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Original Contribution

Coenzyme Q10 in human blood: Native levels and determinants of oxidation during processing and storage

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ABSTRACT

Coenzyme Q10 (Q10) is present in the circulation mainly in its reduced form (ubiquinol-10; UL10), but oxidizes quickly ex vivo to ubiquinone-10 (UN10). Therefore, native UL10:UN10 ratios, used as markers of redox status and disease risk, are difficult to measure. We established an RP-(U)HPLC method with coulometric detection to measure natively circulating UL10 and UN10 concentrations by adding a ubiquinol/ubiquinone mixture as an internal standard immediately after plasma preparation. This allowed adjustment for unavoidable artificial UL10 oxidation as well as for total losses (or gains) of analytes during sample storage, processing, and analysis because the internal standards exactly paralleled the chemical behavior of Q10. This technique applied to blood ($n = 13$) revealed Q10 levels of 680–3300 nM with a mean UL10:UN10 ratio of 95:5, which was inversely associated with total Q10 ($r = -0.69$; $p = 0.004$). The oxidation of UL10 to UN10 was equimolar, increased by O_2, and decreased by lower temperatures or various degassing methods. Although UL10 was stable in blood or when pure in organic solvents at 22 °C, its oxidation was catalyzed dose dependently by α-tocopherol and butylated hydroxytoluene, particularly when present in combination. Key structural features for the catalytic pro-oxidant properties of phenolic antioxidants included two substituents vicinal to the phenolic hydroxyl group.

Coenzyme Q10 (Q10; Fig. 1), a lipophilic cell membrane component and the predominant member among the coenzyme Q species in humans, functions as an electron carrier in the mitochondrial respiratory chain as well as an intracellular antioxidant [1–3]. It is synthesized by human cells, but exposure occurs also through the diet with daily Q10 and Q9 doses estimated (in Scandinavians) to be 4–5 and 0.4–0.6 mg, respectively [4].

Ubiquinol-10 (UL10), the chemically reduced form of Q10, is a free radical scavenger, prevents peroxidation damage to cell membranes, regenerates α-tocopherol, and minimizes the effects of oxidative stress by dehydrogenating to ubiquinone-10 (UN10) [5–7]. The UL10:UN10 ratio is therefore postulated to be a good marker of oxidative stress [8], whereas total Q10 (TQ10) may indicate general physiologic events, such as cell death, because dying cells would leak Q10 into the circulation [5,9–11]. Lower UL10:UN10 ratios have been reported in older individuals and patients with liver disease, coronary artery disease, neurodegenerative disease, dyslipidemia, trisomy 21, and chronic obstructive pulmonary disease [2,9,11–13]. Significant deficiency in skeletal muscle Q10 has been reported in patients with multisystem mitochondrial encephalomyopathies [14], whereas low circulating TQ10 levels were associated with cardiovascular diseases [15,16], ataxia [17], and patients receiving statins [18]. Interestingly, lower cell TQ10 levels are associated with older age [19] but higher blood TQ10 levels were observed in adults versus older children [2].

Ubiquinols oxidize quickly in the presence of oxygen and increasingly at temperatures above −70 °C, which renders native levels difficult to quantitate [20–22]. This has led many investigators to exclusively report TQ10 [23], by either chemically reducing samples with $NaBH_4$ [24] or oxidizing them with $FeCl_3$ or $CuCl_2$ [20] before analysis or by precolumn electrochemical reduction or oxidation [5]. The most popular routine analysis employs reversed-phase HPLC with absorbance readings at 275 nm [25] or electrochemical detection to achieve higher sensitivity. Mass spectrometric detection has also been applied more recently and is a method that is highly selective, but requires the availability of expensive instrumentation [3,26], whereas fluorescence monitoring is not recommended because of its low sensitivity [27]. Simultaneous analysis of UL10 and UN10 can be achieved by chromatographic separation followed by electrochemical (preferably coulometric) detection via oxidation of UL10 at positive voltage and reduction of UN10 with negative voltage [5,26]. This is easier to perform than employing a reduction column after chromatographic separation and monitoring by electrochemical detection with positive voltages [25].

Internal standards of various Q10 analogs (Q6, Q7, Q9) have been employed [5,20,28], including alkoxy analogs of Q10 [26,29]. However,

Abbreviations: HPLC, high pressure liquid chromatography; UV, ultraviolet; ECD, electrochemical detection; LC/MS, liquid chromatography/mass spectrometry; UL, ubiquinol; UN, ubiquinone; BHT, butylated hydroxytoluene; αT, α-tocopherol; γT, γ-tocopherol; Q, coenzyme Q.

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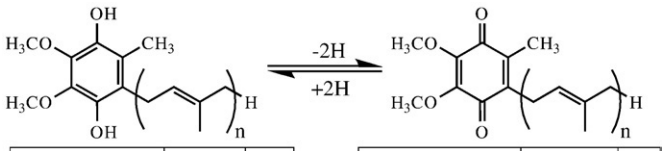

	code	n
Ubiquinol-10	UL10	10
Ubiquinol-9	UL9	9

	code	n
Ubiquinone-10	UN10	10
Ubiquinone-9	UN9	9

Fig. 1. Molecular structure of ubiquinols and ubiquinones. Interconversion occurs readily by redox reaction and is easily achieved electrochemically by dehydrogenation (positive voltage) and by hydrogenation (negative voltage).

only the oxidized forms were used, which adjusts exclusively for the absolute loss of analytes during sample preparation and measurement and not for the conversion of native UL during these processes or during storage.

