Rapid and sensitive analysis of reduced and oxidized coenzyme Q10 in human plasma by ultra performance liquid chromatography-tandem mass spectrometry and application to studies in healthy human subjects

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Abstract

Background—Coenzyme Q10 is an endogenous antioxidant as well as a popular dietary supplement. In blood circulation, coenzyme Q10 exists predominantly as its reduced ubiquinol-10 form, which readily oxidizes to ubiquinone-10 *ex vivo*. Plasma concentrations of coenzyme Q10 reflect net overall metabolic demand, and the ratio of ubiquinol-10:ubiquinone-10 has been established as an important biomarker for oxidative stress. However, the lability of ubiquinol-10 makes accurate determination of both forms of coenzyme Q10 difficult. *Ex vivo* oxidation of ubiquinol-10 to ubiquinone-10 during sample collection, processing and analysis may obfuscate the *in vivo* ratio.

Methods—We developed a rapid and sensitive method for the determination of ubiquinol-10 and ubiquinone-10 in human plasma, using coenzyme Q9 analogues as internal standards. Single-step protein precipitation in 1-propanol, a lipophilic and water-soluble alcohol, allowed for rapid extraction.

Results—Analysis by ultra performance liquid chromatography-tandem mass spectrometry provided rapid run-time and high sensitivity, with lower limits of quantitation for ubiquinol-10 and ubiquinone-10 of 5 μg/L and 10 μg/L, respectively.

Conclusions—This method is suitable for clinical studies with coenzyme Q10 supplementation in various disease states where this lipid-antioxidant may be beneficial. We have applied this method to >300 plasma samples from coenzyme Q10 research studies in chronic haemodialysis patients and postsurgical patients.

Keywords

UPLC-MS/MS; reduced Coq10; oxidized CoQ10; stability; plasma concentrations

Introduction

Coenzyme Q10 (ubiquinone, CoQ10) is an endogenous lipophilic benzoquinone and a vital component of aerobic cellular respiration, coupling electron transport to oxidative phosphorylation in mitochondria. It is synthesized in the human body by a pathway similar to that of cholesterol; systemic concentrations may be affected by diet, disease and drugs that inhibit the mevalonate pathway such as statins. Approximately 95% of CoQ10 in circulation and tissues exists in its reduced form (ubiquinol, UL10), which functions as a potent antioxidant by preventing lipid peroxidation in cellular membranes and low-density lipoproteins (LDL) through its own oxidation to ubiquinone (UN10).[1,2] Therefore, the ratio of UL10:UN10 is considered a biomarker for oxidative stress,[3] and the accurate determination of both forms of CoQ10 is needed in several clinical research areas.

While many analytical methods exist for determining the UL10:UN10 ratio in plasma, gaining an accurate snapshot of a ratio that represents *in vivo* proportions is highly challenging. Oxidation of UL10 during sample collection, storage, processing and analysis can obfuscate a meaningful interpretation of redox status. Therefore, a number of reported methods avoid separation of UL10 and UN10, instead chemically oxidizing UL10 and measuring total CoQ10 (TQ10) only.[4–7] In these cases, a very fast and highly sensitive assay is not required, and HPLC coupled with UV detection is sufficient. To effectively measure both UL10 and UN10 simultaneously and with high sensitivity, HPLC coupled with electrochemical detection (ECD) or tandem mass spectrometry is the preferred approach.[4,8,9] The specificity of tandem mass spectrometry is superior to that of ECD,[4] while yielding a similar lower limit of detection (LOD).

Methods for extracting CoQ10 from human plasma have also varied considerably, ranging from solid-phase extraction procedures[10] to liquid–liquid extractions using hexane[8,11,12] to protein precipitation using 1-propanol.[7,13–15] We found that liquid–liquid extraction in hexane, followed by evaporation and reconstitution, resulted in significant oxidation of UL10. Single-phase extraction through protein precipitation offers the fastest and most accurate method in determining plasma UL10:UN10 ratio. The high lipophili-city of 1-propanol, as well as its miscibility with water, allows for more efficient extraction of CoQ10 than 2-propanol or other short-chain alcohols.[16] With these considerations, we developed a quantitative method combining extraction of human plasma in 1-propanol (containing CoQ9

analogues as internal standards) and direct injection of the extract onto an ultra performance liquid chromatography-electrospray-tandem mass spectrometry (UPLC-ESI(+)-MS/MS) system. Through use of a rapid resolution chromatographic column and high flow rate, the run time per sample was kept under 5 min, optimizing analysis of UL10 and UN10 within a limited time window of stability.

