10}H_2$), is a lipid-soluble antioxidant, which prevents lipid peroxidation in plasma lipoproteins and biological membranes [1, 11]. Major component of the total CoQ_{10}, which is the sum of CoQ_{10} and $CoQ_{10}H_2$ concentration, in human blood exists in the reduced form, and decrease of $CoQ_{10}H_2$/total CoQ_{10} ratio may be a useful biomarker of oxidative stress [12, 13].

Several procedures have been developed to analyze CoQ_{10} in biological samples by reverse-phase high-performance liquid chromatography (HPLC) coupled with UV [14, 15], electrochemical [12, 16], or mass spectrometry [13, 17] detection. HPLC methods employing electrochemical and mass spectrometry detectors have higher selectivity and sensitivity than those with a UV detector; however, these methods are not available in many laboratories because of a lack of specialized equipment. On the other hand, the

conventional pretreatment method of human plasma or serum for HPLC analysis of CoQ_{10} is complicated. This method needs steps of deproteinization with extraction by alcohol and hexane, and evaporation of hexane layer under nitrogen to prevent oxidation of $CoQ_{10}H_2$ to CoQ_{10}; finally, the residue is dissolved in solvent as sample solution. The simple pretreatment requires a precise but convenient method since $CoQ_{10}H_2$ is oxidized easily when exposed to air [16]. It was reported that the $CoQ_{10}H_2$ is unstable in whole blood, plasma, hexane or 1–propanol extract of human plasma [12, 18]. Therefore, several investigators improved pretreatment method [16]. Abdul-Rasheed *et al.* developed a method that involves HPLC coupling with a UV detector using a one-step ice-cold 1-propanol extraction step from plasma [14]. However, a plasma sample volume of 150–300 μL was needed for total CoQ_{10} analysis, and the blood was taken from the antecubital vein. Nowadays, many portable analyzers are available for hemoglobin A_{1c} [19], glucose [20], or lactate [21] in blood obtained by finger prick, which uses a small blood volume of 1–5 μL. The sample of blood obtained by finger prick is necessary for rapid assay, because the blood sampling is easy to correct.

HPLC coupled with a fluorophotometric detector has been widely used in the determination of various substances in biological samples because of high selectivity and sensitivity. It was reported that ethyl cyanoacetate was used for pre-column derivatization of CoQ_{10} in serum for fluorimetric detection in HPLC; however, the method took 20 h for fluorescence derivatization [22]. CoQ_{10} needs fluorescence derivatization for the development of fluorimetric detection because it is not a fluorescent substance itself. 2-Cyanoacetamide, which has been used as a post-column derivatization reagent of fluorimetric HPLC for catecholamines [23], 3,4-dihydroxyphenylalanine [24], and unsaturated disaccharides [25, 26], is similar in chemical structure to ethyl cyanoacetate. We found that CoQ_{10} and $CoQ_{10}H_2$ reacted with 2-cyanoacetamide under alkaline conditions with heating to form fluorescence product. This reaction was applied to HPLC with a post-column fluorescence derivatization of CoQ_{10} and $CoQ_{10}H_2$ in blood. In addition, simple pretreatment of human blood adopted in the HPLC with fluorescence detection of CoQ_{10} and $CoQ_{10}H_2$ was investigated. Moreover, the application of this method to the detection of CoQ_{10} and $CoQ_{10}H_2$ in plasma obtained by finger prick is described in this paper.

Materials and Methods

Materials

Water was purified using a MILLI–Q Labo from Nihon Millipore Kogyo (Yonezawa, Japan). 2-Cyanoacetamide was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). CoQ_{10} (99.5%), potassium trifluoroacetate and HPLC–grade acetonitrile were from Wako Pure Chemical Industries (Osaka, Japan). Potassium hydroxide, potassium formate, sodium borohydride, HPLC–grade ethanol and 2-propanol were from Kanto Chemical (Tokyo, Japan). All other chemicals were of analytical grade.