We intended to establish a simple, fast, sensitive, and accurate HPLC method to measure TQ10 as well as natively circulating UL10 and UN10 concentrations. Realizing that the latter levels change continuously after blood is drawn, our aim was to find an internal standard that allows adjustment for artificial UL10 oxidation as well as for total losses (or gains) of analytes during sample storage, processing, and analysis. We also aimed to evaluate determinants for the oxidation of UL10.

Materials and methods

Chemicals and reagents

Ubiquinone-4, ubiquinone-6, UN10, and α-tocopherol (αT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ubiquinone-9 (UN9) was obtained from Fluka (Sigma–Aldrich). Tocol was purchased from Matreya (Pleasant Gap, PA, USA). Sodium borohydride, dimethylamino pyridine, sodium carbonate, and dodecanoic anhydride were obtained from Sigma–Aldrich. Methanol, hexane, and acetonitrile were HPLC grade from Fisher (Pittsburgh, PA, USA). Hexane and methanol were kept refrigerated before and during extractions. Peroxide test strips (WaterWorks Peroxide Check, Industrial Test Systems, Rock Hill, SC, USA) with a sensitivity <0.5 ppm were used for the determination of peroxide levels.

Plasma preparation and extraction

Venous blood was collected into green-top Li-heparin Vacutainers, twice from 3 and once from 13 healthy volunteers (ages 23–55; mean 41 years). This was approved by the Committee on Human Subjects of the University of Hawaii and all volunteers signed the consent form. The blood was kept at room temperature to examine UL10 oxidation rates during various times followed by processing or the blood was processed immediately by centrifugation at 1050×*g* for 20 min at 4 °C. The supernatant plasma was aliquotted into individual cryogenic vials and either frozen at −80 °C as such or after adding 200 µl UL9: UN9 mixture (95:5 in refrigerated methanol; total ca. 0.6 µM) to 200 µl plasma in 1-ml polypropylene tubes. Plasma preparation followed by storage in −80 °C freezers could be performed as fast as within 40 min.

HPLC

System 1 (extraction and analysis of UN10 with other lipid-phase micronutrients)

Carotenoids, retinoids, tocopherols, and UN10 were analyzed by a well-established HPLC assay with photodiode array detection [30], which is validated by participation in quality assurance programs organized by the U.S. National Institute of Standards and Technology (Gaithersburg, MD, USA) [31] and has been successfully applied in numerous epidemiological and other studies [32–47].

In brief, all procedures before storage of extracted analytes in amber vials were carried out under yellow light to avoid degradation of analytes. Plasma (0.20 ml) was mixed with 0.20 ml ethanol containing butylated hydroxytoluene (BHT) as antioxidant and four internal standards (tocol, retinyl laurate, δ-tocopheryl laurate, and *n*-butyl-β-apo-8′-carotenoate [30]) followed by partitioning into 1.0 ml hexane. The hexane layer was evaporated in amber vials at room temperature under a stream of nitrogen. The dry extracts were redissolved in 0.2 ml HPLC mobile phase (see below) followed by injection of 20 µl into an RP-HPLC system (Model Surveyor; ThermoFisher, San Jose, CA, USA) consisting of a Spherex C18 analytical column (150×3.2 mm, 3 µm) coupled to a Spherex C18 precolumn (4.0×3.0 mm, 10 µm; both columns from Phenomenex, Torrance, CA, USA) using isocratic elution with a mobile phase of 650 ml methanol/250 ml dichloromethane/100 ml acetonitrile/2 ml aq Bis–Tris propane (0.5 M, pH 6.8) and containing 0.25 g/L BHT (1.14 mM) at 0.3 ml/min. Interassay variability of these analytes varied between 3 and 9% [30]. UN10 concentrations were determined from external calibration curves after adjustment for recovery of tocol.

System 2 (extraction and analysis of TQ10 after precolumn electrochemical oxidation and postcolumn UV detection as UN10)

Analysis of total plasma coenzyme Q10 was carried out by HPLC (Model Spectra; ThermoFisher) using the hexane extract available from the procedure described above after redissolving in the mobile phase of HPLC system 1. After coulometric precolumn oxidation using a guard cell Model 5020 at +0.8 V (ESA, Chelmsford, MA, USA) 20 µL were separated on a Gemini C18 precolumn (4.0×2.0 mm, approximately 10 µm) directly connected to a Gemini C18 analytical column (150×2.0 mm, 5 µm; both columns from Phenomenex) using an isocratic mobile-phase mixture of sodium acetate trihydrate (6.8 g)/glacial acetic acid (15 ml)/2-propanol (15 ml)/hexane (275 ml)/methanol (695 ml) [5]. Detection was performed at 275 nm, peak areas were used for quantitation applying external calibration (range 50–2000 nM determined by absorbance readings), and final values were adjusted for internal standard (δ-tocopheryl laurate) recovery. Interassay variability of TQ10 was found to vary between 5 and 7% at levels of 400 nM.

UL10 levels were calculated by subtracting UN10 from TQ10 levels.

