High-Sensitivity Simultaneous Analysis of Ubiquinol-10 and Ubiquinone-10 in Human Plasma

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Abstract

A method to determine ubiquinol-10 and ubiquinone-10 in human serum was developed by using high-performance liquid chromatography consisting of a semi-microcolumn switching system and an electrochemical detector (ECD), which requires minimized sample pre-treatments. A linear dynamic range was obtained from 1.0 to 5000 ng/mL, and recovery values of 89–105% were observed in a low-concentration region of 10–50 ng/mL. In a long operation test, a good precision was maintained during 5100 runs without any maintenance on ECD or columns. In addition, retention behaviors of other ubiquinone homologues were examined.

Introduction

Coenzyme Q10 (CoQ10), a cofactor of ATP production and an effective antioxidant (1), has been obtaining recent attention. Because its cellular concentrations are known to decrease with age (2), it is now being attempted to artificially supply it in different ways. Numerous CoQ10-related products, such as drinks and food supplements, are in the market. CoQ10 is also being used as an ingredient for cosmetic products. Skin applied CoQ10 was found to reduce the depth of wrinkles (3).

CoQ10 has two forms, ubiquinol-10 (reduced) and ubiquinone-10 (oxidized). Yamamoto and coworkers have found that vitamin C and ubiquinol-10 are the most responsive to oxidative stresses in our body. Their amounts in the human body were found to decrease faster than other naturally occurring antioxidants when an oxidative stress was given to the human body (4,5). Many reports have been published its relation to diseases (6), such as Parkinson's disease (7) and congestive heart failure (8,9).

A ratio of the reduced and the oxidized forms has been used as a new oxidative stress marker, and their simultaneous determination is strongly demanded (10,11). Among reported strategies to analyze CoQ10 are high-performance liquid chromatography (HPLC)–UV absorption (12–14), ECD (10,15–19), and mass spectrometry (MS) (20).

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Although a UV detector is relatively inexpensive, easily available, and suitable to run samples in the quality control of CoQ10-contaning products, its not sensitive enough to determine ubiquinol-10 and ubiquinone-10 in biological matrices simultaneously. MS is highly sensitive and selective, but it requires a large amount of investment. ECD is based on oxidation (or reduction) of analytes on its working electrode, and detects only substances that are oxidized (reduced) within a limited potential range, and provides an extremely high selectivity towards such substances.

Ubiquinol-10 is readily detectable with ECD at a common oxidative potential, while ubiquinone-10 does not respond to the detector at the same condition. Although ubiquinone-10 can be converted to ubiquinol-10 beforehand by the addition of sodium borohydride, which makes it possible to measure the total CoQ10 amount (17), the ratio of the two forms will be unknown.

The so called "coulometric" ECD, using a working electrode having a large surface area (a high reaction yield) and multi–potential capability, has been reported to detect two forms of CoQ10 sensitively (18,19). However, it is known that the detector requires a longer baseline stabilization process and a higher cost to replace its working electrode when one wants to keep it in its best condition, in comparison with common ECDs using a glassy carbon-working electrode.

Column switching methods using semi-microcolumns, which clean and concentrate substances of interest, have been proven powerful when a high sensitivity is required and an analyte is contained in complex biological matrices. Stress hormones in urine (21) and methamphetamine in human urine (22) were successfully analyzed in the column-switching format. A column switching method with columns of conventional diameters was applied to the analysis of ubiquinone-10 (14), and it might be interesting to see if the use of columns of reduced diameters leads to further improvement in sensitivity.

In this paper, it is attempted to build a column switching system having a glassy-carbon ECD and a ubiquinone-reducing column, and to apply the system to the high-sensitivity simultaneous determination of the reduced and oxidized forms of CoQ10 in human plasma. In addition, its practical aspects, such as stability in a long-run test, will be discussed.

Experimental

Materials

Ubiquinone-6, ubiquinone-9, ubiquinone-10, α-tocopherol, lycopene, β-carotene, and sodium perchlorate were purchased from Sigma-Aldrich (St. Louis, MO). Ubiquinone-7 was donated by Kaneka (Osaka, Japan). Ubiquinol-10 was prepared according to the method of Yamamoto and coworkers (10). HPLC-grade methanol and 2-propanol (IPA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Water was purified with a Milli-Q system (Nihon Millipore Kogyo, Tokyo, Japan).

