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Short communication

Automated high-performance liquid chromatographic method with precolumn reduction for the determination of ubiquinol and ubiquinone in human plasma

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Abstract

We developed a gradient HPLC method with automated precolumn reduction for direct electrochemical detection of ubiquinol-10 ($CoQ_{10}H_2$) and total coenzyme Q_{10} (TQ_{10}) in human plasma. The concentration of ubiquinone-10 (CoQ_{10}) was calculated by subtraction of $CoQ_{10}H_2$ from TQ_{10}. Preparation of reducing agent and precolumn reduction was performed by a programmable auto-injector. The two mobile phases used were: A, 100% of methanol containing 50 m*M* sodium perchlorate and 10 m*M* perchloric acid; and B, a mixture of ethanol and *tert.*-butanol (80:20, v/v). Sample preparation was simply a deproteinisation process with 10-fold ethanol. A good linear relationship was obtained for $CoQ_{10}H_2$ concentration from 0.1 to 3 μmol/l. The detection limit was 2.5 nmol/l with an injection volume of 20 μl. The analytical recovery and reproduciblity were generally >90%. To validate the method, 18 freshly collected plasma samples of normal healthy subjects were analysed. The mean ratio of $CoQ_{10}H_2/CoQ_{10}$ was 93:7. The proposed method is sensitive, reliable and can be used for clinical investigation.

Keywords: Ubiquinol; Ubiquinone

1. Introduction

Ubiquinol-10 ($CoQ_{10}H_2$), the reduced form of coenzyme Q10, is a powerful lipid-soluble antioxidant present in human plasma [1,2]. The oxidized form is known as ubiquinone-10 (CoQ_{10}). Oxidation of plasma lipoproteins was regarded as a crucial step in atherogenesis [3] and in other diseases linked to increased free radical formation [4]. $CoQ_{10}H_2$ can protect low-density lipoprotein (LDL) from lipid peroxidation by scavenging peroxyl radicals and reducing α-tocopheryl radicals [5–7]. It is the first antioxidant to be oxidized when exposed to oxidants despite its lower concentration compared to that of α-tocopheryl, another principal antioxidant in plasma [8,9]. The antioxidative activity of coenzyme Q10 depends not only on its concentration but also on its redox status. The ratio of $CoQ_{10}H_2$ to CoQ_{10} in plasma may represent an early biomarker of oxidation. It has been reported that this ratio was significantly lower for patients with coronary artery

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disease (CAD) than that of the healthy controls [10]. An altered $CoQ_{10}H_2/CoQ_{10}$ ratio may be the most sensitive indicator of oxidative stress in vivo.

Several HPLC methods for the determination of total coenzyme Q_{10} (TQ_{10}: the sum of $CoQ_{10}H_2$ and CoQ_{10}) have been described recently [11–17]. Simultaneous measurement of CoQ_{10} and $CoQ_{10}H_2$ with in-line ultraviolet and electrochemical (EC) detection has also been reported [12,17]. Nevertheless, the determinations of either $CoQ_{10}H_2$ or CoQ_{10}, and simultaneous determinations of both analyses are scarce. An EC detector is preferred for the determination of $CoQ_{10}H_2$ due to its high sensitivity, although it is not applicable for CoQ_{10}. However, owing to the low concentration of CoQ_{10} in plasma, the sensitivity of UV absorbance detection is inadequate [11]. Thus several on-line post-column reduction methods of CoQ_{10} to $CoQ_{10}H_2$ were reported for simultaneous measurement by EC detection. These methods, however required either on-line reduction column [13,14] or double cell EC detector (to achieve post-column electrochemical reduction) [15–17], coupled-column with column-switching valves [18] or post-column two-way valve [15]. The complication of the instrumentation as reported in the literature limits the practical application of these methods in clinical use.

This paper describes an automated gradient HPLC system using pre-column reduction with EC detection for direct determination of $CoQ_{10}H_2$ and TQ_{10} in deproteinized plasma sample. The sample treatment procedure is simple and the HPLC method is relatively sensitive, reproducible, and is useful for clinical investigation.

