Determination of Ubiquinone-9 and 10 Levels in Rat Tissues and Blood by High-Performance Liquid Chromatography with Ultraviolet Detection

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Abstract

A high-performance liquid chromatographic (HPLC) method is presented for the simultaneous detection of ubiquinone-9 and 10 in rat tissues such as blood, myocardium, and muscle. After liquid–liquid extraction, the ubiquinones are subsequently analyzed by HPLC with ultraviolet (UV) detection at their maximum absorbance (275 nm). Reference calibration curves in ethanol are used to determine tissular levels of ubiquinones. Because a treatment with HMG–CoA reductase inhibitors is expected to decrease the ubiquinone levels, reference calibration curves are performed to ensure that the ratios (ubiquinone/internal standard) observed in such an experiment could be evaluated directly on a calibration curve. The assay is sensitive (0.0625 µg/mL), reproducible (4% coefficient of variation for ubiquinone-9 and 6% for ubiquinone-10), and linear up to 20 µg/mL (or 100 mg of tissue) for ubiquinone-9 and up to 10 µg/mL (or 100 mg of tissue) for ubiquinone-10. The ubiquinone levels in control tissues or blood are within the ranges of those previously reported.

Introduction

Peroxidation of membrane lipids, a complex process known to occur in both animals and plants, has been implicated in many processes of tissue injury. Ubiquinone is an essential lipophilic component of the electron transport chain of oxidative phosphorylation (1). Aside from its role in mitochondrial bioenergetics, ubiquinone may also affect membrane fluidity (2) and protect membrane phospholipids against peroxidation (3). It appears that ubiquinone could be active in the inner mitochondrial membrane where it is present in significantly greater molar concentration than other components of the electron transfer chain (4,5).

Ubiquinone is present in all cellular membranes, but its function there depends on its localization (6–8). In the inner mitochondrial membranes, a normal respiratory rate requires the maintenance of a high ubiquinone concentration, and even a limited decrease is deleterious for maintaining a normal function (9). Ubiquinone-10 has been established to be indispensable to the cardiac function (9–13).

The major form of ubiquinone in rats contains nine isoprene residues, but a relatively high level of the lipid with a side-chain of 10 isoprene units is present in rat tissues (14). Its distribution in various organs is quite different from that of the other lipid products of the mevalonate pathway and does not vary more than 10-fold. In the brain, spleen, and lung, the level of ubiquinone is similar in humans and rats, but in most of the other human organs, the amounts are considerably lower than those in rats. In humans, the amount of the ubiquinone-9 form is only 2–5% of the total, whereas the ubiquinone-10 form is dominant. However, smaller or larger amounts of ubiquinone-10 are also present in all rat organs. Levels of ubiquinone could be an important factor in a number of human pathologies.

Analytical techniques have been described for the determination of ubiquinone (15–17). However, some of these methods utilized external standards not taking into account the potential reactions occurring during sample preparation (18). This is the main reason why we have decided to use an internal standard.

Because the physico-chemical properties of Q_{11} are very similar to those of Q_{10} and Q_9, potential reactions that would affect Q_{10} and Q_9 would likely also affect Q_{11} to a similar extent, thereby minimally influencing the Q_9–Q_{11} and Q_{10}–Q_{11} ratios. The analytical procedure described here enables simultaneous determination of ubiquinone-9 and ubiquinone-10 in either blood, heart, or muscle. The high-performance liquid chromatographic (HPLC) separation was based on the procedures described by J.K. Lang (15) and Ikenoya et al. (16) and was designed to simplify the analytical process in selecting a single mobile phase for the different tissues in order to simplify and optimize the analysis with a maximal resolution. Previous methods (15,16) required different HPLC conditions (e.g., mobile phase) for the determination of ubiquinone-9 and 10 in different tissues. This method combines the optimization of the liquid–liquid extraction method adapted from J.K. Lang (15) and G.W. Burton (19), the HPLC analysis conditions, and the use of Q_{11} as the internal standard.

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Experimental

Chemicals and reagents

Ubiquinone-9, ubiquinone-10, and the internal standard ubiquinone-11 were provided by the Nisshin Chemicals Company (Tokyo, Japan). BHT (2,6-di-*tert*-butyl-*p*-cresol) and ascorbic acid were obtained from Sigma (St. Louis, MO). Sodium dodecylsulfate (SDS) was obtained from BDH Biochemicals (Toronto, Canada). HPLC-grade solvents and all other chemicals of analytical grade were purchased from Anachemia (Montréal, Canada).