System 3 (extraction and analysis of UL and UN by UV and electrochemical detection)

After centrifugation of blood, 200 µl plasma was immediately mixed with 200 µl of a mixture of known concentrations of UL9 and UN9 in refrigerated methanol (ca. 0.6 and 0.03 µM, respectively) as internal standards. This mixture was immediately extracted and analyzed by HPLC or stored at −80 °C. The thawed plasma, spiked with the UL9/UN9 mixture, was extracted with 200 µl of chilled hexane by vortexing 1.5 min followed by centrifugation for 5 min at 4 °C and 5082×*g*. A 100-µl sample of the hexane layer was transferred to a 200-µl conical insert in an amber HPLC autosampler vial and injected into a Hypersil Gold C18 column (50 mm×2.1 mm×, 1.9 µm; ThermoFisher) using a model Surveyor HPLC system (ThermoFisher) with a 20-µl loop injector using a mobile phase of (v/v) 83% methanol, 14% acetonitrile, 3.5% water, 0.04% glacial acetic acid, and (w/v) 0.06% aq lithium acetate at a flow of 0.25 ml/min modified from a previous report [5]. UL and UN detection was performed by UV absorbance at 295 nm and coulometrically using a CoulArray Model 5600A detector (ESA) with consecutive cell voltages of +0.75, −0.70, and +0.65 V for maximum response of UL10 and UN10 at the last cell. To achieve faster turnaround times, ultra-HPLC was performed on a Model Accela UHPLC system (ThermoFisher) with a Coulochem III

detector and 5011A analytical cells (ESA) set at −0.70 and +0.65 V using the above-listed mobile phase at 0.5–0.7 ml/min.

UL10 and UN10 concentrations were determined from external calibration curves after adjustment for recovery of the internal standards UL9 and UN9, respectively. This adjusted for the absolute loss due to pipetting, injection, spillage, or other factors; for possible extract concentration due to solvent evaporation; and also for the conversion from the reduced to the oxidized forms of Q10 during sample storage, workup, and analysis.

Calibration of UN10 and UL10 was required on each day of analysis to ensure good between-day precision. Because of the fast oxidation, UL9 and UN9 concentrations in the internal standard mixture were analyzed on each day of blood processing to determine the references for recovery calculation purposes.

Calibration

Stock solutions for calibration were prepared by dissolving 10 mg UN10 in 10 ml hexane followed by absorbance readings using $\epsilon = 14{,}200$ at 275 nm in isopropanol [48]. UL10 stock solutions were prepared accordingly after reduction from UN10 with $NaBH_4$ (see below) using $\epsilon = 3510$ at 290 nm in isopropanol, which was determined after synthesis as shown below. All stock solutions were confirmed to be of >99% purity by HPLC analysis with coulometric detection.

Calibrators were prepared by dissolving stock solutions to five to seven concentrations in the range of approximately 30–1800 nM in the mobile phase for HPLC system 1 or in chilled hexane for coulometric detection in system 3 or the mobile phases of the respective HPLC systems. UL10 and UN10 response factors were used for UL9 and UN9, respectively, because of identical UV-chromophores and the same coulometrically responsive structural features plus extremely similar retention times. Calibration curves were obtained by plotting detector responses versus calibrator concentrations using best-fit regressions with Excel 2004 software (Microsoft). Calibration curves were accepted if r^2 values were greater than 0.998.

Syntheses

UL10

UL10 was prepared by mixing 5 µl UN10 (1 mg/ml in hexane) with 200 µl water containing 10 mg sodium borohydride and 4 ml ethanol [49]. After incubation for 10 min in the dark, 1 ml water was added, followed by partitioning into 5 ml hexane. The hexane was then kept at −20 °C until needed for calibration. UL9 was prepared similarly by mixing 5 µl UN9 (1 mg/ml in hexane) with 200 µl water containing 10 mg sodium borohydride and 4 ml ethanol. UL:UN ratios were usually >200:1, as determined by HPLC analysis.

When needed for spiking, an aliquot (usually 1–1.5 ml) was removed, dried under nitrogen, redissolved in chilled methanol, and analyzed by HPLC to determine exact Q9 levels that were subsequently used for spiking. Ideally, the UL9:UN9 ratio was kept at 20:1 for spiking purposes.

For the determination of UL10's extinction coefficient (molar absorptivity), the hexane phase described above was dried, then weighed, and redissolved in hexane followed by dilutions with isopropanol for absorbance readings. After absorbance was read the samples were confirmed to contain UL at >99% purity. Three independent experiments with multiply repeated absorbance readings yielded a molar extinction coefficient of 3510 at 290 in isopropanol (cv 3%).

δ-Tocopheryl laurate

δ-Tocopheryl laurate was prepared by mixing δ-tocopherol (25 mg) and lauric acid anhydride (70 mg) with dimethylaminopyridine (10 mg) and triethylamine (0.5 ml) in dichloromethane (2 ml). The mixture was refluxed at 60 °C for 3 h and washed with 1 ml water, and the dichloromethane layer was separated and condensed under reduced pressure. The resulting oil was subjected to SPE C18 column purification and was washed with water and methanol and eluted with dichloromethane to give the final product.

Results

Because of the lack of availability of UL10 from commercial sources we synthesized it by reduction of UN10 with $NaBH_4$ [49] and determined UL10's hitherto unknown molar absorptivity (3510 at 289 nm in 1-propanol; cv 3%) in three independent syntheses with repeated absorbance readings. This allowed calibrations of all HPLC systems and the reporting of all coenzyme Q10 levels in molar units.

When we applied our traditional HPLC system for lipid-phase micronutrients (HPLC system 1) we discovered that plasma hexane extracts stored at room temperature for several days in the methanol-based HPLC mobile phase led to a decrease in UL10 concentrations with a concomitant increase in UN10 levels (Fig. 2A). Our attempts to quantitate this conversion in this HPLC system were unsuccessful because the UL10 peak could not be separated from lycopene (and α-carotene) isomers. As shown by the UV/Vis scan of the UL10 peak a carotenoid-specific absorbance pattern at 400–470 nm was present in addition to the UL10 absorbance at 290 nm (see insets of Fig. 2A). To provide an affordable alternative for Q10 quantitation, we developed HPLC system 2, which reliably measures TQ10 after quantitative precolumn coulometric oxidation of UL10 to UN10 followed by fast HPLC analysis and UV detection of UN10 (native + precolumn oxidized forms) at 275 nm. This system was applied successfully (interassay cv 4–11%) for measurement of plasma hexane extracts that had been stored at −80 °C for several months and were known to be stable regarding TQ10 levels [20]. This system allowed quantitation of Q9 levels by using tocol or preferably δ-tocopheryl laurate as internal standard (Fig. 2B).