2. Experimental

2.1. Chemicals and reagents

Coenzyme Q10 was obtained in oxidized form (CoQ_{10}) from Fluka Chemie (Buchs, Switzerland). Methanol (gradient grade), absolute ethanol, 1-propanol, 2-propanol and hexane (pro-analysis grade) were purchased from E. Merck (Darmstadt, Germany). Sodium perchlorate, perchloric acid and sodium borohydride ($NaBH_4$) were also from E. Merck.

2.2. Sample collection

Eighteen hospital staff members who have no known disease, were used for baseline evaluation of various parameters. Venous blood with EDTA as anticoagulant were kept in icebox immediately after collection and the plasma was separated from erythrocytes by centrifuging at 1500 *g* for 10 min at 4°C. The plasma, if not analysed, was frozen at −80°C within 30 min after collection.

2.3. HPLC apparatus and conditions

A Hewlett-Packard Model 1050 HPLC pump system was installed with a continuous seal-wash kit (Palo Alto, CA, USA). The seal-wash solution was simply a mixture of 2-propanol and water (20:80, v/v). The two mobile phase solutions used for gradient separation were: (A) 100% of methanol containing 50 m*M* sodium perchlorate and 10 m*M* perchloric acid; and (B) a mixture of ethanol and *tert*.-butanol (80:20, v/v). The initial conditions were 90% A and 10% B for 4 min and the mobile phase B was changed linearly over 6 min to 50%, and then the system reverted immediately to the initial conditions for 3 min. The flow-rate was 1.5 ml/min for 6.5 min and it was reduced to 1.0 ml/min from 7 min onwards. A Gilson autoinjector (Model 231-401) equipped with a programmable microcomputer, a sample loop (size, 20 μl) and a sample cooler (Model 832, set at 4°C) (Villiers-le-Bel, France) was used for reducing agent preparation, sample injection and pre-column reduction. In between every three samples, a sealed sample vial containing 2.5 mg $NaBH_4$ would be added with 500 μl of methanol. The determination of $CoQ_{10}H_2$ and TQ_{10} were performed by injections of the sample before and after mixing with 10 μl of this reducing agent for 1 min prior to HPLC analysis. All analyses were duplicated. The $CoQ_{10}H_2$ was separated on an analytical column (Hewlett-Packard Hypersil C_{18}, 5 μm, 125×4.0 mm I.D.) with a guard cartridge (LiChrosper 100 RP-18, 5 μm, 4×4 mm I.D.). The analyte was detected by an amperometric EC detec-

tor (Model 1049A, Hewlett-Packard) with a solid state Ag/AgCl reference electrode over a glassy carbon-working electrode (potential +700mV, full recorder scale at 50 nA). The results were computed by using a Millennium 2010 software (Waters, Milford, MA, USA).

2.4. Standard preparation

The preparation work was carried out under a dim environment to avoid photochemical decomposition of CoQ_{10}. All reagents used were kept cold on ice. To obtain a 1 mmol/l stock standard solution, we dissolved 10 mg of CoQ_{10} in 1 ml of hexane and mixed with 10.58 ml of ethanol. A 1 ml volume of this stock was kept in each of the crimp cap amber vials and stored at −80°C. Each week, we diluted this 1 ml stock 10-fold to 100 μmol/l with ethanol and stored at −80°C. Each day, we prepared fresh solution (10 μmol/l) in ethanol from the 100 μmol/l solution. The working standard solutions were further diluted in ethanol to give concentrations of 0.1, 0.2, 0.5, 1.0, 2.0 and 3.0 μmol/l. To have the same dilution factor as in the plasma sample, we mixed 40 μl of these working standards with 400 μl of ethanol. The peak height of $CoQ_{10}H_2$ determined was plotted against the corresponding concentration of CoQ_{10} prepared in various working standards.

2.5. Sample preparation

A 50 μl aliquot of plasma was deproteinized with 500 μl of ethanol and vortex-mixed for 30 s. After being kept on ice for 5 min, the sample was centrifuged at 14 000 *g* for 2 min at 4°C. We transferred 490 μl of supernatant into the crimp cap amber vial and placed in the sample cooler without delay. For each sample, we loaded 25 μl of this extract twice before and after adding the reducing agent sequentially.