Fluorophotometric Determination

Fifty μL of 1 mmol/L 2-cyanoacetamide in ethanol, 50 μL of 0.5 mmol/L CoQ_{10} in ethanol, and 800 μL of organic solvent, were mixed, and then 100 μL of 40 mmol/L potassium hydroxide in ethanol was added to the mixture. The mixture was heated at 60°C for 5 min, then after cooling, the excitation and emission spectra were measured using a spectrofluorometer (RF–5000, Shimadzu, Kyoto, Japan).

Apparatus

The liquid chromatograph consisted of two isocratic pumps (PU–2080 Plus, JASCO, Tokyo, Japan), manual sample injector (7725i, Rheodyne), column oven (655–A52, Hitachi, Tokyo, Japan), reaction oven (S–3850, Soma Optics, Tokyo, Japan), and integrator (807–IT, JASCO, Tokyo, Japan). Detection was performed with a fluorescence detector (FP–2020 Plus, JASCO, Tokyo, Japan).

Chromatographic Conditions

Figure 1 shows a flow diagram of the HPLC with a post-column fluorescence derivatization system for the analysis of CoQ_{10} and $CoQ_{10}H_2$. The eluent was a mixture of acetonitrile:2-propanol (45:55 v/v%) containing 1 mmol/L 2-cyanoacetamide and 3 mmol/L potassium trifluoroacetate. It was delivered from pump A at a flow rate of 0.8 mL/min. A 20 μL sample was injected via the injector. The CoQ_{10} and $CoQ_{10}H_2$ from other fluorescent substances in plasma were separated in the ODS column (Inertsil ODS-3, 150×4.6 mm i.d., particle size 5 μm) at 25°C. The eluate was mixed with 20 mmol/L potassium hydroxide in ethanol as alkaline solution delivered from pump B at a flow rate of 0.2 mL/min. The mixture was reacted in a coil (5 m×0.5 mm i.d.) then placed in the oven at 85°C. After cooling in a coil (3 m×0.5 mm i.d.), the fluorescence intensity was monitored with the fluorescence detector (excitation at 460 nm, emission at 550 nm, slit: 40 nm, response: slow).

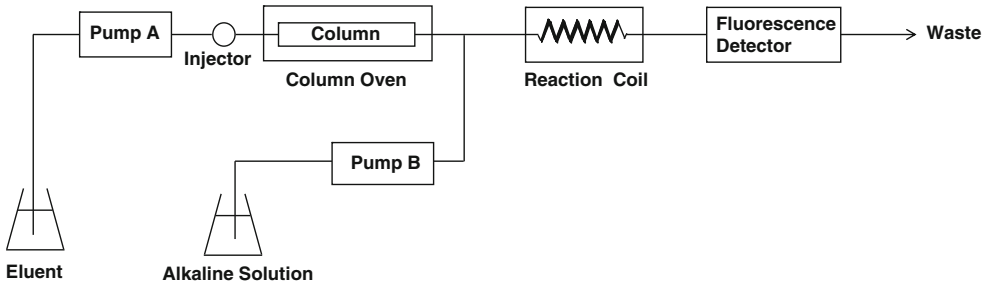

Fig. 1 A flow diagram of the HPLC–fluorescence detection of CoQ_{10} and $CoQ_{10}H_2$. Eluent: mixture of acetonitrile:2–propanol (45:55 v/v%) containing 1 mmol/L 2-cyanoacetamide and 3 mmol/L potassium trifluoroacetate. Alkaline solution: 20 mmol/L ethanolic potassium hydroxide solution. Pump A and B: flow rate at 0.8 and 0.2 mL/min, respectively. Injector: manual sample injector. Column: ODS column (Inertsil ODS-3, 150×4.6 mm i.d., particle size 5 μm). Column Oven: temperature at 25°C. Oven: reaction coil (5 m×0.5 mm i.d.) at 85°C with cooling coil (3 m×0.5 mm i.d.). Fluorescence Detector: excitation and emission wavelengths at 460 nm and 550 nm, respectively