HPLC system 3 was established to quantitate simultaneously UL10 and UN10 without interference from other plasma lipid phase micronutrients by employing a HypersilGold C18 column that separated ULs and UNs in an empty and late part of the chromatogram, after elution of retinoids, carotenoids, and tocopherols. Detection was performed by absorbance at 295 nm and by the approximately 40-fold more sensitive coulometric detection at +0.7 and −0.7 V for UL and UN, respectively, as optimized by hydrodynamic voltammograms. Faster run times could be achieved by increasing the solvent flow, which required ultra-HPLC hardware (data not shown). UL10 was relatively stable in blood and also alone in organic solvents at 22 °C and in the presence of Bis–Tris propane (0.5–1 mM), tocol (<10 µM), or γ-tocopherol (γT; 2–28 µM); however, it oxidized efficiently and dose dependently to UN10 when BHT or αT was present in in vitro experiments (Fig. 3, Table 1). The oxidation was particularly fast when BHT was combined with αT or γT and was complete at tocopherol levels of >13 µM before 24–26.5 h postinitiation (our last measuring point). In the latter cases hourly conversion rates could not be calculated because the exact time at which 100% conversion occurred was unknown (any time in the 8- to 24-h period). Whenever oxidation occurred the conversion rates were usually much faster in the first 1–3 h than at later hours. The reaction speed was 2- to 4-fold faster when BHT was combined with αT versus combined with γT. Interestingly, whenever oxidation of UL10 occurred in the presence of αT or γT the tocopherol levels stayed at the initial concentrations at any time monitored as measured by HPLC system 1. This suggested that αT and γT acted catalytically. Also, the conversion of UL10 to UN10 occurred in an equimolar fashion as evidenced by the molar changes of UL10 and UN10 measured by HPLC/UV/ECD after adjustment for absolute losses by using tocol as an internal standard (Table 1). This in addition to LC/MS measurements identifying the ECD signals as predicted (data not shown) excluded the formation of any other products than UN10 during this reaction of UL10.