For comparison study, four sets of samples (each, $n=12$), were individually treated with 10-fold of ethanol, 1-propanol and 2-propanol; or deproteinized with 5-fold of methanol prior to extraction with 10-fold of hexane as reported [13]. After automated pre-column reduction, 10 μl of the reduced sample was used for HPLC analysis.

3. Results and discussion

3.1. Chromatographic analysis

In our initial experiment, we found that the chromatographic behavior of $CoQ_{10}H_2$ relied greatly on the composition of ethanol and *tert*.-butanol of the mobile phase. However, increasing the ethanol composition resulted in poor resolution of $CoQ_{10}H_2$ from unidentified components. Furthermore, the mobile phase crystallized easily with the use of more than 20% (v/v) of *tert*.-butanol and was harmful to the piston seal. On the other hand, the baseline of EC detection was very noisy when a Ag/AgCl reference electrode with internal electrolyte was used. The fluctuation of background current could be due to the solubility of the internal electrolyte in 100% organic mobile phase. Thus it was replaced with a solid-state reference electrode. The addition of 50 m*M* sodium perchlorate into mobile phase served as a supporting electrolyte. Nevertheless, it was noted that using this mobile phase, the background current gradually elevated after a few injections of reduced samples. The addition of 10 m*M* of perchloric acid was found useful in keeping the background current constant. Using an isocratic elution system of methanol–ethanol–*tert*.-butanol (70:25:5, v/v/v), containing 50 m*M* sodium perchlorate and 5 m*M* perchloric acid, $CoQ_{10}H_2$ standard was detected at 10 min. However, it was observed in actual sample that, there were negative electrolysis current generated from some of the unknown components eluted before $CoQ_{10}H_2$. The huge negative peak tailing caused precipitous baseline and seriously interfered with the measurement of $CoQ_{10}H_2$ (figure not shown). Thus gradient elution method was used for the present investigation. The specimen was analysed without adding any reducing agent for the measurement of $CoQ_{10}H_2$, and after reduction for the measurement of TQ_{10}. The CoQ_{10} concentration was calculated by subtraction of the determined level of $CoQ_{10}H_2$ from TQ_{10}. Using the gradient elution, the elution of $CoQ_{10}H_2$ was free from interference and could be detected at

9.05 (±0.05) min. Fig. 1(a)–(d) show the chromatograms of standards with ubiquinol concentration of 0.1 μmol/l, 2.0 μmol/l, plasma sample of a healthy subject (TQ_{10}, 0.72 μmol/l), and its spiked sample (+CoQ_{10}, 1.0 μmol/l); respectively.

3.2. Extraction efficiency

Coenzyme Q10 is readily soluble in hexane, insoluble in methanol, and slightly soluble in ethanol, 1-propanol and 2-propanol. Due to the incompatibility of hexane with the methanol based mobile phase, the injection volume had to be limited to 5 μl [13]. Traditionally, to improve the detectability, the plasma sample after deproteinisation with alcohol was extracted with hexane, evaporated to dryness and reconstituted in alcoholic solvent [11,14,16,17], or purified on SPE cartridges [19] prior to HPLC analysis. These complicated and time-consuming procedures may result in artefactual oxidation of $CoQ_{10}H_2$ to CoQ_{10} [13,15]. It was also noted that the extraction efficiency of ethanol was not as good as 2-propanol and 1-propanol. In order to minimize artefactual oxidation, sample preparation was kept to a minimum and consisted only of 2-propanol-mediated protein precipitation from an aliquot of plasma. To avoid coulometric overload, Edlund used a sophisticated dual column system [19] and Lagendijk et al. [15] applied postcolumn-valve switching technique. Both methods were considered tedious and complex for clinical screening of a large number of samples. In our comparison study, no significant differences on peak height values were observed for extraction of twelve different plasma samples (TQ_{10} range, 0.3–1.8 μmol/l), using ethanol, 1-propanol and 2-propanol. On the contrary, significantly lower values were obtained for samples treated with hexane (Fig. 2). This finding confirmed that hexane was not