Pretreatment of Plasma Samples Collected by Finger Prick

Forty μL of blood was collected in a heparin-treated hematocrit capillary tube (VC-H075H, TERUMO, Tokyo, Japan) by finger prick using a lancing device with a lancet (FINETOUCH, TERUMO, Tokyo, Japan). The blood was then centrifuged at 10,000 rpm for 10 s at room temperature, and 20 μL of the supernatant was used for analysis immediately. The plasma samples of healthy volunteers were included after informed consent had been obtained.

A 20 μL sample of plasma was vortex-mixed with 40 μL of 1-propanol for 10 s, and then 5 mg of potassium formate was added to the mixture. The mixture was vortex-mixed for 20 s, and was then centrifuged at 10,000 rpm for 60 s. The upper layer of 20 μL was injected onto an HPLC directory.

Preparation of $CoQ_{10}H_2$

The $CoQ_{10}H_2$ standard solution was prepared from reduction of CoQ_{10} by sodium borohydride [27]. First, 890 μL of ethanol and 100 μL of CoQ_{10} in ethanol were mixed in a tube, and then 10 μL of 0.25% sodium borohydride aqueous solution was added to the mixture. The mixture was left to stand for 10 min at room temperature, and the $CoQ_{10}H_2$ standard solution was used within 2 h. The reduction rate of CoQ_{10} to $CoQ_{10}H_2$ was determined by HPLC using UV detection at 275 nm.

Results and Discussion

Fluorophotometric Detection

Figure 2 shows excitation and emission spectra of CoQ_{10} after the reaction with 2-cyanoacetamide containing ethanol and 2-propanol under alkaline conditions. The excitation and emission maximum wavelengths from the mixture after the reaction were 442 nm and 549 nm, respectively. Figure 3 shows a scheme of the production of fluorescent substance by the reaction of CoQ_{10} and $CoQ_{10}H_2$ with 2-cyanoacetamide under alkaline conditions. Craven's reaction, which is a specific color reaction for quinone, produces transitory blue-colored products by the displacement of methoxyl substituents from the CoQ_{10} with active methylene compounds such as ethyl cyanoacetate under alkaline conditions [28–31]. Moreover, Craven's reaction gives fluorescent products at a later stage of the reaction. Rokos reported that CoQ_{10} incubated at room temperature for 12–24 h in ethyl cyanoacetate with methanolic potassium hydroxide solution resulted in fluorescent products with excitation at 430 nm and at emission 530 nm [30]. 2-Cyanoacetamide is an active methylene compound with a cyano group, such as ethyl cyanoacetate, and the reaction

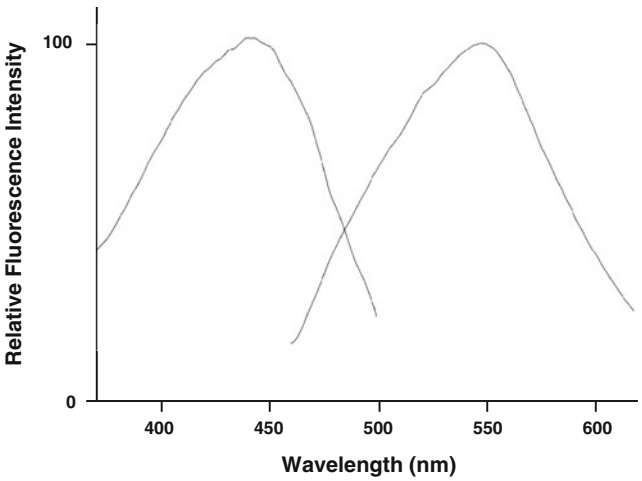

Fig. 2 The fluorescence excitation and emission spectra of 0.03 mmol/L of CoQ_{10} allowed to react with 1.25 mmol/L of 2-cyanoacetamide and 4 mmol/L of potassium hydroxide in ethanol:2–propanol (1:4 v/v%) at 60°C for 5 min