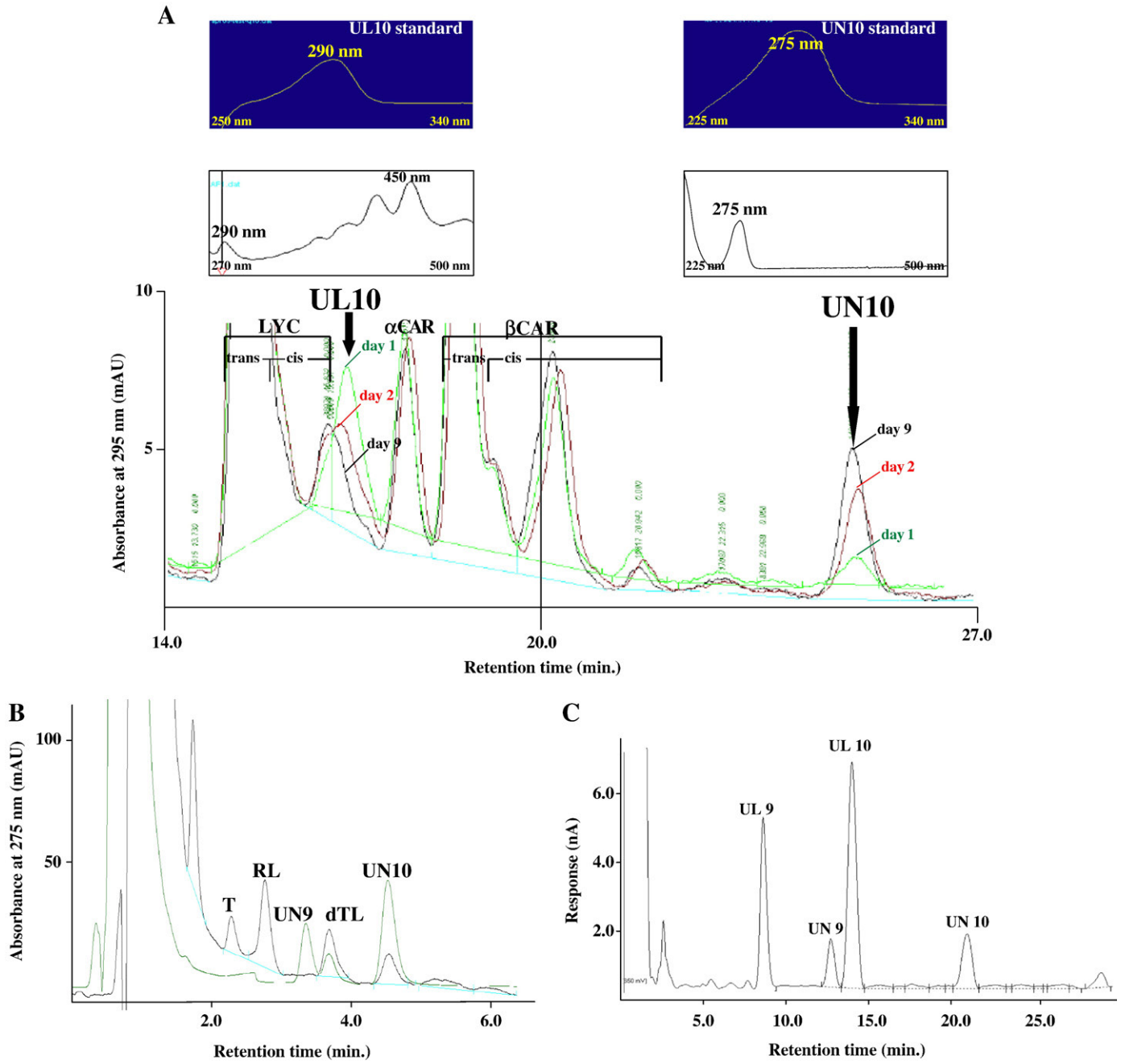

Fig. 2. (A) HPLC trace of a plasma hexane extract obtained from a Q10 supplemented subject after storage at 22 °C for several days using system 1 resolving tocopherols and retinoids (not shown) and also carotenoids and Q10. UL10 and UN10 in the plasma trace can be identified by typical absorption at 290 and 275 nm, respectively (standard and plasma analytes are shown in the upper and lower UV/Vis scan panels, respectively). UL10 coelutes with carotenoids, indicated by absorption in the 450 nm range. Over several days oxidation of UL10 to UN10 is observed by a decreasing UL10 peak, whereas the UN10 signal increases. LYC, lycopene; αCAR, α-carotene; βCAR, β-carotene. (B) HPLC trace of a plasma hexane extract (black) using HPLC system 2 after precolumn oxidation and postcolumn monitoring at 275 nm. UN10 and the internal standard δ-tocopheryl laurate (dTL) are resolved from UN9, retinyl laurate (RL), and tocol (T) (overlaid gray/green traces are standards). All other lipophilic antioxidants are eluted in the first 2 min and do not interfere. (C) HPLC trace of a plasma hexane extract using HPLC system 3 resolving ULs and UNs after all other lipophilic antioxidants (carotenoids, tocopherols, retinoids) are eluted in the first 5 min. Other lipophilic plasma components (fats, cholesterol) cause a noisy baseline after elution of UN10. A two- to threefold increase in flow rates decreases run times two- to threefold but requires ultra-HPLC equipment.

Lowering the temperature and various methods of degassing the solvents used in the UL10 oxidation reaction diminished the speed of UL10 conversion (Table 1). Oxidation rates were approximately 20-fold lower in plasma stored at −80 °C vs room temperature. Similarly, they were 1.1- to 2.2-fold lower after membrane filtering or by argon or nitrogen purging of solvents, whereas they were 1.9- to 4.0-fold less after combined membrane filtering and argon purging. In contrast, conversion rates were increased by a factor of 1.2–1.3 by blowing oxygen into the solvents before using them for dissolving UL10 followed by monitoring its oxidation. This suggested that oxygen plays a key role during this UL10 conversion. The oxidation pattern described above for UL10 was, as expected, almost identical for UL9 (Fig. 3, Table 1). Therefore, adjustment of UL10 or UN10 for UL9 and UN9, respectively, led to almost unchanged Q10 levels over time (Fig. 3).

In specimen from Q10 unsupplemented subjects the UL oxidation rates at 22 °C were minimal in unprocessed blood (<0.1%/h after 1 day), moderate once processed to plasma (0.4–0.6%/h after 8 h),

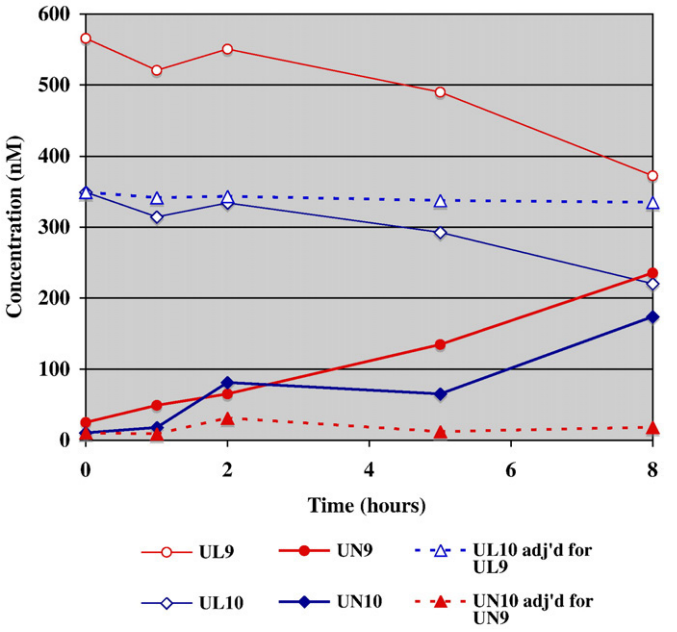

Fig. 3. Decrease in UL9 (spiked as internal standard) and UL10 (native) concentrations and concomitant increase in UN9 and UN10 levels in a plasma hexane extract over time at 22 °C. Very similar oxidation rates of UL9 to UN9 and UL10 to UN10 were observed. Therefore, UL10 levels adjusted for UL9 and UN10 levels adjusted for UN9 remained little changed. This pattern was also observed with authentic Q9 and Q10 standards when dissolved in solvents containing α-tocopherol or BHT. However, almost no change over time at room temperature occurred when ubiquinols were dissolved in organic solvents alone (see Table 1).