Instrumentation and HPLC conditions

The HPLC system used in this study consisted of LC modules of Nanospace SI-2 series (Shiseido, Tokyo, Japan), including a 3009 degassing unit, two 3001 inert pumps, a 3033 autosampler, a 3004 column oven, a 3011 high pressure valve, and a 3005 electrochemical detector. The ECD has a double-layered reference electrode and provides a high stability towards non-aqueous mobile phases (Figure 1). EZChrom for Shiseido (Shiseido, Tokyo, Japan) was used as a data system and device controller.

The HPLC columns used were a SHISEIDO CQ-C (cleanup column, C18 reversed-phase, 2.0 mm i.d. × 35 mm, 5 µm, Shiseido), a SHISEIDO CQ-S (analytical column, C18 reversed-phase, 2.0 mm i.d. × 150 mm, 5 µm, Shiseido), and SHISEIDO CQ-R (ubiquinone-reducing column, 2.0 mm i.d. × 20 mm, Shiseido), whose inner structure and packing materials are designed to minimize oxidation of ubiquinol. A schematic of the column switching system is shown in Figure 2. Also, CAPCELL PAK C8 UG (2.0 mm i.d. × 35 mm, 5 mm, Shiseido) was tested as another cleanup column candidate in an optimization study.

Mobile phases used in the study were 50mM $NaClO_4$ in methanol–IPA (90:10, v/v) at a flow rate of 240 µL/min for the analytical column and 50mM $NaClO_4$ in methanol–water (95:5, v/v) at a flow rate of 200 µL/min for the cleanup column. The oxidation potential for ECD was 650 mV. Temperatures of column oven and autosampler were maintained at 40°C and 4°C, respectively. Aliquots (40 µL) of the plasma extract sample (to be described later in the text) were injected automatically into the HPLC system. During the first 2 min, the analyte molecules in the sample solution were concentrated at the top of the first (cleanup) column, at status A of the switching valve (Figure 2). Then, with the valve status switched to B, the concentrated analytes were transferred to, and separated in, the secondary analytical column. When the detection of the analytes was over, the switching valve was returned to status A (initial state) to prepare for the next sample.

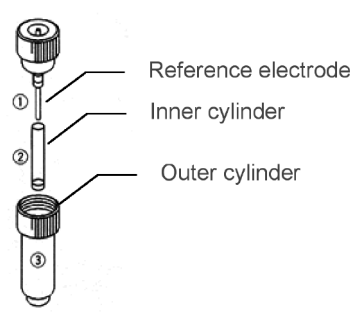

Figure 1. A double-layered reference electrode of ECD.

Preparation of standard solutions

Ubiquinone-6, -7, -9, and -10 were dissolved in IPA–water (93:7, v/v) at concentrations, 3000 ng/mL, respectively. The 93% IPA was used for the dilution of the standard solutions.

Preparation and extraction of samples

Heparinized human plasma (100 µL) collected from healthy volunteers was centrifuged at 3500 rpm for 10 min at 4°C. It was, thereafter, mixed vigorously with 900 µL of IPA in a polypropylene tube. After centrifugation at 12000 rpm for 5 min at 4°C, the IPA layer was injected directly to the HPLC system. The remaining sample was stored at –80°C for a reproducibility study.

Optimization in column-switching conditions

Using the human plasma extract, retention behaviors of ubiquinol-10 and other substances were examined by changing a length of cleanup time zone from 0.3 min to 2.0 min (0.3-min increment) and a reversed-phase column type used for cleanup (C18 or C8).

Linearity, recovery, and durability

Ubiquinone-10 standard samples in a range of 1.0–5000 ng/mL were used to study a linearity of the method. Signal-to-noise (S/N) ratios of detection and quantitative limits were defined as 3 and 10, respectively.