Experimental

Chemicals and reagents

Hexane, 1-propanol, 2-propanol, ethanol and methanol were obtained from Fisher Biosciences (Fair Lawn, NJ). EDTA, ammonium formate, oxidized form of CoQ10 and coenzyme Q9, sodium borohydride and 1,4-benzoquinone were obtained from Sigma-Aldrich (St. Louis, MO).

Preparation of standards

Oxidized CoQ10 (ubiquinone UN10) was dissolved in hexane to produce a primary stock standard at a concentration of 500 mg/L. The stock was stored in aliquots at −80°C. A new stock was prepared monthly. A working CoQ10 standard was prepared daily by diluting the primary stock in ethanol to produce a final concentration of 1 mg/L and kept on wet ice during preparation of the standard curve.

Reduced CoQ10 (ubiquinol, UL10) standard was prepared fresh daily as follows. Oxidized CoQ10 stock of 100 μL (above) was mixed with 1900 μL of hexane in a screw-cap glass tube. Fifty microlitres of methanol and 20 mg of sodium borohydride were added. The tube was capped and shaken by hand for 3 min, and then placed in the dark for 5 min to complete the reduction. One millilitre of water containing 100 μM EDTA was added and the tube was shaken for 1 min. The mixture was centrifuged (1500 g, 5 min, 4°C). The upper hexane layer was transferred to a clean glass tube. Hexane layer of 200 μL was diluted with 800 μl of ethanol to produce a final reduced CoQ10 concentration of 5 mg/L. The standard was kept on wet ice during preparation of the calibration set.

Preparation of internal standards

Internal standard coenzyme Q9 (oxidized form), was dissolved in hexane to produce a primary stock standard at a concentration of 500 mg/L. The stock was stored in aliquots at −80°C. A new stock was prepared monthly.

Coenzyme Q9 (reduced form) standard was prepared daily as follows. Coenzyme Q9 (oxidized form) 100 μL of stock (above) was mixed with 1900 μL of hexane in a screw-cap glass tube. Fifty microlitres of methanol and 20 mg of sodium borohydride were added. The tube was capped and shaken by hand for 3 min, and then placed in the dark for 5 min to complete the reduction. One millilitre of water containing 100 μM EDTA was added and the tube was shaken for 1 min. The mixture was centrifuged (1500 g, 5 min, 4°C). The upper hexane layer was transferred to a clean glass tube.

A working internal standard solution was prepared by diluting 5 μL of coenzyme Q9 (oxidized form) primary stock and 100 μL of coenzyme Q9 (reduced form) standard to 10

mL with 1-propanol to produce a final concentration of 250 μg/L each. The working internal standard was prepared daily and kept on wet ice during sample preparation.

Preparation of standard curves

Separate sets of calibration standards were prepared for the oxidized and reduced forms of CoQ10; this allowed ready detection of unacceptable redox conversion. Ethanol was used as the matrix for the calibration standards; both oxidized and reduced CoQ10 were stable in ethanol for the duration of a typical analysis. Two sets of 2-mL microcentrifuge tubes labelled 0, 0.5, 1, 2.5, 5, 10, 25 and 50 μL were prepared. To the first set of tubes, labelled amounts of oxidized CoQ10 were added along with ethanol to a total volume of 100 μl (calibration range of 5–500 μg/L). To the second set of tubes, labelled amounts of reduced CoQ10 were added along with ethanol to a total volume of 100 μL (calibration range of 25–2500 μg/L). Two hundred microlitres of working internal standard solution were added to each tube, thoroughly mixed and then transferred to a 96-well plate or autosampler vial for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

Blood sample collection

Whole blood sample was collected in Vacutainers™ containing sodium heparin as an anticoagulant. Collected blood samples were immediately placed on wet ice and centrifuged (4°C, 5000 *g*, 5 min). Blood plasma was transferred to chilled cryo-tubes and immediately capped and frozen at −20°C, until its later transport to a −80°C freezer within 48 h.