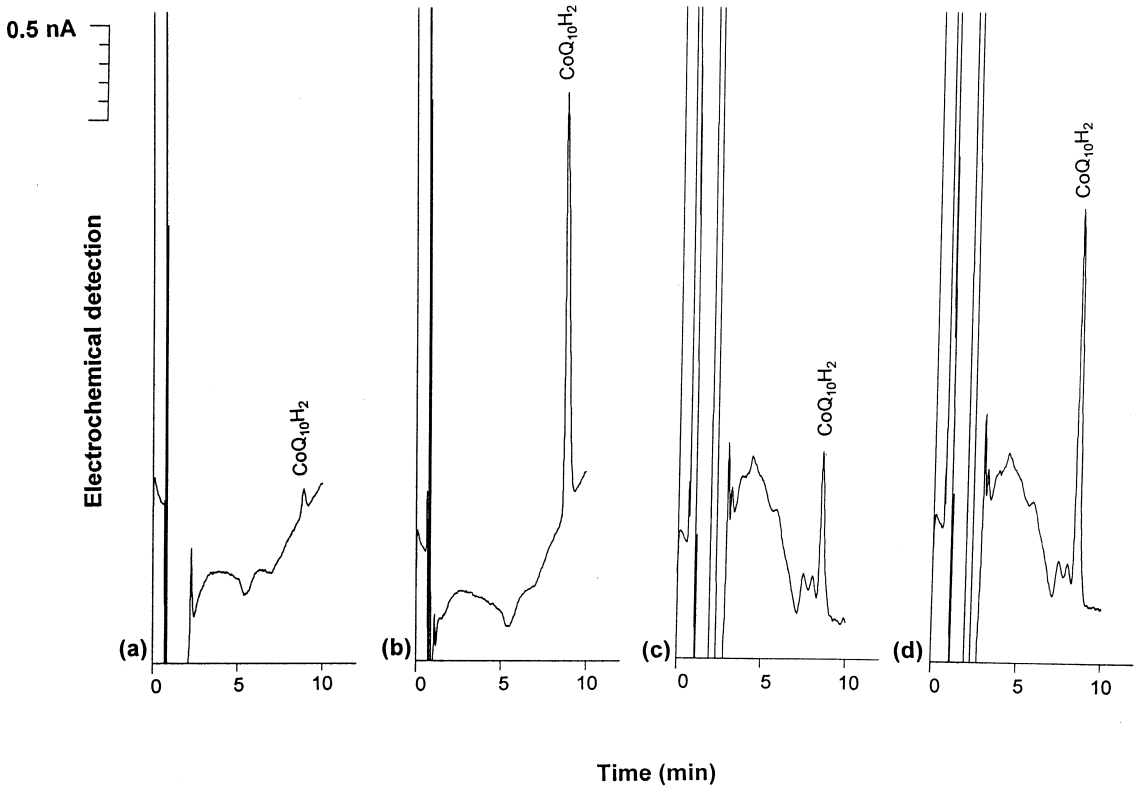

Fig. 1. Chromatograms of (a) and (b), $CoQ_{10}H_2$ reduced from 0.1 and 2.0 μmol/l of CoQ_{10} standard, (c) plasma sample of a healthy subject with TQ_{10} concentration 0.72 μmol/l, and (d) the same sample supplemented with 1.0 μmol/l of CoQ_{10}.

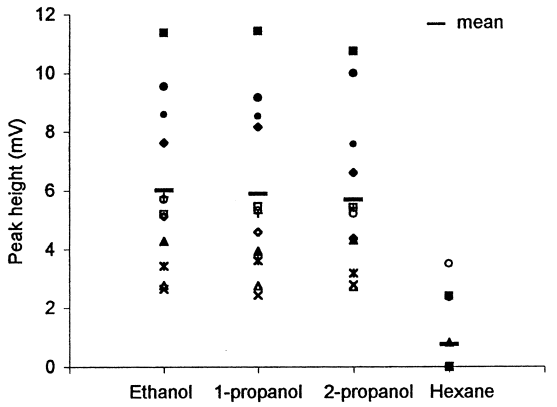

Fig. 2. Extraction efficiency comparison among four different solvents.

an effective extraction agent for the present HPLC analysis. In the present method, no complicated HPLC system is required. One part of plasma sample was simply deproteinized with 10 parts of ethanol and analysis could proceed immediately using an auto-injector. This 10-time dilution factor provided the best sample recovery and minimized the problem of detector cell overloading. No significant changes in chromatographic and EC performances were observed when the system was performed on a 24 h basis for up to 6 days.