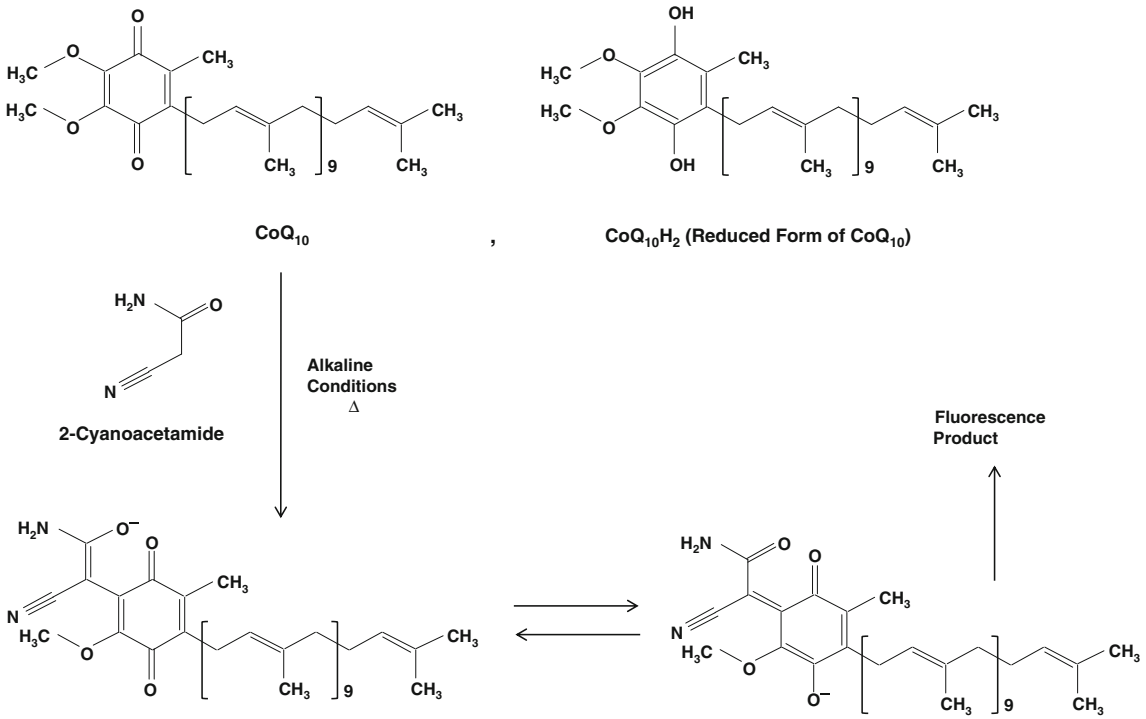

Fig. 3 Possible mechanism of fluorescence derivatization by the reaction of CoQ_{10} and $CoQ_{10}H_2$ with 2-cyanoacetamide under alkaline conditions

with CoQ_{10} under alkaline conditions showed fluorescent products with similar wavelength of excitation and emission. Therefore, it is considered that the reaction proceeds through Craven's reaction.

Fluorescence derivatization using ethyl cyanoacetate with CoQ_{10} was applied to pre-column HPLC by Abe *et al.* in 1977 [22]. However, this method was not useful for rapid analysis because of the long duration of fluorescence derivatization. The fluorescence intensity of the reaction mixture at 50°C peaked at 20 h. In contrast, our method using 2-cyanoacetamide was available for HPLC with post-column fluorescence derivatization because the fluorescence intensity peaked at 5 min (Fig. 4).