The working standards for HPLC calibration were prepared by combining individual stock solutions. The stock solutions were prepared by dissolving pure ubiquinones in ethanol at concentrations of 100 µg/mL. The working solutions were prepared by diluting the stock solutions to give final concentrations of 1.25–40.0 µg/mL. These solutions, when kept at –80°C, are stable for several months. All containers used in these experiments were glassware with the exception of the storage of animal tissues and plasma, which were kept in plastic tubes.

Photosensitivity

Because the potential photodegradation of ubiquinone (15) cannot be completely ruled out, all extraction steps were performed in the absence of direct sunlight or incandescence.

Instrumentation

The HPLC system consisted of an isocratic pump (LDC Milton Roy, Riviera Beach, FL), a model 7125 injector (Rheodyne, Cotati, CA), and an ultraviolet (UV) detector (Milton Roy) set at a wavelength of 275 nm. The column (ODS) was packed with 5 µm Spherisorb C_{18} (Hichrom, Reading, UK). The mobile phase consisted of a mixture of methanol and ethanol (70:30). The solvent flow rate was 1.2 mL/min, and the procedure was performed at room temperature.

Animal study

Tissue from adult male Sprague Dawley rats weighing approximately 250 g were used to develop this analytical method. The animals were euthanized with ether, and samples of myocardium, muscle (these two being "freeze-clamped"), and heparinized blood were frozen immediately, stored in liquid nitrogen, and subsequently transferred in a –80°C freezer (freeze-clamping is a rapid freezing technique used to minimize changes in metabolite composition once the tissue has been dissected).

Tissue and blood extraction

Direct extraction with organic solvents was selected in order to avoid ubiquinone exposition to strong alkali (therefore this method could also be used for the determination of ubiquinone in tissues). The most efficient solvent mixture proved to be the ethanol–*n*-hexane (16). Ethanol was added to remove protein by partition and denature enzymes (16). In order to ensure maximal extraction of ubiquinone, the extraction was performed twice.

Approximately 100 mg of freeze-clamped tissue was accurately weighed in the frozen state and subsequently homogenized with 1 mL of water in a Potter-Elvehjem homogenizer (Polytron, Kinematica GMBH, Luzern, Switzerland) with a motor-driven pestle. A 50-µL volume of a BHT-in-ethanol solution (10 mg/mL) was added to each sample to prevent auto-oxidation. (For the blood, 0.2-mL samples of whole blood were mixed with 0.8 mL of 0.1M SDS. Then 1.0 mL of 5mM ascorbate in 5mM phosphate buffer [pH 7.4] was added, and the mixture was vortex-mixed for 1 min.)

The samples (after addition of 1 mL 0.1M SDS and brief homogenization) were then transferred to a 10-mL glass tube fitted with a poly(tetrafluoroethylene) (PTFE)-lined screw-cap, 2 mL of ethanol was added, and the mixture was vortex-mixed for 30 s. Then 2 mL of hexane was added, and the tightly capped test tube was vigorously vortex-mixed for 2 min. It was then centrifuged for 5 min at 2200 rpm, and the hexane organic supernatant layer was transferred to a small vial. The extraction with hexane was then repeated a second time. The combined extracts were evaporated with a Speed VAC (Savant Instruments, Farmingdale, NY) and kept frozen at –80°C. Samples were injected shortly after reconstitution with 200 µL of mobile phase because the ubiquinols could be rapidly oxidized and decomposed within 2 h.

Calibration curves

Tissues (3 g) were mixed and homogenized with 30 mL of water and 1.5 mL of BHT. Working solutions of ubiquinone standards in ethanol were spiked into tissue homogenates (containing the internal standard at concentrations of 10 µg/mL [or 100 mg tissue]) to obtain final concentrations varying from

Table I. Intra-Assay Precision of the Method*

Q	Concentration spiked (µg/mL or 100 mg)	Recovery (%) (mean ± SD)	Intra-assay CV (%)
Q_{10} (ethanol)	0.0625	108.4 ± 8.4	3.5
	0.5	112.8 ± 3.5	1.7
Q_9 (ethanol)	0.0625	102.4 ± 1.3	6.1
	2.0	90.2 ± 3.3	2.0
Q_{10} (blood)	0.0625	96.2 ± 6.0	6.0
	1.0	94.3 ± 5.5	1.5
Q_9 (blood)	0.0625	95.6 ± 5.0	3.5
	1.0	106.8 ± 7.2	6.0
Q_{10} (myocardium)	0.0625	117.0 ± 7.1	3.3
	10.0	114.4 ± 6.5	2.9
Q_9 (myocardium)	0.0625	93.5 ± 6.5	5.7
	20.0	112.8 ± 7.1	2.7
Q_{10} (muscle)	0.0625	109.8 ± 7.1	2.5
	1.0	90.0 ± 6.5	4.0
Q_9 (muscle)	0.0625	112.5 ± 8.3	8.0
	2.0	112 ± 7.5	4.5

* Three replicates.