95 µL of plasma was mixed with 5 µL of IPA, containing 2000, 4000, and 10000 ng/mL of ubiquinone-10. Then, 900 µL of IPA was added to each mixture. The IPA layer was extracted by the same method described earlier. The resultant samples were supposed to contain 10, 20, and 50 ng/mL of ubiquinone-10, respectively. The recovery values of ubiquinone-10 were calculated by dividing peak area values obtained from the spiked samples by those obtained from standard solutions of the corresponding concentrations.

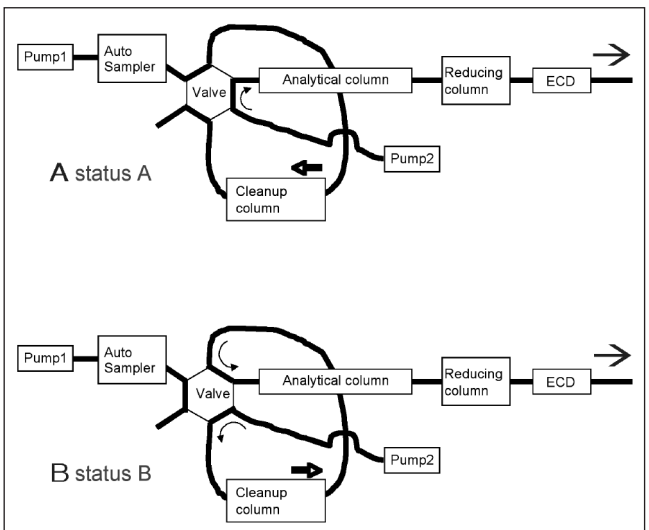

Figure 2. A schematic of the column switching system.

The durability was evaluated by running 5100 samples extracted from human plasma.

Results and Discussion

Typical chromatograms of a human plasma extracts (three subjects) are shown in Figure 3. The substances shown in the figure, having conjugate double bonds (lycopene and β-carotene) or a phenolic hydroxyl group (α-tocopherol and ubiquinol-10), could be selectively detected with ECD. UV detection would not be selective enough to see the similar separation profile of these substances, responding to many other coexisting substances.

Ubiquinone-10, an oxidized form of CoQ10, is not observed in the figure. The reducing column attached to the end of the analytical column is supposed to convert ubiquinone-10 to ubiquinol-10, the detectable form of CoQ10. In fact, samples run right after being prepared from healthy volunteers hardly showed a recognizable ubiquinone-10 peak. The simultaneous analysis of both forms of CoQ10 will be discussed in experiments with ubiquinone-10-spiked samples described later in the text.

In the column-switching system (Figure 2), the first column is filled with the mobile phase containing 5% buffer when the valve is switched to initiate the backflush and transfer (using Pump 2). The mobile phase initially flowing backwards on the first column is the same weak mobile phase, which was already on the column. After a little time, the front of the non-aqueous mobile phase will wash through the solute, lowering its k value and causing spatial focusing to occur.

A series of chromatograms of the human serum extract used for optimization of column-switching conditions are shown in Figure 4, where a C18 cleanup time is altered with a 0.3-min increment. A period shorter than 1.3 min failed to adequately remove unwanted components (Figure 4A), and sent a part of them to the analytical column, and a period of 0.6 min or longer guarantees the complete collection of α-tocopherol and ubiquinol-10 (Figure 4B). At 0.3 min, the column switching seemed to take place before the main components reached the cleanup column, which resulted in small peak areas for the major components.

A similar study was carried out with a C8 cleanup column, aiming at time saving by the use of the column of smaller overall retention (Figure 5). Similarly, a cleanup period of 1.0 min or shorter resulted in incomplete removal of unwanted components. On the contrary, a part of α-tocopherol will be lost at a period longer than 1.0 min (Figure 5A). The C18 column did not seem to have a capability strong enough to keep α-tocopherol, while ubiqunol-10 was completely collected at 0.6 min and longer (Figure 5B) because of its large hydrophobicity. Assuming a possiblility that α-tocopherol will be used as an indicator of efficiency in ECD, or that α-tocopherol itself will be determined or used as an internal standard, the C18 column to be used at 1.6 min or longer was considered to be an appropriate cleanup column, capable of removing unwanted components adequately and collecting CoQ10 completely.