The fluorescence spectra and intensity were affected by organic solvents and water in the fluorescence reaction. Fluorescence was shown in the presence of CoQ_{10}, and the spectra were measured at an excitation wavelength of 330 nm. The reaction mixture containing acetonitrile showed an emission maximum wavelength of 560 nm (Fig. 5a), and 1-propanol and 2-propanol showed the same spectrum. However, the reaction mixture containing ethyl acetate showed an emission maximum wavelength of 500 nm (Fig. 5b), and methanol and ethanol showed the same spectrum. The addition of water, acetone or 2-butanol to the reaction mixture inhibited the fluorescence reaction, and spectra showed no fluorescence (their solvent volume was 80 v/v%). The eluent composition of acetonitrile and 2-propanol was used for HPLC because they showed the same fluorescence spectra, and excellent separation of CoQ_{10} and $CoQ_{10}H_2$ from other fluorescent substances in blood. Moreover, acetonitrile is useful at low column pressure.

Fluorimetric HPLC for CoQ_{10}

We applied the fluorescence derivatization of 2-cyanoacetamide with CoQ_{10} under alkaline conditions to fluorimetric HPLC. The chromatographic conditions were optimized using a standard solution of CoQ_{10}. The optimal concentration of reagents for the fluorescence derivatization was investigated over the ranges of 0.1–3 mmol/L for 2-

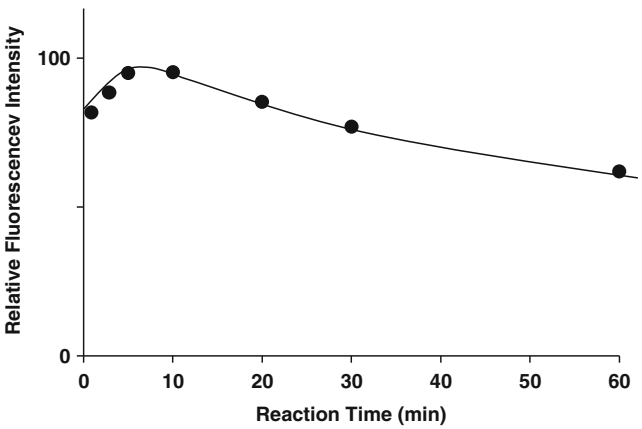

Fig. 4 The effect of reaction time on the reaction of CoQ_{10} with 2-cyanoacetamide under alkaline conditions. Conditions: 0.03 mmol/L of CoQ_{10}, 1.25 mmol/L of 2-cyanoacetamide, 4 mmol/L of potassium hydroxide reacted at 60°C in ethanol:2–propanol (1:4 v/v%)