0.0625 to 20 µg/mL (or 100 mg tissue). Calibration curves for ubiquinone-9 and ubiquinone-10 were generated by a least squares linear regression of the analyte–internal standard peak height ratio versus the ubiquinone or analyte concentration and were performed for each tissue (i.e., blood, myocardium, and muscle).

Reference calibration curves

Because a treatment with HMG–CoA reductase inhibitors is expected to decrease the ubiquinone levels, reference calibration curves were performed to ensure that the ratio observed in such an experiment could be evaluated directly on a calibration curve. For each tissue, a corresponding calibration curve was performed in ethanol, after liquid–liquid extraction, at the same concentrations. To validate the ethanol calibration curves used for the determination of the ubiquinone content, these curves had to be parallel to the corresponding tissue calibration curve.

Recovery

Recoveries of ubiquinone-9 and ubiquinone-10 were evaluated with tissue samples spiked with concentrations varying from 0.0625 µg/mL (or 100 mg tissue) to 10.0 µg/mL (or 100 mg tissue). The internal standard (ubiquinone-11) was added at the beginning of the extraction process. In one series, the ubiquinone standards were spiked before extraction, and in the other series, only after the extraction (Table I). The recovery estimates were based on the comparison of the standard–internal standard peak height ratios obtained in both series of samples.

Intra-assay precision

Intra-assay precision was evaluated by repeated measures (three replicates) in ethanol and tissue spiked with the analytes. The extraction procedure was then performed as described earlier. Each concentration on the calibration curve was analyzed in triplicate (mean ± coefficient of variation [$\bar{x}$ ± CV]).

Accuracy

To evaluate accuracy, the tissues were spiked with ubiquinone standard solutions at different concentrations ranging from 0.0625 to 80 µg/mL. The samples were assayed blindly, and concentrations were derived from the calibration curves. Accuracy was evaluated by comparing the estimated concentrations with the known concentrations of ubiquinone. This procedure was performed for both ubiquinone-9 and 10.