When CoQ10 standard samples were run in the column switching system, the solvent used for preparing them needed some consideration. The plasma-IPA extract prepared by the method described earlier in the text is supposed to contain a small portion of water. The existence of water, which makes a sample solution close in polarity to the mobile phase, was found to play an important role in the sample focusing process when an

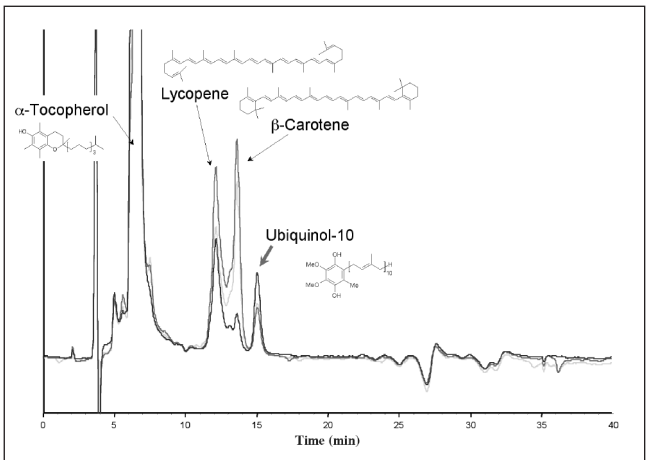

Figure 3. Typical chromatograms of a human plasma extracts (three subjects).

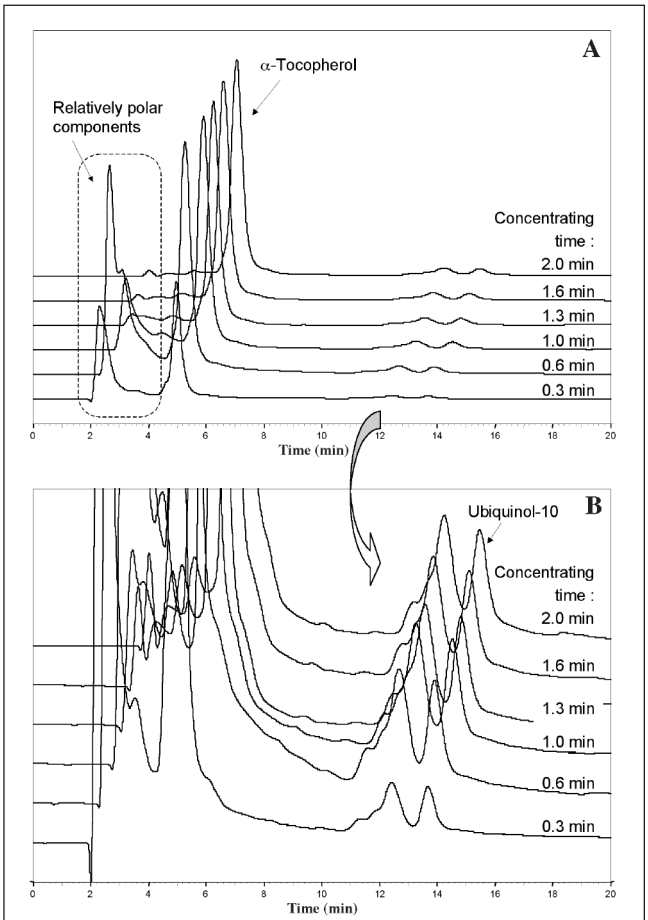

Figure 4. Separation profiles of human plasma extract with C18 cleanup column; overall profile (A) and magnified around elution position of ubiquinol-10 (B).

injection volume was relatively large (40 µL). Standard solutions for making a calibration curve were prepared by using 93% IPA as a solvent to keep a nice peak profile.

Calibration curves obtained with standard ubiquinone-10, showed a good linearity over the range of 1.0–5000 ng/mL. A coefficient of determination (r^2) was calculated as 0.9994. Figure 6 shows the chromatograms obtained with standard ubiquinone-10, where the detection and the quantitation limits were 1.0 and 3.0 ng/mL, respectively. Since a typical value for a sum of reduced and oxidized forms of CoQ10 in human plasma ranges from a few hundreds ng/mL to one thousand and a few hundreds ng/mL, the linear dynamic range observed here seems appropriate for the purpose.

