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 has been developed for the simultaneous detection of ubiquinone-9 and 10 in rat tissues such as blood, heart and muscle. After liquid-liquid extraction, the ubiquinones were subsequently analyzed by HPLC with UV detection at their 275 nm maximum absorbance. Reference calibration curves in ethanol were used to determine tissular levels of ubiquinones. Since the expected effect of a treatment with HMG-CoA reductase inhibitors is likely to be a decrease in the ubiquinone levels we have performed reference calibration curves 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 (c.v. of 4% 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]. Besides 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[6,7,8]. 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 rat contains 9 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 brain, spleen and lung, the level of ubiquinone is similar in human and rat, but in most of the other human organs, the amounts are considerably lower than those in the rat. In human, the amount of the ubiquinone-9 form is only 2-5% of the total whereas the ubiquinone-10 form is dominant. Smaller or larger amounts of ubiquinone-10 are, however, also present in all rat organs. Levels of ubiquinone could be an important factor in a number of human pathology.

Analytical techniques have been described for the determination of ubiquinone[15-17]. However, some of these methods utilized external standards not taking account of 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 HPLC separation is based on the procedures described by L.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 L.K. Lang[15] and G.W. Burton[19], the HPLC analysis conditions as well as the use of Q_{11} as the internal standard.

EXPERIMENTAL

Chemicals and Reagents

Ubiquinone-9 and ubiquinone-10 and the internal standard ubiquinone-11 were kindly 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, U.S.A.). Sodium dodecylsulfate (SDS) was obtained from BDH Biochemicals (Toronto, Canada). Solvents of HPLC grade and all other chemicals of analytical grade were purchased from Anachemia (Montreal, 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^{o}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 Since the potential photodegradation of ubiquinone[15] cannot be completely ruled out all extraction steps were performed in the absence of direct sunlight or incandescent.

Instrumentation

The HPLC system consisted of a isocratic pump (LDC Milton Roy, Riviera Beach, FL, U.S.A.), a model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) and a 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-ethanol (70:30). The solvent flow rate was 1.2 mL/min and the procedure was performed at room temperature.

Animal Study

Tissue of 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 heart, muscle (these two being "freeze-clamped") and heparinized blood were frozen immediately, stored in liquid nitrogen and subsequently transferred in a -80^{o}C freezer (freeze clamping is a rapid freezing techniques used to minimize changes in metabolite composition once the tissue has been dissected).

Tissue/Blood Extraction

Direct extraction with organic solvents has been 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 is 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 volume of 50 μL of a solution of BHT in ethanol (10 mg/mL) was added to each sample to prevent auto-oxidation. [N.B. For the blood, 0.2 mL samples of whole blood were mixed with 0.8 mL of 0.1 M SDS. Then 1.0 mL of 5 mM ascorbate in 5 mM phosphate buffer (pH 7.4) was added and the mixture was vortex-mixed for 1 min.]

The samples (after addition of 1 mL of 0.1 M SDS and brief homogenization) were then transferred to a 10 mL glass tube fitted with a 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 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, U.S.A.) and kept frozen (at -80^{o}C). Samples were injected shortly after reconstitution with 200 µL of mobile phase, as the ubiquinols could be rapidly oxidized and decomposed within 2 hours.

Calibration Curves

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

Reference calibration curves Since the expected effect of a treatment with HMG-CoA reductase inhibitors is likely to be a decrease in the ubiquinone levels we have performed reference calibration curves 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

Recovery of ubiquinone-9 and ubiquinone-10 was 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 (n=3) 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 (@ ± CV).

Accuracy

To evaluate accuracy the tissues were spiked with ubiquinone standard solutions at different concentrations ranging from .0625 μg/mL to 80 μg/mL. The samples were assayed blindly and concentrations 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.

Interassay Reproducibility

Interassay reproducibility was determined by assaying several samples (n:4-6) 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 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 hours after. The stability of ubiquinone-9 solution in ethanol was also studied over a period of 2 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 (1a), heart (1b) and muscle (1c) tissues are illustrated in Figure 1. The reference and tissue calibration curves are 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 ratio ubiquinone 9 or 10/internal standards) obtained for each sample.