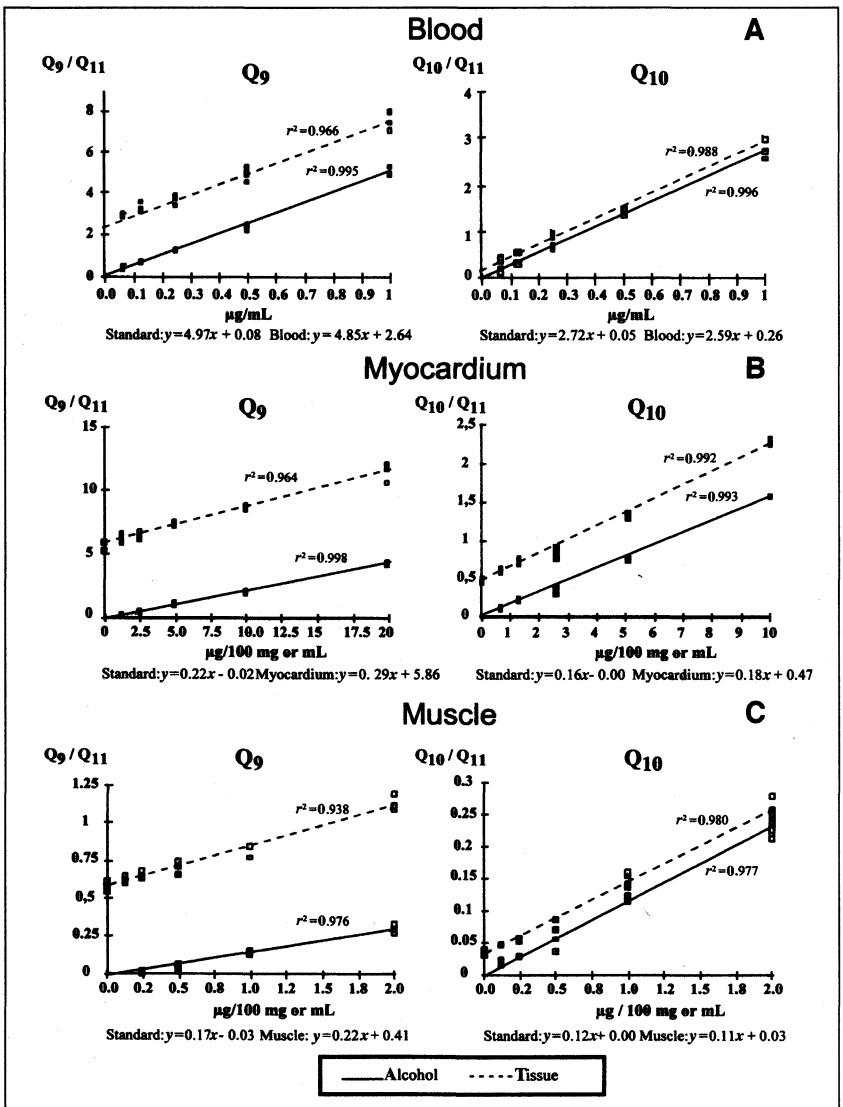

Figure 1. Reference calibration curves for ubiquinone-9 (Q_9) and ubiquinone-10 (Q_{10}) in alcohol with the corresponding calibration curves in spiked blood (A), myocardium (B), and muscle (C) tissues.

Interassay reproducibility

Interassay reproducibility was determined by assaying several samples (four to six) of the same stock solutions used for the calibration curves. It was evaluated on repeated measures of the stock solutions (after extraction and on different days) consisting of a mixture of ubiquinone-9, ubiquinone-10, and ubiquinone-11 in ethanol.

Stability

The overnight stability of ubiquinone-9 was studied with spiked blood, muscle, myocardium, and ethanol. Samples were assayed in triplicate on the day of preparation and 24 h afterward. The stability of the ubiquinone-9 solution in ethanol was also studied over a period of two weeks.

Results and Discussion

Calibration curves

All calibration curves were linear. The reference calibration curves for ubiquinone-9 and ubiquinone-10 in alcohol with the corresponding calibration curves in spiked blood, myocardium, and muscle tissues are illustrated in Figures 1A, 1B, and 1C, respectively. The reference and tissue calibration curves were parallel, and therefore the linear regression of the ethanol curves was used to derive the ubiquinone-9 and ubiquinone-10 content in experimental samples. The linear regression of the reference calibration curve was

used to derive the tissular concentration corresponding to the ratio of the peak height between ubiquinone-9 or 10 and the internal standard obtained for each sample.

Examples of chromatograms obtained after extraction of ethanol standard, blood, muscles, and myocardium samples are illustrated in Figure 2. The overall HPLC analysis took approximately 12 min; the retention time of ubiquinone-9 was 5.5 min, that of ubiquinone-10 was 7.3 min, and that of the internal standard ubiquinone-11 was 9.5 min. The selectivity of the UV detection allowed for a good resolution of ubiquinone-9 and ubiquinone-10 in the analyzed tissues. The response was linear from 1.25 to 20 µg/mL (or 100 mg tissue) for ubiquinone-9 and from 1.25 to 10 µg/mL (or 100 mg tissue) for ubiquinone-10.

In order to increase the recovery of the procedure, the extraction with hexane was repeated a second time. The recovery of ubiquinone-9 and 10 ranged from 90 to 112% for the different tissues (Table I). Based on an estimate of the minimum amount of analyte detectable with a signal-to-noise ratio of 3, the detection limit was 2 ng for ubiquinone-9 and 10.

Intra-assay precision

The intra-assay variability was assessed by triplicate extraction and measures of ethanol and tissue samples spiked with the analytes at concentrations varying from 0.0625 to 20.0 µg/mL (or 100 mg tissue). Intra-assay variability ranged from 2 to 6% for ubiquinone-9 and from 2 to 4% for ubiquinone-10 in standard solutions in ethanol. In the different tissues, the coefficient of variation ranged from 3 to 6% for ubiquinone-9 and from 2 to 8% for ubiquinone-10 (Table I).

Accuracy

Blindly assayed spiked samples (six replicates) of ubiquinone at concentrations covering the standard curves showed mean accuracies for ubiquinone-9 of 99.7% ± 6.4 (myocardium), 90.4% ± 7.8 (muscle), and 82.8% ± 3.5 (blood); for ubiquinone-10, the mean accuracies were 103.9% ± 5.8 (myocardium), 88.5% ± 3.2 (muscle), and 117.3% ± 15.4 (blood).