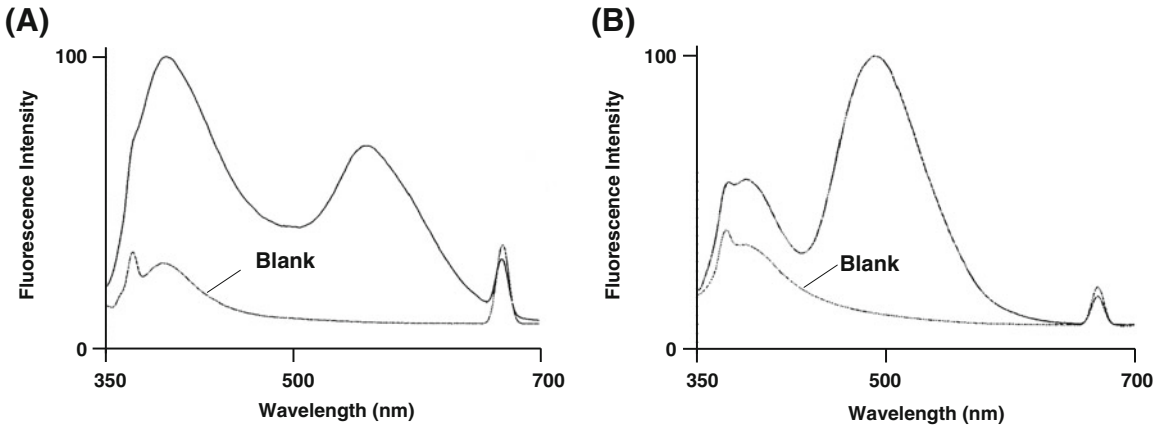

Fig. 5 The fluorescence spectrum (excitation at 330 nm) of the reaction mixture from the reaction of CoQ_{10} with 2-cyanoacetamide under alkaline conditions. Conditions: 0.03 mmol/L of CoQ_{10}, 1.25 mmol/L of 2-cyanoacetamide, 4 mmol/L of potassium hydroxide reacted at 60°C for 5 min in **a** ethanol:acetonitrile (1:4 v/v%) or **b** ethanol:ethyl acetate (1:4 v/v%). Blank: absence of CoQ_{10}

cyanoacetamide, and 5–100 mmol/L for potassium hydroxide. The maximum peak areas for the fluorescence of CoQ_{10} were obtained at concentrations of 1 mmol/L 2-cyanoacetamide, and 20 mmol/L potassium hydroxide. The reaction temperature was investigated over the range of 70–100°C. The maximum peak area was observed at a temperature of 85°C. The optimal wavelengths of detector were investigated over the ranges of 440–470 nm for excitation, and 530–565 nm for emission. The maximum peak areas were obtained at wavelengths of 460 nm, and 550 nm, respectively. In addition, 3 mmol/L potassium trifluoroacetate in the eluent was effective for excellent separation of CoQ_{10} and $CoQ_{10}H_2$ from other fluorescent substances in blood. On the other hand, 2-cyanoacetamide was soluble and stable in the eluent. Therefore, this post-column derivatization HPLC system was simplified because only two pumps were used to deliver the eluent containing 2-cyanoacetamide and the alkaline solution.

The sample pretreatment by deproteinization and extraction of CoQ_{10} and $CoQ_{10}H_2$ in plasma using 1-propanol with potassium formate was effective for excellent separation of CoQ_{10} and $CoQ_{10}H_2$ from other fluorescent substances in blood. The addition of a salt to the mixture of water and aliphatic alcohol occurs in two phase equilibrium of water and alcohol layer, for instance, the combination of 1-propanol and water containing sodium chloride or potassium chloride [32]. This salting out effect is useful as a simple and rapid method for the pretreatment of biological samples. Hasegawa *et al.* reported that cortisol and cortisone in umbilical cord blood were prepared using acetonitrile for deproteinization with anhydrous sodium carbonate and sodium chloride for separation of water layer and acetonitrile layer as a sample for chemiluminescence HPLC [33].

Figure 6a shows a chromatogram of standard solution of CoQ_{10}. CoQ_{10} showed a linear response in the range of 0.32–1276 ng (0.032–127.6 μg/mL) and the correlation coefficients of the curve was more than 0.9998. The detection limit (S/N=3) of CoQ_{10} was 0.16 ng.

Figure 6b shows a chromatogram of standard solution of $CoQ_{10}H_2$. It was found that $CoQ_{10}H_2$ reacted with 2-cyanoacetamide under alkaline conditions the same as CoQ_{10}. $CoQ_{10}H_2$ in the reaction coil of HPLC may react with 2-cyanoacetamide via CoQ_{10}, because $CoQ_{10}H_2$ was oxidized under alkaline conditions with heating [34].