Quality control samples

Whole blood was collected from two sets of healthy volunteers. One set of volunteers had been taking CoQ10 supplements and the second group had taken no supplements. Blood from each group of volunteers were pooled; aliquots of supplemented (high-QC) and non-supplemented (low-QC) plasma were frozen at −80°C and two aliquots from each pool were analysed alongside each set of samples.

Preparation of blood samples and controls

Plasma samples were thawed on ice and 100 μL of each sample was added to a 2-mL microcentrifuge tube. A total of 200 μL of working internal standard solution were added to each tube. Tubes were capped, vortexed well and centrifuged for 10 min (25,000 *g*, 10 min, 4°C). The supernatant was transferred to a 96-well plate and placed in the LC autosampler, which was kept at 4°C for the duration of the run. Injection volume ranged from 2.5 to 5 μL.

It was found during method development that to assure stability and reproducibility, the sample set should be allowed to equilibrate in the 4°C autosampler for at least 30 min before commencement of injection and no more than 12 clinical samples along with the calibrators and controls should be prepared during each run to avoid significant oxidation of the reduced CoQ10 in plasma extracts. While one set of samples and controls is being analysed on the LC-MS/MS, a second set of samples and controls may be prepared for analysis.

LC-MS/MS instrumentation and operating conditions

An Agilent G6410B triple-quadrupole mass spectrometer (equipped with `Hotbox' upgrade) was coupled to an Agilent 1290 UPLC with binary pump, autosampler and column compartment. The LC column was an Agilent Zorbax SB-C18, 30 mm × 2.1 mm × 3.5 μm maintained at 40°C. The mobile phase was methanol containing 5 mM ammonium formate pumped at a rate of 0.8 mL/min. The run time was 4.2 min per injection.

The mass spectrometer source was operated in the positive ion electrospray mode. Drying gas was 10 L/min of nitrogen at 350°C. Nebulizer gas was nitrogen at 35 psig. Capillary voltage was 4000 V. The mass spectrometer was operated in the MRM mode with the following transitions. Coenzyme Q10 (oxidized): m/z 863.7→197.1, fragmentor = 200 V, CE = 41 V, dwell = 200 ms. Coenzyme Q10 (reduced): m/z 882.7→197.1, fragmentor = 160 V, CE = 25 V, dwell = 200 ms. Coenzyme Q9 (oxidized form): 795.6→197.1, fragmentor = 190 V, CE = 33 V, dwell = 200 ms. Coenzyme Q9 (reduced form): 814.7→197.1, fragmentor = 150 V, CE = 25 V, dwell = 200 ms.

Calibration curves were generated with peak heights and analysed with a second order polynomial fit by the MassHunter WorkStation.

Results and discussion

Extraction solvents and UL10:UN10 ratio

In the present study, several extraction procedures were evaluated, including a hexane liquid–liquid extraction and single-step protein precipitation using 1-propanol or 2-propanol. As other authors have concluded,[4,15–17] 1-propanol was the best solvent for CoQ10 extraction, both in terms of analyte recovery and stability in plasma UL10:UN10 ratio.

Optimization of the chromatographic method

The chromatographic method of Ruiz-Jiménez et al.[8] was adapted to our analytical system with minimal changes considering the similarity of our instrumentation and the robustness of the method for rapid separation of the four compounds of interest. A representative mass-ion chromatogram of a plasma sample is shown in Figure 1. UL9 eluted at approximately 1.1 min, followed by UL10 at 1.9 min, UN9 at 2.3 min and UN10 at 4.0 min. The retention times exhibited good stability over a 5 h run with ≤ 5% coefficient of variation.

Optimization of the detection method

The triple quadrupole mass spectrometer was optimized to the following conditions: drying gas temperature was 350°C with a flow rate of 10 L/min, nebulizer pressure was 35 psig, quadrupole temperature was 100°C and capillary voltage was 4000 V. Four precursor ions determined to be highest in abundance were m/z 882.7 for UL10 $[M + NH_4]^+$, m/z 863.7 for UN10 $[M + H]^+$, m/z 814.7 for UL9 $[M + NH_4]^+$ and m/z 795.6 for UN9 $[M + H]^+$. It is unclear why UL10 and UL9 formed ammonium adducts more readily than UN10 and UN9; ubiquinones formed predominantly a proton adduct. Ruiz-Jiménez et al. in contrast found $[M + NH_4]^+$ adducts for all four species.[8] All compounds fragmented to a common benzylium ion at m/z 197.1 through loss of the lipophilic isoprenyl side-chain. Operating

conditions of the collision cell were optimized for each transition. These optimization mass spectrometry parameters are summarized in Table 1.