Interassay reproducibility

The interassay mean coefficient of variation ranged from 3 to 7% for ubiquinone-9 and from 3 to 5% for ubiquinone-10 (Table II).

Stability

Dry residues of extracted samples spiked with ubiquinone-9 and 10 were stable when stored overnight at –5°C (Table III). Stock solutions of ubiquinone-9 (0.0625 and 2.0 µg/mL) in ethanol showed no significant decrease in their concentrations when stored for two weeks under the same conditions ($\bar{x}$ 94–106%).

Applications

This assay was directly used in a preclinical study evaluating the effects of different HMG–CoA reductase inhibitors on the ubiquinone levels. Application results for the controls were within the ranges previously reported in the rat and were as follows: for ubiquinone-9, 0.52 µg/mL in blood, 26.4 µg/g in muscle, and 267 µg/g in the myocardium; for ubiquinone-10, 0.07 µg/mL in blood, 2.5 µg/g in muscle, and 29.4 µg/g in the myocardium.

Conclusion

This method allows the separation of endogenous ubiquinone-9 and ubiquinone-10, which were resolved in all tissues studied. It was also demonstrated that this method provides a quantita-

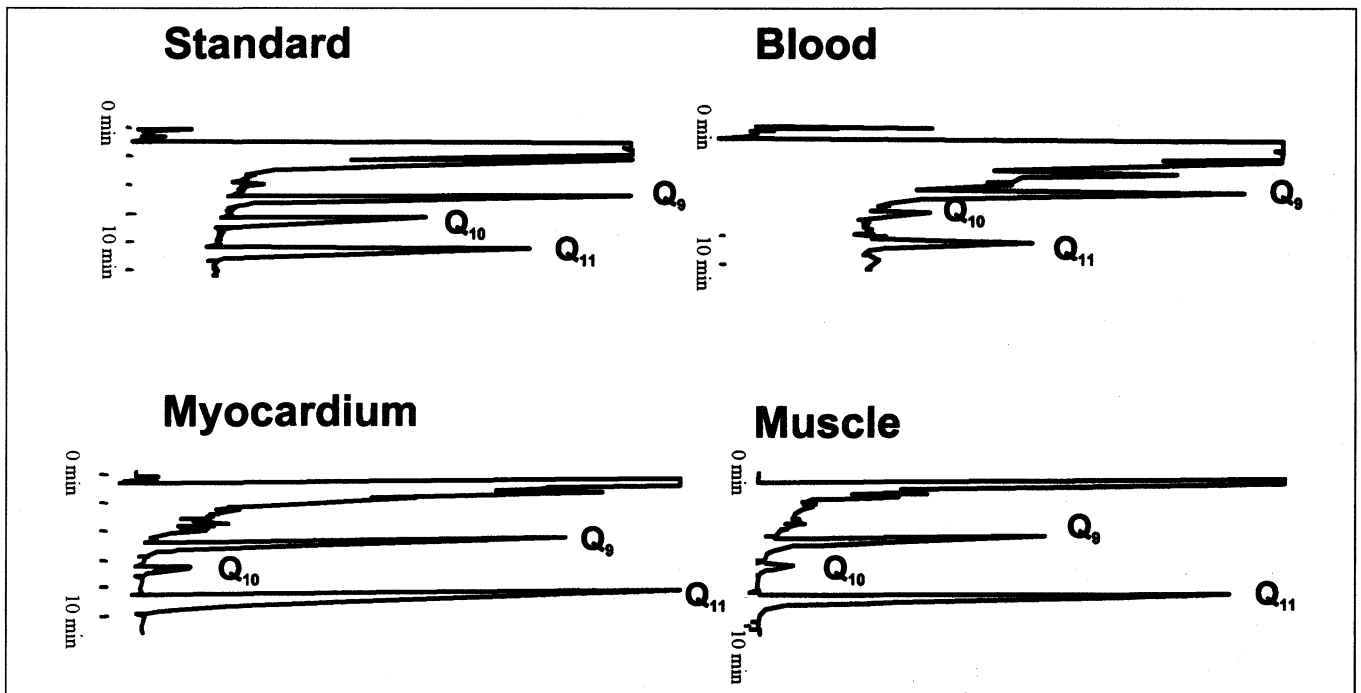

Figure 2. Examples of chromatograms obtained from ethanol standard spiked with ubiquinone-9 (Q_9) and ubiquinone-10 (Q_{10}) and in blood, muscle, and myocardium.

tive evaluation of the ubiquinone-9 and ubiquinone-10 levels in these tissues. The extraction method allows for peaks that are free of interfering endogenous substances.