Figure 6c and d show chromatograms of plasma from the antecubital vein of a healthy volunteer before and after the addition of 2-cyanoacetamide 1 mmol/L to the mobile phase. CoQ_{10} and $CoQ_{10}H_2$ were not detected, and unknown peak of other fluorescent substance was detected shown in Fig. 6c. In contrast, CoQ_{10} and $CoQ_{10}H_2$ were simultaneously detected, and excellent separation of CoQ_{10} and $CoQ_{10}H_2$ from other fluorescent substances was achieved within 15 min shown in Fig. 6d. The coefficient of variation of within-run (n=6) for CoQ_{10} of 99.7 ng and 6250 ng added to 50 μl of plasma were 1.76% and 1.16%, and of day to day (n=5) were 3.52% and 1.79%. The recoveries for CoQ_{10} as the same conditions were 100.3% and 90.6%. CoQ_{10} concentrations were measured in blood plasma of three male non-smoking healthy volunteers. The concentration of CoQ_{10} was found to equal 31.4±24.1 (mean ± S.D.) ng/mL, which was in accordance with the values of 49.1±19.1 ng/mL from plasma of white male non-smokers reported previously [18]. The oxidation of $CoQ_{10}H_2$ to CoQ_{10} was considered to minimize by deproteinization and extraction using 1-propanol with potassium formate for the sample pretreatment.

CoQ_{10} And $CoQ_{10}H_2$ from Plasma Samples Obtained by Finger Prick

Analysis of serum and plasma CoQ_{10} and $CoQ_{10}H_2$ derived from venous blood by HPLC is conventionally used. The

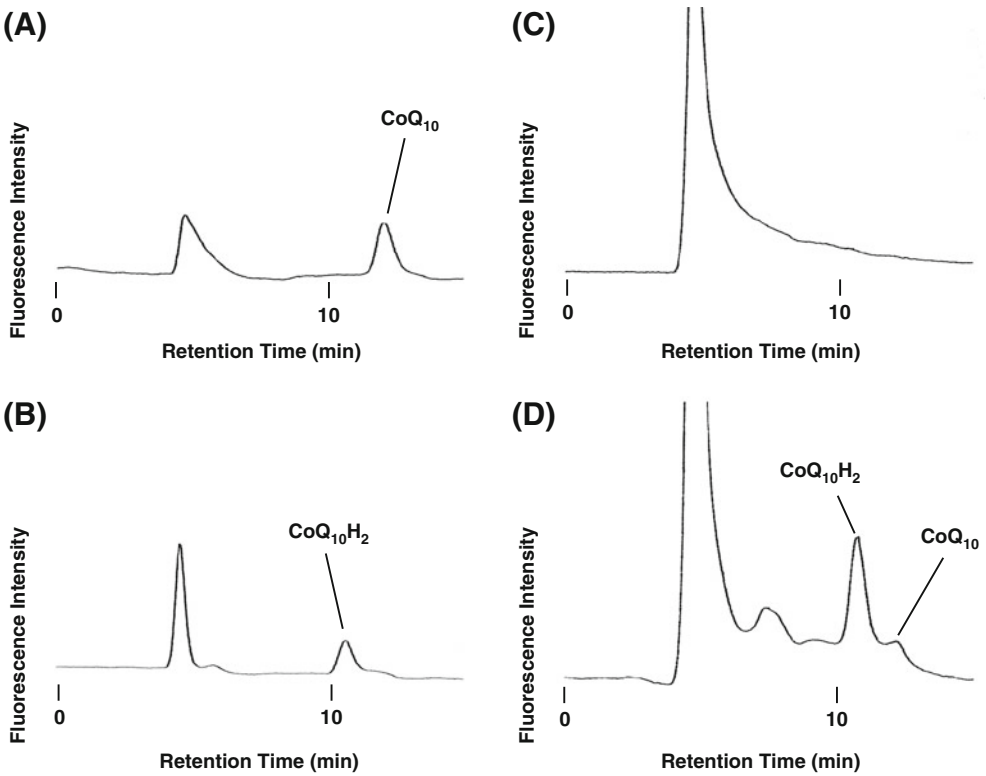

Fig. 6 Chromatograms of standard solution of CoQ_{10} (5.0 ng/injection) (**a**), and $CoQ_{10}H_2$ (2.5 ng/injection) (**b**), plasma from the antecubital vein of a healthy volunteer before (**c**) and after (**d**) the addition of 2-cyanoacetamide 1 mmol/L to the mobile phase