Method validation

Calibration curves were run for UL10 and UN10 with the peak height representing the standard concentration for each compound, using UL9 and UN9 as internal standards. LOD and lower limits of quantitation (LOQ) were set based on signal-to-noise ratios of 3 and 10, respectively; the LOQ has the additional criteria of accuracy $\leqslant$ 15% of the spiked concentration in triplicate samples. The LOD in plasma for this method was 1 μ/L for both UL10 and UN10, while the LOQ for UL10 and UN10 was 5 and 10 μg/L, respectively. The calibration range for UL10 was 5–500 μg/L and for UN10 it was 25–2500 μg/L, which cover the anticipated concentration ranges of plasma samples from both non-supplemented and high-dose supplemented human subjects; both curves consistently exhibited $r^2 \geqslant 0.999$ from run to run.

Pooled plasma samples, stored at −80°C, from non-supplemented and supplemented volunteers were used as low- and high-quality controls (QCs), respectively. Chromatographic precision of the instrumentation was evaluated by the reproducibility of 10 repeated injections of each QC sample. The precision for low QCs was 6.5% and 13.6% for UL10 and UN10, respectively; the precision for high QCs was 3.5% and 10.5% for UL10 and UN10, respectively. Intraday precision of the assay was evaluated by processing 10 samples each of low and high QCs, which were divided into two runs to keep the maximum number of samples per run under 12. Intraday precision for low QCs was 11.6% and 7.7% for UL10 and UN10, respectively; intraday precision for high QCs was 1.6% and 6.1% for UL10 and UN10, respectively. Linearity of sample volume was validated for a range of 0.025–0.1 mL with- $\leqslant$ 15% coefficient of variation.

Benchtop stability of 1-propanol plasma extracts

Stability of UL10 and UN10 during a typical assay run was evaluated by processing the low- and high-QC plasma samples according to the current method and each extract was repeatedly injected onto the LC-MS over 4.5 h following their equilibration in the autosampler at 4°C. Table 2 shows the stability of peak heights of the monitored mass ions for the two analytes and their corresponding internal standards: UL10, UL9, UN10 and UN9. Figure 2 shows the estimated concentration of UL10 and UN10 in the extract over time by converting the peak height ratios to concentration equivalent according to a calibration curve obtained immediately before the sample injections. Each time point represents the mean peak height or concentration of three to four replicate injections over the 0.5 h periods. The internal standards UL9 and UN9 peak heights were reasonably stable over the entire experiment, whereas significant conversion of UL10 to UN10 (>20%) was noted beyond 2 h following sample preparation and loading onto the autosampler. Hence, all routine analyses were limited to 12 clinical samples per batch in order to keep the total run time under 2 h.

Comparison of samples oxidized with 1,4-benzoquinone

To compare the current method with methods that measure TQ10 by chemical oxidation of plasma samples with 1,4-benzoquinone, we followed the procedure outlined by Hansen et al.[4] Low- and high-QC samples were analysed in triplicate for UL10 and UN10 concentration along with samples treated for 10 min with 1 mM 1,4-benzoquinone prior to extraction in 1-propanol. Benzoquinone-treated samples showed a complete disappearance of UL10 and a total CoQ10 recovery of $100.7 \pm 11.8\%$ indicating quantitative oxidation of UL10 to UN10.

Stability upon processing of blood samples

To accommodate multisite clinical studies, we have standardized the blood collection and processing protocol. All blood samples collected in heparinized tubes were immediately chilled in slush ice and centrifuged within 1 h of collection (2500 r/min, 4°C, 15 min) to harvest the plasma. Plasma samples were temporarily held in a −20°C freezer at the study site until transfer to −80°C freezer at the long-term storage site within 24 h. A short-term stability study was conducted with freshly collected plasma samples from a supplemented human subject comparing storage at −20°C versus −80°C. Figure 3 shows that UL10 and UN10 in plasma were stable under either storage condition over 24 h.