Plasma extracts with different spiking levels (10, 20, and 50 ng/mL) all showed good recovery values (89.4–105.8%). Without any maintenance on ECD or columns, 5100 continuous runs of a human plasma extract were performed to study the durability of the system. No significant change in retention time and peak efficiency was observed between the first and the last chromatograms.

The use of the narrow-diameter columns in the study resulted in the reduction of mobile phase consumption to approximately one third the amount that would be consumed in a conventional column system. Besides such a practical feature, it should be noted that a low flow rate typical of a narrow diameter system allows analyte molecules to have more chances to have an electrochemical reaction on the working electrode, leading to an increase in sensitivity. Empirically, a sensitivity increase gained by lowering a flow rate from 1.0 mL/min to 0.2 mL/min is approximately two-fold.

The semi-microcolumn switching system having the ECD with a double-layered reference electrode and the on-line ubiquinone-reducing column seems highly durable in the operation with high-organic-content mobile phase system. The fact that all the hydrophilic components in the plasma extract are discarded prior to the detection seemed to contribute to the durability obtained here. An advantage of the ECD used in the study is that its glassy carbon working electrode can be reactivated by simply polishing its surface even when the sensitivity decreases in a long operation.

Several other ubiquinones with different isoprene chain length were also run in the system. A chromatogram obtained with the ubiquinones is shown in Figure 7. As expected, these substances were eluted in the order of increasing chain length. Ubiquinone-8 was not commercially available, but would probably elute between ubiquinones-7 and -9. The system might be useful to study ubiquinols and ubiquinones, not only from human plasma, but also from other biological sources.

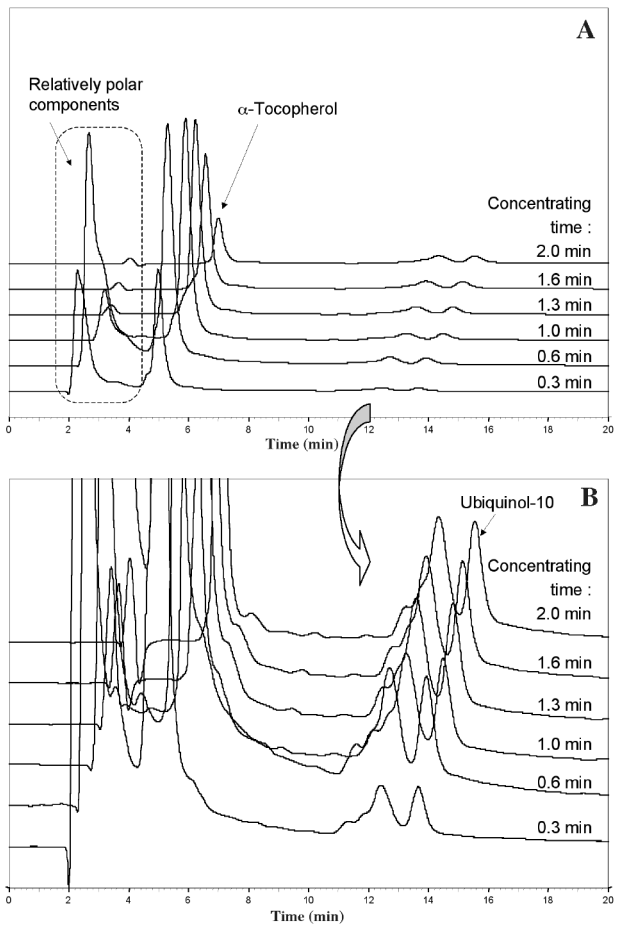

Figure 5. Separation profiles of human plasma extract with C8 cleanup column; overall profile (A) and magnified around elution position of ubiquinol-10 (B).