The advantage of our method compared to others resides in the combination of several features: (*a*) For the extraction, the solvent ratio has been selected to maximize the extractability (16), and the BHT has been added to prevent auto-oxidation during extraction. (*b*) The SDS was used to increase the efficiency of the extraction (19) in view of its ability to dissociate and solubilize membrane proteins. (*c*) The mobile phase has also been selected to simplify and optimize the analysis in the different tissues with maximal resolution. (*d*) The use of Q_{11} as the internal standard (20) is an important factor for the accurate quantitative analysis of Q_9 and Q_{10} in the different tissues pending an exact measurement of the volume of the Q_{11} ethanol solution to be analyzed. (*e*) The use of reference calibration curves in ethanol allows for an accurate measurement of a potential decrease that would be observed in the tissue ubiquinone levels.

The limitation of this method is that it does not allow for a direct measurement of ubiquinol levels. However, BHT is immediately added to the sample after collection in order to avoid oxidation and to keep the ubiquinones in their initial form.

This method was not designed to document changes in ubiquinone levels varying according to oxidative stress (e.g., adrenergic stress) but to measure ubiquinone in a given control environment (i.e., without oxidative stress changes) where it is the synthesis level that varies and where the oxidative form of ubiquinone is the predominant one. This method was designed to allow observation of the long-term effect on ubiquinone in a basal condition with synthesis-inhibitory effects of HMG–CoA reductase inhibitors on ubiquinone levels under nonoxidative conditions. Although the measure of the reduced form could be of importance in some instances, this method could be easily adapted to do so (e.g., by measuring the ubiquinols at a wavelength of 291 nm [21]). In preliminary experiments (data not shown), the ubiquinol levels that we observed in the myocardium and muscle rat tissues were approximately 10-fold lower than the ubiquinone levels and were similar to previous reports (14,17,22). Because the Q_{red}–Q_{ox} ratio should not vary in one condition following a treatment with drugs, we are confident that the method proposed adequately addresses our needs; the objective of our experiments was to study the effect of HMG–CoA reductase inhibitors on the ubiquinone pool for tissues in which the main portion of ubiquinones is in the oxidized form.

The relevance of this method is that it allows for a simultaneous determination of ubiquinone-9 and ubiquinone-10 in blood, myocardium, and muscle rat tissue in one analysis through a relatively rapid and simple method.

Table II. Calibration Curve and Inter-Assay Variability

Concentration in ethanol (µg/mL)	Q	Replicates	Peak height ratio (mean + SD)	CV (%)
0.0625	Q_9	3	0.185 ± 0.007	3.6
	Q_{10}	4	0.08 ± 0.003	4.0
0.125	Q_9	4	0.340 ± 0.025	7.3
	Q_{10}	5	0.168 ± 0.01	5.2
0.25	Q_9	4	0.825 ± 0.05	6.0
	Q_{10}	4	0.334 ± 0.02	5.4
0.5	Q_9	6	1.34 ± 0.07	5.6
	Q_{10}	4	0.661 ± 0.02	2.8
1.0	Q_9	5	2.74 ± 0.08	2.8
	Q_{10}	4	1.326 ± 0.06	4.6

Table III. Overnight Stability*

Q	Concentration (µg/mL or 100 mg)	% at 24 h†	CV (%)
Q_{10} (ethanol)	0.0625	107.1	2.8
	0.5	104.4	3.6
Q_9 (ethanol)	0.0625	107.5	2.7
	2.0	106.2	1.7
Q_{10} (myocardium)	0.0625	103.8	5.3
	5.0	98.7	2.4
Q_9 (myocardium)	0.0625	110.0	2.0
	10.0	97.4	6.7
Q_{10} (muscle)	0.0625	108.8	4.6
	1.0	99.0	6.4
Q_9 (muscle)	0.0625	106.8	5.1
	1.0	99.7	7.2
Q_{10} (blood)	0.0625	102.2	2.7
	1.0	106.2	1.4
Q_9 (blood)	0.0625	100.3	3.4
	1.0	101.0	3.5

* Three replicates.
† $(Q \text{ at } 24h/Q_{initial}) \times 100$.

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