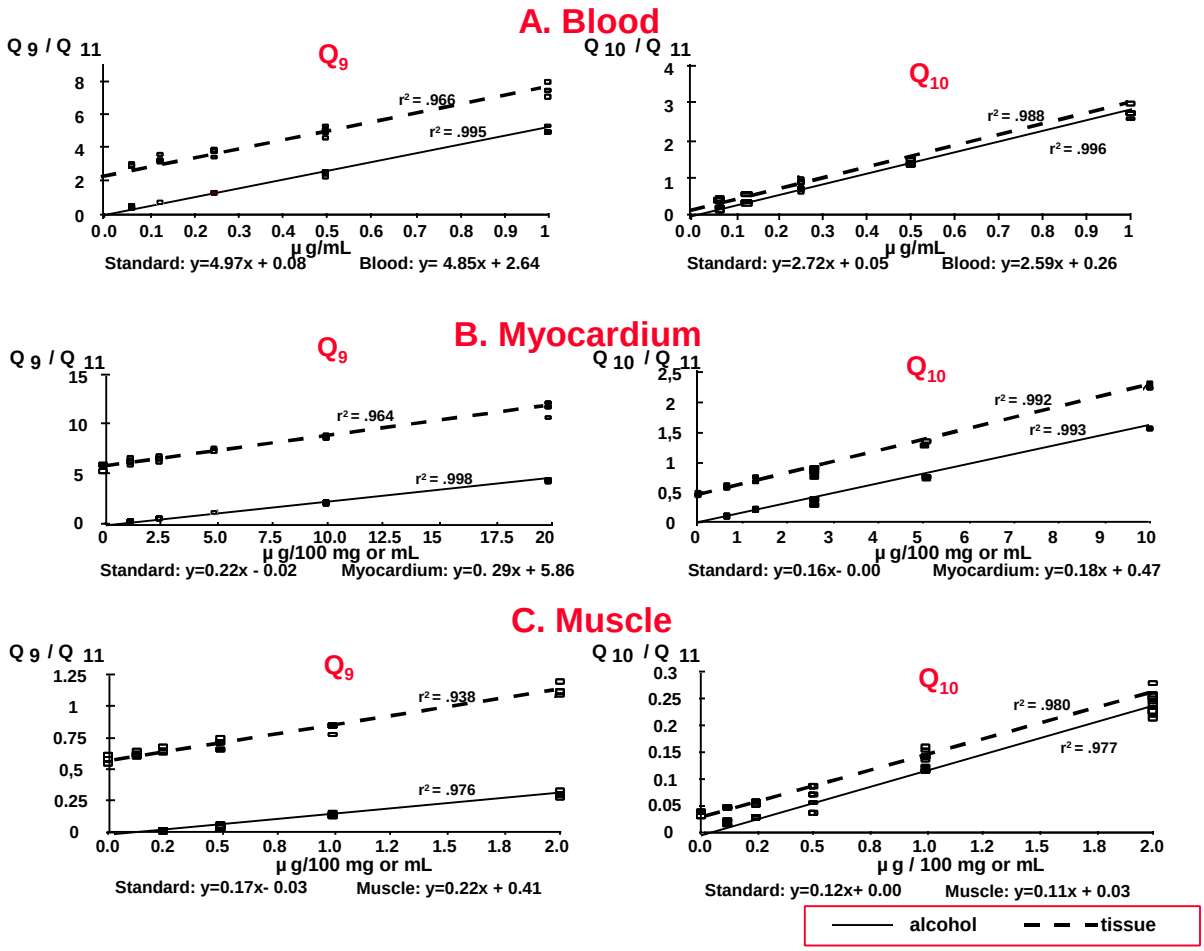

Figure 2: Examples of chromatograms obtained in ethanol standard spikes with ubiquinone-0 (Q9) and ubiquinone-10 (Q10) and in blood, muscles and myocardium.

Examples of chromatograms obtained after extraction of ethanol standard, blood, muscles and heart samples are illustrated in Figure 2. The overall HPLC analysis takes approximately 12 minutes with retention times of 5.5 min for ubiquinone-9, 7.3 minutes for ubiquinone-10 and 9.5 minutes for ubiquinone-11, the internal standard. The selectivity of the UV detection allowed for a good resolution of ubiquinone-9 and ubiquinone-10 in the tissues analyzed. 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.