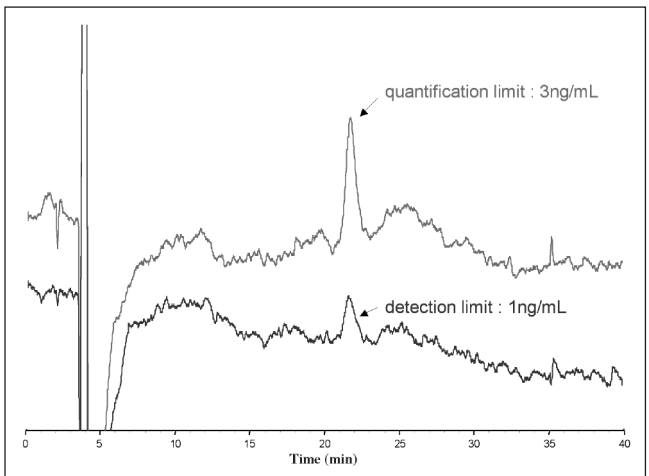

Figure 6. Detection and quantitation limits of standard ubiquinone-10.

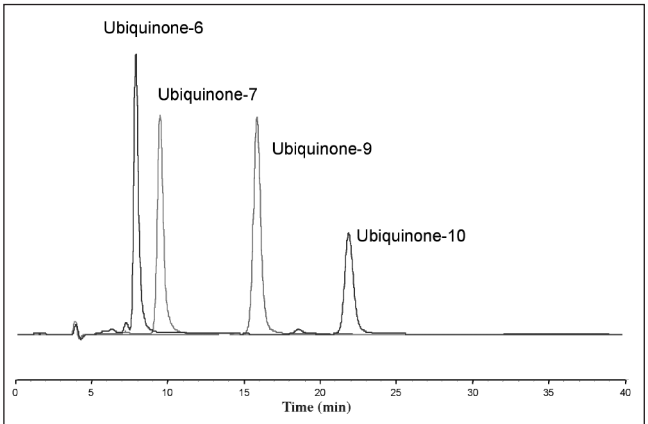

Figure 7. Retention profile of other ubiquinones.

Conclusions

The column-switching system with ECD having a double-layered reference electrode, after optimization of its switching conditions, was found to provide a highly sensitive and selective method towards analysis of ubiquinol-10 and ubiquinone-10. Its linear dynamic range, recovery, and durability were suitable for running routine samples. Retention behaviors of other ubiquinones with different isoprene chain length were also observed.