Table 1
Rate of in vitro UL10 oxidation to UN10 (%/h)

Hours	BTP or BHT					
	No addition[a]	0.5 mM BTP	1.0 mM BTP	No addition[a]	1.1 mM BHT	2.3 mM BHT
1	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%
3	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	1.8%
5	<0.1%	<0.1%	<0.1%	<0.1%	0.9%	1.0%
8	<0.1%	<0.1%	<0.1%	<0.1%	0.6%	0.8%
24–26.5	<0.1%	<0.1%	<0.1%	<0.1%	0.8%	1.1%
	αT					
	No addition[a]	2.6 μM αT	13.2 μM αT	25.5 μM αT	31.3 μM αT	50.4 μM αT
1	<0.1%	1.3%	3.8%	1.0%	4.3%	6.1%
3	<0.1%	0.4%	1.5%	1.6%	2.5%	3.3%
5	<0.1%	0.3%	1.0%	2.2%	2.8%	2.9%
8	<0.1%	0.2%	0.6%	2.0%	3.2%	4.2%
24–26.5	<0.1%	0.2%	0.7%	1.9%	2.7%	3.1%
	αT + 1.1 mM BHT + 0.5 M BTP					
	BHT + BTP alone	2.6 μM αT	13.2 μM αT	25.5 μM αT	50.4 μM αT	
1	1.4%	2.9%	10.6%	18.1%	34.8%	
3	0.8%	2.4%	5.9%	10.0%	17.9%	
5	0.8%	2.6%	6.7%	9.8%	15.2%	
8	0.7%	2.5%	7.4%	10.0%	>12.5%	
24–26.5	1.0%	2.2%	>4.2%	>4.2%	>4.2%	
	γT					
	No addition[a]	1.7 μM γT	2.9 μM γT	5.3 μM γT	13.7 μM γT	27.6 μM γT
1	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%
3	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%
5	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%
8	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%
24–26.5	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%
	γT + 1.1 mM BHT + 0.5 M BTP					
	BHT + BTP alone	2.9 μM γT	6.0 μM γT	13.9 μM γT	27.6 μM γT	
1	<0.1%	2.7%	4.8%	5.5%	7.6%	
3	2.1%	3.2%	3.9%	5.7%	7.9%	
5	1.1%	2.9%	3.4%	6.3%	11.0%	
8	1.3%	2.3%	3.2%	4.7%	7.4%	
24–26.5	0.9%	1.8%	2.4%	>4.2%	>4.2%	

All values are means of two to five measurements. Unless otherwise noted all data measured by HPLC/ECD (system 3) at 22 °C were obtained from samples dissolved in methanol:dichloromethane:acetonitrile (66.5:21.8:11.7; v/v/v) with UL10 concentrations at 1156 nM (1000 ng/ml). All values are lower after solvent degassing (1.1- to 2.2-fold after membrane filtering or argon or nitrogen purging and 1.9- to 4.0-fold after combined membrane filtering and argon purging). If a value is greater than a given number, then calculation was not possible because of 100% conversion before the given time point. Empty cells, no data generated. BHT, butylated hydroxytoluene; BTP, Bis–Tris propane (pH 6.8); αT, α-tocopherol; γT, γ-tocopherol. αT and γT levels never change over time.

[a] UL10 alone at 900–3500 nM; replicated in each experiment; addition of tocol as internal standard (≤10 μM) does not change values.

and high in the plasma hexane extract (3.5%/h after 1 day; Table 2). Blood specimens from one individual (not supplemented with Q10) that were obtained 2 days apart showed native plasma αT and UL10 levels of 45.3 and 1.1 μM in the first specimen and 75.5 and 1.1 μM in the second specimen, applying HPLC systems 1 and 3, respectively (Table 2). After 8 h the first and second specimens showed native UL10 oxidation rates of 0.35 and 0.58%/h, respectively (Table 2). Addition of EDTA to plasma (1 mM) did not change the UL10 oxidation rates.

Six separately aliquotted pooled quality control plasma samples stored at −80 °C were spiked with known amounts of UL9:UN9 (95:5) and analyzed over the course of 3 days. System 3 showed UL10 levels of 634 nM (cv 5.3%) and UN10 levels of 27 nM (cv 7.5%) after adjustment of UL10 for UL9 and UN10 for UN9. The adjusted mean ratio of UL10:UN10 was 96:4 (cv 2.7%). The same analysis of another quality control set of plasma from 13 healthy subjects revealed mean UL10 and UN10 levels of 1466 nM (median 1479 nM, SD 660 nM) and 77 nM (median 63 nM, SD 57 nM), respectively, with a mean UL10:UN10 ratio of 95:5 (median 96:4). A clear trend of larger UL10:UN10 ratios with lower TQ10 levels ($r = -0.69$; $p = 0.004$) was observed. The limit of quantitation for UL10 and UN10 from plasma was 23 nM.

Discussion

The molar extinction coefficient of UL10 determined in this study, 3510, is in good agreement with that of UL6 (3850) reported previously [49] and validates our results. Our coulometric settings to detect ULs and UNs, which were optimized using hydrodynamic voltammogram results applied the oxidation–reduction–oxidation mode with readings at the last cell [26], thereby detecting only redox-reversible compounds leading to increased signal-to-noise ratios, were similar to those of previous reports [5,28]. Using the same response factors for Q9 as for Q10 in our HPLC system when converting coulometric responses (peak area) to molar concentrations is in agreement with the very similar HPLC retention times of these analogs and with UL9 and UL10 or UN9 and UN10 exhibiting superimposable hydrodynamic voltammograms [20].