Figure 2

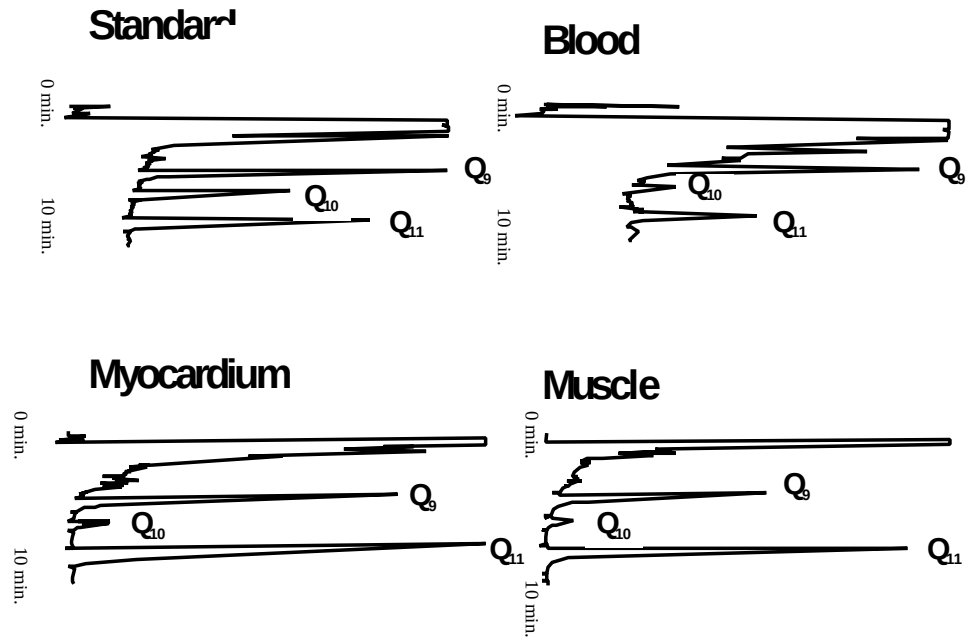

FIGURE 2: Examples of chromatograms obtained in ethanol standard spiked with ubiquinone-9 (Q_9) and ubiquinone-10 (Q_{10}) and in blood, muscles and myocardium.

In order to increase the recovery of the procedure, the extraction with hexane is repeated a second time. The recovery of ubiquinone-9 and 10 from different tissues 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 limit of detection is 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).

TABLE I. Intra-Assay Precision of the Method*

Q	Concentration spiked (mg per mL or 100 mg)	Recovery (%) x ± 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

Accuracy

Blindly assayed spiked samples of ubiquinone at concentrations covering the standard curves showed, for ubiquinone-9 a mean accuracy of (% ± SD, n=8), 99.67 ± 6.4 (myocardium), 90.4 ± 7.8 (muscle), 106.1 ± 13.7 (liver) and 82.8 ± 3.5 (blood); for ubiquinone-10 the mean accuracy was: 103.9 ± 5.8 (myocardium), 88.5 ± 3.2 (muscle), 109.2 ± 11.8 (liver) and 117.3 ± 15.4 (blood).

Interassay Reproducibility

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

TABLE II Calibration Curve and Inter-Assay Variability

Concentration (μg/mL) in ethanol	**Q**	**n**	**Peak Height Ratio x ± SD**	**CV (%)**
0.0625	Q_9	3	.185 ± .007	3.6
	Q_{10}	4	.08 ± .003	4.0
0.125	Q_9	4	.340 ± .025	7.3
	Q_{10}	5	.168 ± .01	5.2
0.25	Q_9	4	.825 ± .05	6.0
	Q_{10}	4	.334 ± .02	5.4
0.5	Q_9	6	1.34 ± .07	5.6
	Q_{10}	4	.661 ± .02	2.8
1.0	Q_9	5	2.74 ± .08	2.8
	Q_{10}	4	1.326 ± .06	4.6

Stability

Dry residues of extracted samples spiked with ubiquinone-9 and 10 were stable when stored overnight at -5 ^{o}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 (@ 94 - 106%).

TABLE III Overnight Stability[+]

Q	Concentration (mg per 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 at 24h/$Q_{initial}$) x 100

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: ubiquinone-9: 0.52 μg/mL in blood, 26.4 μg/g in muscle and 267 μg/g in the heart; ubiquinone-10: 0.07 μg/mL in blood, 2.5 μg/g in muscle and 29.4 μg/g in the heart.

DISCUSSION

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 quantitative evaluation of the ubiquinone-9 and ubiquinone-10 levels in these tissues. The extraction method allows to obtain peaks 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 autooxidation during extraction. b) The SDS was used to increase the efficiency of the extraction [19] in view of its property 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 a maximal resolution. d) The use of Q_{11} as 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 and e) the use of reference calibration curves in ethanol to allow for an accurate measurement of a potential decrease that would be observed in the tissue ubiquinone levels.

The limitation of this method resides in the fact 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 has not been 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 observe the long term effect on ubiquinone in a basal condition with synthesis inhibitory effects of HMG-CoA reductase inhibitors on ubiquinone levels in non-oxidative conditions. Although the measure of the reduced form could be of importance in some instances this method could be easily adapted to do so (for example by measuring the ubiquinols at a wavelength of 291 nm[21]). In preliminary experiments (data not shown) in rat tissues the ubiquinol levels that we observed in the myocardium and muscle were approximately ten-fold lower than the ubiquinone levels and were similar to previous reports.[17, 14, 22] Because Qred/Qox ratio should not vary in one condition following a treatment with drugs we are confident that the method proposed adequately addresses our needs since the objective in our experiments was mainly in the observation of an effect on the ubiquinone pool, in tissues where the main portion of ubiquinones (in the tissues of interest) is in the oxidized form.

The relevance of this method resides in the fact that it allows for a simultaneous determination of ubiquinone-9-ubiquinone-10 in one analysis by a relatively rapid and simple method to evaluate the concentrations of ubiquinone-9 and ubiquinone-10 in blood, myocardium and muscle rat tissue.

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