Plasma UL10 and UN10 in non-supplemented healthy volunteers

We applied the current method to analyse plasma samples from 10 non-supplemented, young adult healthy volunteers (Table 3). The average UL10 concentration was 1353 ± 629 µg/L and the average UN10 concentration was 32.7 ± 12.6 µg/L. The UL10:UN10 ratio ranged from 22.3 to 64.4, with a mean of 41.7. Table 4 summarizes the previously reported plasma UL10:UN10 ratio in healthy, non-supplemented subjects from 14 studies using a variety of analytical methods. The reported ratios span a large range. Our group found UL10:UN10 ratios higher than that those reported by most of the earlier studies, although the mean is quite similar to the study of Miles et al.[18] (41.0) and the range overlaps with several other studies that uses a single-step extraction method (i.e. 1-propanol protein precipitation or methanol-hexane extraction followed by direct injection of hexane phase). We attribute our higher basal UL10:UN10 ratio in plasma to the advantage of our method to avoid the commonly encountered postextraction, bench-top instability problem.

Conclusions

A highly sensitive and selective method for the simultaneous determination of UL10 and UN10 concentrations in human plasma was developed, with the advantage of combining LC-MS/MS with an efficient single-step extraction in 1-propanol. Although the stability of UL10 in 1-propanol plasma extract limits the number of samples that should be processed per batch to 12, the simple extraction procedure and fast instrument run time (4.2 min) allow for multiple runs per day. This method is suitable for clinical studies with CoQ10 supplementation in various disease states where this lipid-antioxidant may be beneficial. We have applied this method to >300 plasma samples from CoQ10 research studies in chronic haemodialysis patients and postsurgical patients.

Acknowledgments

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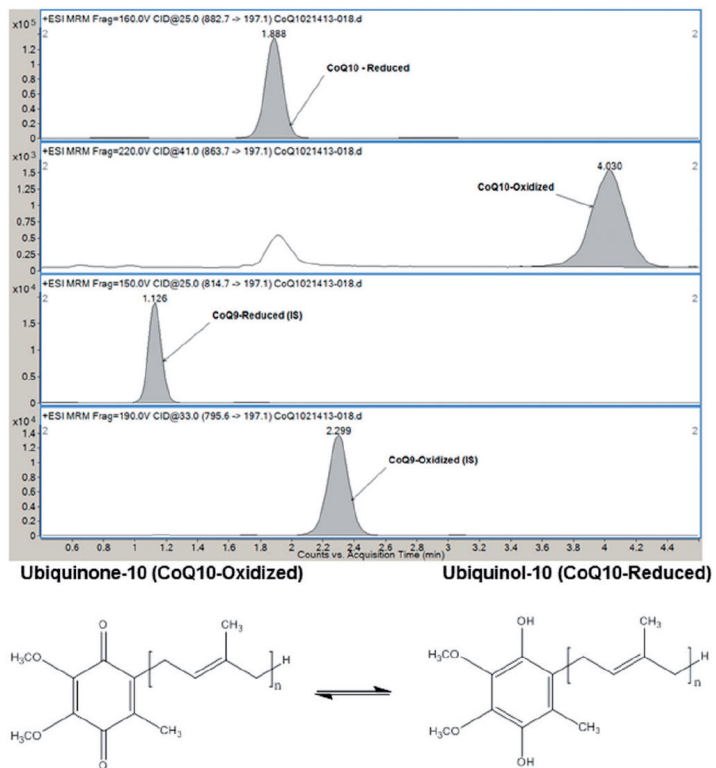
Figure 1.

Representative mass-ion chromatogram showing separation of UL10, UL9, UN9 and UN10. Structures of ubiquinone and ubiquinol, where n is the number of isoprene units in the side chain. UL10 and UN10 have 10 isoprene units, while UL9 and UN9 have 9 isoprene units; otherwise they are structurally identical.