Unexpectedly, UL10, when dissolved in organic solvents (methanol, acetonitrile, and dichloromethane mixtures), was stable at 22 °C if no other chemicals were present. However, when BHT or αT alone, or particularly when BHT in combination with αT or γT, was added, UL10 oxidized quickly to UN10 in an equimolar fashion without formation of other products and without consumption of αT or γT. Two aliphatic substituents (methyl or tertiary butyl residues) vicinal to

Table 2
Ex vivo UL10 oxidation rate to UN10 (%/h)

	Hours	UL10	UL9
Blood[a]: 1.0 μM UL10, 33.3 μM αT	2[b]	<0.1%	
	24	<0.1%	
Plasma			
1.1 μM UL10, 45.3 μM αT[c]	3	0.07%	
	8	0.35%	
1.1 μM UL10, 75.5 μM αT[c]	3	0.89%	
	8	0.58%	
	Weeks at −80 °C		
0.8 μM UL10, 29.3 μM αT[d]	2	0.019%	0.024%
	4	0.016%	0.026%
	Hours		
Plasma extract in hexane: 0.8 μM	1	11.4%	13.4%
UL10; 29.3 μM αT[d]	3	6.5%	6.4%
	5	7.8%	7.3%
	8	2.3%	2.1%
	24–26.5	3.5%	3.4%

Unless otherwise noted all data measured after exposure to 22 °C by HPLC/ECD (system 3) were from the same subject (not supplemented with Q10); all concentrations are in micromolar for corresponding native plasma levels. UL10, ubiquinol-10 (native); UL9, ubiquinol-9 (spiked); αT, α-tocopherol.

[a] Blood was stored at the given times followed by plasma preparation and immediate extraction into hexane and HPLC analysis (system 3).

[b] Same results at 6 °C.

[c] Collected 2 days apart.

[d] Using pooled plasma; no changes were observed after addition of EDTA (1 mM; pH 6).

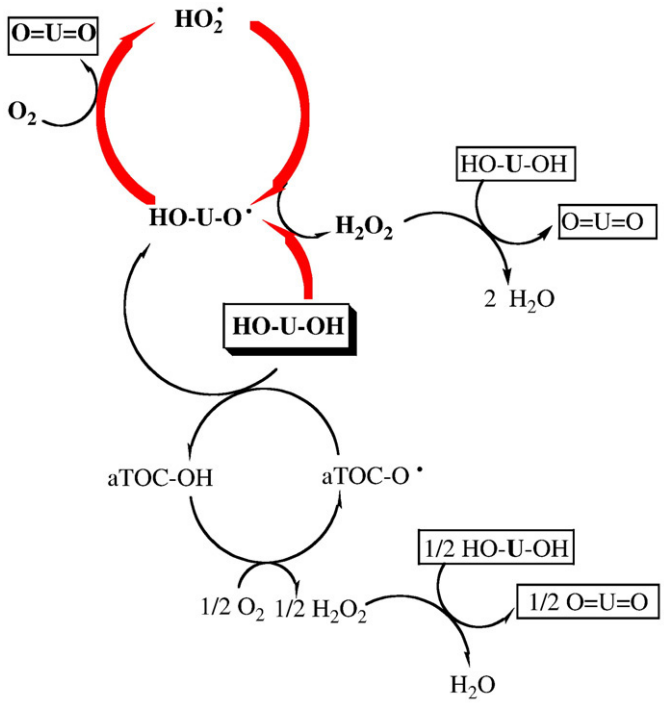

Fig. 4. Hypothesized mechanism of αT (aTOC-OH) catalyzing the redox cycling of UL (HO-U-OH) to UN (O=U=O). The αT radical produced during αT's antioxidant action toward oxidants (oxygen) is reduced by UL to αT by the formation of a UL radical. The latter reacts quickly with oxidants (oxygen) and redox cycling of UL with oxygen is initiated (bold reaction cycle). Phenols other than αT can act as catalysts alone or synergistically if aliphatic (or possibly other) residues are present on both α-positions of the phenolic hydroxyl group.

phenolic hydroxyls seemed to be key structural features of the observed catalytic action on UL oxidation, because aliphatic alcohols such as BTP or nonalkylated phenols such as tocol had no effect, whereas BHT, αT, and other phenols with two aliphatic residues on both vicinal carbons of the aromatic, such as 2,6-di(propan-2-yl)phenol (data not shown), caused fast UL oxidation. The latter molecular structure is known to enhance resonance stabilization of phenoxy radicals [50], which are therefore likely intermediates in the observed catalytic reaction. Phenols with other than aliphatic residues (amines, hydroxyls, thiols) on the vicinal carbons can most likely result in the same catalytic effect.

Because of the usual processing sequence of blood to plasma and finally to a plasma extract we investigated the UL10 oxidation rates in each of these three states. The UL oxidation rate was minimal in unprocessed blood, moderate once processed to plasma, and high once plasma was extracted into organic solvents (Table 2). The latter finding is in good agreement with our in vitro results (Table 1), and our overall findings are similar to those of previous reports from lipid-phase antioxidants [51–53]. We hypothesize that during the above-mentioned processing events the compartmentalization of lipophilic antioxidants is increasingly destroyed through the increasing breakup from the lipoproteins. Although relatively well preserved when natively bound to lipoproteins, UL10 is believed to oxidize once lipoprotein binding is broken, which allows UL10 to come into contact with other molecules, particularly oxygen. As reported for other micronutrients, the moderate oxidation occurring in plasma is further diminished in unprocessed blood because of the antioxidant effects of blood components [51–53]. Interestingly, and in agreement with our in vitro data, we found, however, that increasing native αT levels led to faster UL10 oxidation in native plasma (Table 2). In contrast, free metals do not seem to contribute to the UL10 oxidation because addition of EDTA to plasma did not change the oxidation rates.