References

1. M. Turunen, J. Olsson, and G. Dallner. Metabolism and function of coenzyme Q. *Biochim. Biophys. Acta.* **1660:** 171–99 (2004).
2. A. Kalen, E. Appelkvist, and G. Dallner. Age-related changes in the lipid compositions of rat and human tissues. *Lipids* **24:** 579–94 (1989).
3. U. Hoppe, J. Bergemann, W. Diembeck, J. Ennen, S.Gohla, I. Harris, J. Jacob, J. Kielholz, W. Mei, D. Pollet, D. Schachyschabel, G. Sauermann, V. Schreiner, F. Stab, and F. Steckel. Coenzyme Q10, a cutaneous antioxidant and energizer. *BioFactors* **9:** 371–78 (1999).
4. Y. Yamamoto, M. Kawamura, K. Tatsuno, S. Yamashita, E. Niki, and C. Naito. Oxidative Damage and Repair, Ed. K.J.A. Davies, Pergamon Press, New York, 1991, pp. 287–91.
5. R. Alleva, M. Tomasetti, S. Bompadre and G.P. Littarru. Oxidation of LDL and their subfractions: Kinetic aspects and CoQ10 content. *Molec. Aspects Med.* **18:** s105–s112 (1997).
6. S.L. Molyneux, C.M. Florkowski, M. Lever, and P.M. George. Biological variation of coenzyme Q10. *Clin. Chem.* **51:** 455–57 (2005).
7. C.W. Shults, M.F. Beal, D. Song, and D. Fontaine. Pilot trial of high dosages of coenzyme Q10 in patients with Parkinson's disease. *Exp. Neurol.* **188:** 491–94 (2004).
8. P.H. Langsjoen and A. M. Langsjoen. The clinical use of HMG CoA-reductase inhibitors and the associated depletion oh coenzyme Q10. A review of animal and human publications. *BioFactors* **18:** 101–11 (2003).
9. M.A. Silver, P. H. Langsjoen, S. Szabo, H. Patil, and A. Zelinger. Effect of atovastatin on left ventricular diastolic function and ability of coenzyme Q10 to reverse that dysfunction. *Am. J. Cardiol.* **94:** 1306–10 (2004).
10. S. Yamashita and Y. Yamamoto. Simultaneous detection of ubiquinol and ubiquinone in human plasma as a marker of oxidative stress. *Anal. Biochem.* **250:** 66–73 (1997).
11. K. Hara, S. Yamashita, A. Fujisawa, S. Ishiwa, T. Ogawa, and Y. Yamamoto. Oxidative stress in newborn infants with and without asphyxia as measured by plasma antioxidants and free fatty acids. *Biochem. Biophys. Res. Commun.* **257:** 244–48 (1999).
12. F. Mosca, D. Fattorini, S. Bompardre, and P.G. Littarru. Assay of coenzyme Q10 in plasma by a single dilution step. *Anal. Biochem.* **305:** 49–54 (2002).
13. T. Okamoto, K. Fukui, M. Nakamoto, T. Kishi, T. Okishio, T. Yamagami, N. Kanamori, H. Kishi, and E. Hiraoka. High-performance liquid chromatography of coenzyme Q-related compounds and its application to biological materials. *J. Chromatogr.* **342:** 35–46 (1985).
14. P. Jiang, M. Wu, Y. Zheng, C. Wang, Y. Li, J. Xin, and G. Xu. Analysis of coenzyme Q10 in human plasma by column-switching liquid chromatography. *J. Chromatogr. B* **805:** 297–301 (2004).
15. H. Wakabayashi, S. Yamato, M. Nakajima, and K. Shimada. Simultaneous determination of oxidized and reduced coenzyme Q and a-tocopherol in biological samples by high performance liquid chromatography with platinum catalyst reduction and electrochemical detection. *Biol. Pharm. Bull.* **17:** 997–1002 (1994).
16. T. Okamoto, Y. Fukunaga, Y. Ida, and T. Kishi. Determination of reduced and total ubiquinones in biological materials by liquid chromatography with electrochemical detection. *J. Chromatogr.* **430:** 11–19 (1988).
17. Q. Wang, B.L. Lee, and C.N. Ong. Automated high-performance liquid chromatographic method with precolumn reduction for determination of ubiquinol and ubiquinone in human plasma. *J. Chromatogr. B* **726:** 297–302 (1999).
18. P.H. Tang, M.V. Miles, A. DeGrauw, A. Hershey, and A. Pesce. HPLC analysis of reduced and oxidized coenzyme Q10 in human plasma. *Clin. Chem.* **47:** 256–65 (2001).
19. N. Hermans, P. Cos, D.V. Berghe, A.J. Vlietinck, and T. Bruyne. Method development and validation for monitoring in vivo oxidative stress: Evaluation of lipid peroxidation and fat-soluble vitamin status by HPLC in rat plasma. *J. Chromatogr. B* **822:** 33–39 (2005).
20. K. Teshima and T. Kondo. Analytical method for ubiquinone-9 and ubiquinone-10 in rat tissues by liquid chromatography/turbo ion spray tandem mass spectrometry with 1-alkylamine as an additive to the mobile phase. *Anal. Biochem.* **338:** 12–19 (2005).
21. T. Kanda, O. Shirota, Y. Ohtsu, and M. Yamaguchi. Direct analysis of cortisol and cortisone in human urine by semi-microcolumn liquid chromatography with mixed-function precolumn. *J. Microcolumn Separations* **7:** 445–49 (1995).
22. Y. Makino, A. Suzuki, T. Ogawa, and O. Shirota. Direct determination of methamphetamine enantiomers in urine by liquid chromatography with a strong cation-exchange precolumn and phenyl-β-cyclodextrin-bonded semi-microcolumn. *J. Chromatogr. B* **729:** 97–101 (1999).

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