UL10: ubiquinol-10; UN10: ubiquinone-10; CoQ9: coenzyme Q9; CoQ10: coenzyme Q10.
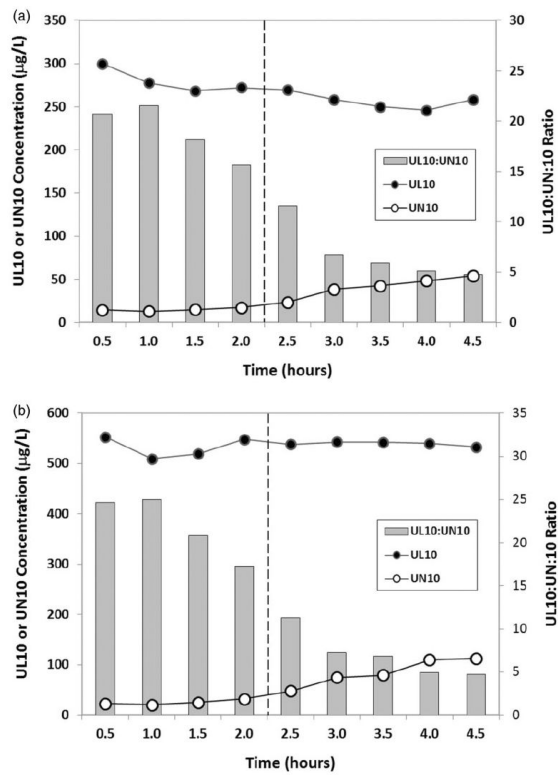
Figure 2.
Stability of 1-propanol extract of low (a) and high (b) plasma quality controls in autosampler at 4°C over 4.5 h. Injection of samples commenced after 30 min of equilibration. Significant oxidation of UL10 to UN10 in the 1-propanol extracts of human plasma spike occurred beyond 2 h.
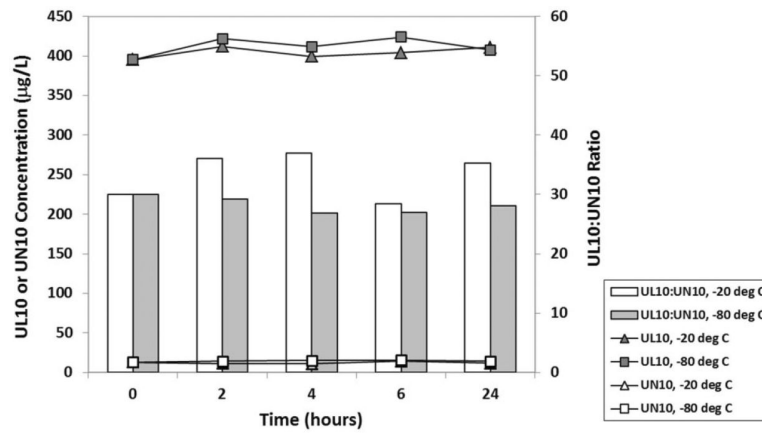
Figure 3.
Comparative short-term storage stability of UL10 and UN10 in freshly collected plasma samples from a supplemented human subject at −20°C and −80°C.

Table 1

Chromatographic and detection parameters of UL10, UN10, UL9 and UN9.

LC-ESI(+)-MS/MS	UL10	UN10	UL9	UN9
R_t (min)	1.9	4.0	1.1	2.3
Molecular adduct	$[M+NH_4]^+$	$[M+H]^+$	$[M+NH_4]^+$	$[M+H]^+$
MRM transition (m/z)	882.7→197.1	863.7→197.1	814.7→197.1	795.6→197.1
Fragmentor voltage	160 V	220 V	150 V	190 V
Collision energy	25 V	41 V	25 V	33 V

UL10: ubiquinol-10; UN10: ubiquinone-10; UL9: ubiquinol-9; UN9: ubiquinone-9; LC/MS: liquid chromatography-tandem mass spectrometry; ESI: electrospray ionization.

Table 2

Stability of the analytes and corresponding internal standards in autosampler at 4°C over 4.5 h as indicated by their monitored mass ion peak heights (mean ± SD, n = 3–4).