blood sampling process by venipuncture is invasive and requires blood-drawing techniques using syringes. Self-collection of blood by finger prick with an automatic lancet is less invasive and much easier; therefore, it is useful for self-monitoring such as blood glucose level and hemoglobin A_{1c}. Moreover, dried blood spots obtained by finger prick are used for the therapeutic drug monitoring (TDM) of anti-epileptics [35], benzodiazepines [36], antibiotics [37], analgesics [38], and others. Therefore, the sample of blood obtained by finger prick is useful for rapid and easy assay. Figure 7 shows a chromatogram of plasma from a healthy volunteer obtained by finger prick. The sample solution was obtained by deproteinization and extraction using 1-propanol with potassium formate. CoQ_{10} and $CoQ_{10}H_2$ were simultaneously detected, and the chromatogram was the same as the chromatogram of plasma from the antecubital vein shown in Fig. 6d. The average concentration of CoQ_{10} in plasma from three male non-smoking healthy volunteers obtained by finger prick was found to equal 46.0±29.8 ng/mL. This value is similar to the one obtained for plasma from the antecubital vein of healthy volunteers. The percentage of $CoQ_{10}H_2$ in total CoQ_{10} is a candidate marker of oxidative stress because the ratio may be lower in patients with certain conditions, such as heart disease, DNA damage, and chronic active hepatitis [12, 13, 39]. However, the rapid sample preparation is required during the measurement process to minimize $CoQ_{10}H_2$ oxidation [16]. The present HPLC procedure may be useful for determination of $CoQ_{10}H_2$/total CoQ_{10} ratio using simple and rapid pretreatment of plasma obtained by finger prick.

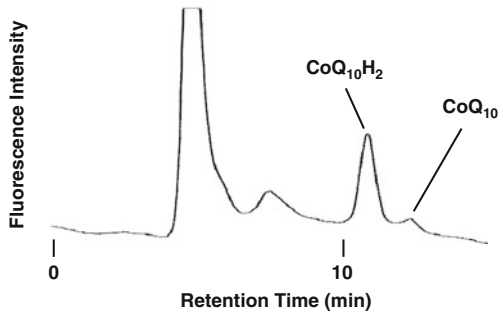

Fig. 7 Chromatogram of plasma from a healthy volunteer obtained by finger prick

Conclusion

It was found that the reaction of CoQ_{10} and $CoQ_{10}H_2$ with 2-cyanoacetamide under alkaline conditions generates fluorescent products, and the reaction was considered to proceed via Craven's reaction. The fluorescence intensity from the reaction mixture at 60°C peaked at 5 min; therefore, it was adopted for HPLC with post-column fluorescence derivatization of CoQ_{10} and $CoQ_{10}H_2$ in the blood. This HPLC system required only two pumps to

deliver the eluent and the alkaline solution, because 2-cyanoacetamide was soluble in the eluent. CoQ_{10} showed a linear response in the range of 0.32–1276 ng, and the detection limit (S/N=3) was 0.16 ng. Moreover, the sample pretreatment by deproteinization and extraction of CoQ_{10} and $CoQ_{10}H_2$ in plasma using 1-propanol with potassium formate gave excellent separation of CoQ_{10} and $CoQ_{10}H_2$ from other fluorescent substances in blood, and the simple and rapid pretreatment of blood samples was considered to minimize the oxidation of $CoQ_{10}H_2$ to CoQ_{10}. On the other hand, the plasma sample obtained by finger prick gave a similar chromatogram to that of venous blood obtained by venipuncture, and CoQ_{10} was at the same level. The simple and rapid collection of plasma by finger prick and extraction pretreatment using 1-propanol with potassium formate may be useful for determination of the $CoQ_{10}H_2$/total CoQ_{10} ratio as a biomarker of oxidative stress.

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