Our findings of phenolic antioxidants catalyzing an oxidation reaction seemed paradoxical. However, pro-oxidant properties of this group of compounds, particularly of αT oxidizing co-antioxidants such as quinols, are well documented [54]. Similar to the redox cycling of lipids caused by αT [54], we hypothesize that αT can catalyze the redox cycling of UL as shown in Fig. 4. The αT radical produced during αT's antioxidant action toward oxidants (oxygen) is reduced by UL to αT under formation of a UL radical. The latter reacts quickly with oxidants (oxygen) to form UN and redox cycling of UL to UN is initiated (bold reaction cycle in Fig. 4). The decreased conversion of the UL10 oxidation with increased efficiency of solvent degassing in combination with the increased conversion caused by saturating solvents with oxygen suggests that oxygen is the reaction partner during the oxidation of UL10. This mechanism is supported by earlier observations on UL10 scavenging αT radicals and reacting quickly with oxygen [55]. The catalytic function of tocopherols during the observed UL oxidation was confirmed by repeated analyses and the fact that an αT change of 1–2 μM (if αT were a reaction participant) was never observed, although well within the sensitivity of our measurements.

We were unable to detect hydrogen peroxide by commercial test strips (WaterWorks Peroxide Check) designed to give semiquantitative peroxide levels in organic solvents (data not shown). The possibility of this test not being sensitive enough to detect H_2O_2 seems unlikely, again considering that if the reaction occurred, concentrations of 1–2 μM would have been generated that would have been well within the sensitivity of the applied method (<0.5 ppm). Therefore, the reduction of O_2 was probably continued via H_2O_2 to the formation of water as shown in Fig. 4.

The unavoidable oxidation tendency of UL in plasma, or particularly once present in solution in plasma extracts with other phenolic antioxidants, poses a critical challenge for the accurate determination of native UL:UN ratios in the circulation. The oxidation rate is particularly fast during the first hours and increases by difficult-to-control factors such as temperature, storage time of plasma and particularly its extracts, and type and concentration of phenolic plasma antioxidants (which are unknown in part or entirely for most plasma extracts). The former factors were realized previously [25], including the good stability of total Q10 in plasma when stored at −80 °C over 2 years with or without several thaw–freeze cycles [20] or when plasma crashed with isopropanol is stored at 0 °C up to 4 h [5].

We now report for the first time the catalytic effect of tocopherols and other phenolic antioxidants on UL oxidation. Therefore, previous reports have probably underestimated native plasma UL10:UN10 ratios, and assays for TQ10 probably measured the latter accurately, assuming that sound analytical methods were applied. For TQ10 analysis we recommend the coulometric precolumn oxidation method as established in HPLC system 2 (see Materials and methods) because of its robustness using UV detection, high speed, and resolution of various internal standards including tocol, retinyl laurate, and δ-tocopheryl laurate from UN10 and also UN9 (Fig. 2B).

We propose for the first time that the application of HPLC system 3 can determine accurately native UL10 and UN10 concentrations in the circulation by spiking known UL9 and UN9 amounts immediately after blood draws. Because of the high similarity of the overall molecular structures (only one isoprene unit difference in the side chain) and the identical structural elements involved during redox reactions, Q9 mirrors the fate of Q10 ideally, as supported by the similar HPLC retention times, the same hydrodynamic voltammograms, and particularly by the highly similar oxidation rates when investigated as single agents or when present in plasma extracts (Tables 1 and 2). Therefore, the added UL9 and UN9 act as superb internal standards in our proposed method not only by reflecting absolute analyte losses but also by reflecting the UL oxidation rate. After adjustment of measured UL10 to UL9 and UN10 to UN9 native UL10 and UN10 levels, as occurring in the circulation, can be calculated accurately (see Fig. 1). The key step is to add known amounts of the internal standards to known volumes of blood or plasma immediately after collection or preparation, respectively. This could pose a critical challenge in epidemiologic or clinical studies because of the constantly changing UL9:UN9 ratio in the spiking solution, which requires UL9 and UN9 quantitation of the internal standard solution in a calibrated analytical system on each day of blood collection.

Very low plasma concentrations of UL9 (ca 1% vs UL10) have been reported in humans [3] and at these levels little interference is to be expected when UL9 + UN9 are used as the internal standard. If native Q9 needs to be measured we suggest the use of UL7/UN7, UL6/UN6, or alkoxy ubiquinone analogs [26,29], added again in a mixture with their ubiquinols. We showed the feasibility of the latter approach by preparing dipropoxy and dibutoxy UN10 and UL10 analogues because they eluted without interference and baseline separated from all reduced and oxidized forms of Q9 and Q10 in all HPLC systems (data not shown).

The higher plasma UL10 levels we found in the second quality control group was probably due to the large proportion of subjects supplementing with Q10 in that second versus the first volunteer group. Large differences in TQ10 concentrations between Q10 nonsupplementers and supplementers (680–3300 nM) and, interestingly, a trend of a larger UL10:UN10 ratio with lower TQ10 levels were observed. The latter might be due to the body being overwhelmed with the task of keeping Q10 reduced in the circulation when present at very large concentrations or to unknown other factors.

Our findings on the catalytic effects of tocopherols and other phenolic antioxidants on UL oxidation emphasize the high likelihood of native UL:UN ratios to change during processing, storage, and analysis of blood or tissue preparations. This change will be variable and inconsistent between samples because of the variable and in most cases unknown concentrations of catalyzing phenolic antioxidants in addition to unknown other cofounders such as oxygen levels or temperature. The stability of UL10 if kept in its original environment in blood indicates that αT does not cause considerable UL10 oxidation physiologically or that the UL10 oxidation is counteracted by other antioxidant mechanisms. The proposed addition of internal standards tracking the observed changes during blood processing and analysis will be most helpful in future experimental, clinical, or epidemiologic studies evaluating the role of Q10 in health and disease by being able to accurately determine native UL10 and UN10 levels in the matrices investigated.

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