Time period (h)	UL10	UL9	UN10	UN9
Low plasma control				
0–0.5	1504 ± 119	1662 ± 117	38 ± 2	1279 ± 30
0.5–1.0	1357 ± 46	1619 ± 44	35 ± 2	1322 ± 46
1.0–1.5	1288 ± 57	1590 ± 35	39 ± 4	1275 ± 52
1.5–2.0	1286 ± 23	1567 ± 51	44 ± 8	1224 ± 76
2.0–2.5	1329 ± 47	1634 ± 79	57 ± 9	1180 ± 58
2.5–3.0	1281 ± 40	1644 ± 15	88 ± 11	1114 ± 21
3.0–3.5	1305 ± 40	1730 ± 49	102 ± 12	1158 ± 41
3.5–4.0	1254 ± 44	1692 ± 60	120 ± 15	1209 ± 58
4.0–4.5	1238 ± 33	1589 ± 16	132 ± 22	1177 ± 39
High plasma control				
0–0.5	2892 ± 204	1781 ± 32	50 ± 2	1235 ± 34
0.5–1.0	2352 ± 205	1571 ± 39	45 ± 4	1240 ± 32
1.0–1.5	2379 ± 98	1559 ± 35	59 ± 3	1326 ± 64
1.5–2.0	2531 ± 50	1571 ± 10	68 ± 7	1196 ± 58
2.0–2.5	2631 ± 102	1664 ± 60	97 ± 11	1126 ± 39
2.5–3.0	2704 ± 33	1695 ± 39	146 ± 16	1089 ± 6
3.0–3.5	2810 ± 105	1764 ± 41	170 ± 20	1194 ± 35
3.5–4.0	2741 ± 78	1727 ± 45	209 ± 21	1065 ± 31
4.0–4.5	2638 ± 44	1684 ± 8	231 ± 23	1144 ± 73

UL10: ubiquinol-10; UN10: ubiquinone-10; UL9: ubiquinol-9; UN9: ubiquinone-9.

Table 3

Plasma concentrations of UL10 and UN10 in a group of healthy, non-supplemented human subjects.

Sample	UL10 (µg/L)	UN10 (µg/L)	UL10:UN10 ratio
1	650	29.1	22.34
2	1150	17.9	64.36
3	1059	26.1	40.54
4	1632	47.6	34.26
5	1051	21.9	48.01
6	2395	45.8	52.35
7	1615	35.2	45.94
8	2324	53.3	43.63
9	579	18.4	31.42
10	1072	31.6	33.91
Mean ± SD	1353 ± 629	32.7 ± 12.6	41.7 ± 12.0

UL10: ubiquinol-10; UN10: ubiquinone-10; UL9: ubiquinol-9; UN9: ubiquinone-9.

Table 4

Comparison of UL10:UN10 ratio in healthy human subjects between current study and published literature reports using various methods for quantification of UL and UN in plasma or serum.

Reference	UL10:UN10 ratio	Blood anticoagulant	Extraction solvent	Plasma volume, µL	Internal standard	Detection technique
Takada et al.[19]	1.1	Heparin	EtOH/hexane	1000	CoQ9	Amperometric
Okamoto et al.[20]	6.7	Heparin	Hexane	200	CoQ9	Amperometric
Wakabayashi et al.[12]	1.9	Serum	Hexane	100	CoQ9	Amperometric
Finckh et al.[11]	19.0	Heparin	Hexane	10	CoQ9	Coulometric
Lagendijk et al.[17]	16.5	EDTA	1-propanol	300	N/A	Coulometric
Yamashita et al.[21]	21.7	Heparin	MeOH/hexane	50	CoQ9	Amperometric
Kaikkonen et al.[22]	7.8	Heparin/EDTA	MeOH/hexane	50	CoQ7/CoQ9	Coulometric
Wang et al.[23]	13.3	EDTA	EtOH	100	N/A	Amperometric
Menke et al.[24]	7.3	EDTA	Hexane	100	UL9/UN9	Coulometric
Tang et al.[15]	26.0	Heparin	1-propanol	100	UL9/UN9	Coulometric
Ruiz-Jiménez et al.[8]	13.8	Heparin	hexane	100	UL9/UN9	LC-MS/MS
Miles et al.[18]	41.0	Heparin	1-propanol	100	UL9/UN9	Coulometric
Franke et al.[25]	19.0	Heparin	Hexane	200	UL9/UN9	Coulometric
Matsuzaki et al.[26]	24.8	Serum	MeOH/hexane	50	CoQ9	Amperometric
Current study	41.7	Heparin	1-propanol	100	UL9/UN9	LC-MS/MS

CoQ9: coenzyme Q9; LC-MS/MS: liquid chromatography-tandem mass spectrometry.