3.3. Calibration, recovery and precision

To ensure accurate calibration, the CoQ_{10} standard solutions were prepared fresh and introduced just after reduction. The use of automated sampling system allowed us to have uniformity on the reaction time and analytical procedures. The optimum reduction condition was also investigated. From our preliminary investigations, it was found that the reactivity of reducing agent decreased rapidly after 3 h, thus an auto-injection system was programmed to prepare a fresh aliquot of reducing agent to be introduced between every three samples. It was also noted that excessive reducing agent contributed to bubble generation and affected the reproducibility. A 10 μl volume of reducing agent containing 0.5% (w/v) of $NaBH_4$ was found to be effective enough to reduce CoQ_{10} concentration as high as 3 μmol/l. A good linear relationship was obtained between the peak height and the concentration of $CoQ_{10}H_2$ from 0.1–3 μmol/l. The typical regression equation and correlation coefficient (r) were $y=0.432+21.99x$ ($r=0.999$), where y is the peak height (mV) and x is the concentration of $CoQ_{10}H_2$ reduced from CoQ_{10}. The C.V.s of the linearity and slope of the calibration graph for between-day analyses were 0.13% and 3.2%, respectively ($n=10$). The detection limit (signal to noise ratio of 3) was 2.5 nmol/l. Plasma ubiquinol concentration as low as 0.025 μmol/l could be detected. To verify the precision of the method, a pooled plasma sample containing added CoQ_{10} of 0.5, 1.0 and 2.0 μmol/l, was used to determine the within-day and between-day variations. As shown in Table 1 the precision of the method was good. The mean C.V. of within-day and between-day assay was 3.3% and 5.1%, respectively. The mean analytical recovery was 99.2%.

3.4. Stability of ubiquinone and ubiquinol

The stock solution prepared in ethanol at 1.0 mmol/l of CoQ_{10} was stable for at least a month, and at lower concentration of 100 μmol/l, it was stable for up to a week at −80°C. The standard was

Table 1
Precision and analytical recovery ($n=3$ each)

Added to plasma CoQ_{10} (μmol/l)	Mean measured $CoQ_{10}H_2$ (μmol/l)	Recovery (%)	C.V. (%)	
			Within-day	Between-day
Blank	0.699	–	1.1	2.2
+0.5	1.195	99.2	2.4	3.3
+1.0	1.776	107.7	3.8	2.9
+2.0	2.528	90.7	6.0	12.2
Mean	–	99.2	3.3	5.1

found unstable after 24 h at −20°C. In order to evaluate the stability of the analytes we prepared twenty vials of working standard (1.0 μmol/l) and analysed each at hourly intervals; the results showed that CoQ_{10} kept at 4°C was stable for at least 14 h with variation of less than 6%. The stability of $CoQ_{10}H_2$ and CoQ_{10} in the ethanol extract were also investigated. A pooled fresh plasma sample was deproteinized and aliquoted into 12 vials. Analyses were carried out at hourly intervals. The $CoQ_{10}H_2$ and TQ_{10} in the ethanol extract were stable for up to 12 h with C.V.s of <4% and 5%, respectively. With a programmable automated sampling system and sample cooler, eight samples could be analysed in an 8 h working day and twelve samples could be arranged for overnight analysis.

The mean values of $CoQ_{10}H_2$ and TQ_{10} for samples collected from 18 apparently healthy subjects and analysed within 24 h, were 0.857(±0.352) and 0.925(±0.379) μmol/l; respectively. The $CoQ_{10}H_2/CoQ_{10}$ ratio was 93:7 which is in good agreement with the earlier reports of ratios 92:8 [16], 95:5 [13] and 96:4 [15].

4. Conclusion

We developed an automated HPLC system using a pre-column reduction approach that allows the direct measurement of $CoQ_{10}H_2$ and TQ_{10} in a single plasma extract for EC detection. The CoQ_{10} concentration was calculated as the difference of TQ_{10} and $CoQ_{10}H_2$. The rapid and simple sample treatment procedure as proposed here avoided artefactual oxidation. No sophisticated separation and detection devices were needed. The proposed method is reliable and convenient for clinical investigation.

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