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Improved high-performance liquid chromatographic method for the determination of coenzyme Q_{10} in plasma

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ABSTRACT

Coenzyme (Co) Q_{10} was dissociated from lipoproteins in plasma by treatment with methanol and extraction with *n*-hexane. Subsequent clean-up on silica gel and C_{18} solid-phase extraction cartridges with complete recovery (99 ± 1.2%) produced a clean extract. High-performance liquid chromatographic (HPLC) separation was performed on a C_{18} reversed-phase column. Three simple, rapid procedures are presented: HPLC with final UV (275 nm) detection, a microanalysis utilizing a three-electrode electrochemical detector and a microanalysis with column-switching HPLC and electrochemical detection. The methods correlate very well with classical ethanol–*n*-hexane extraction with UV detection. The identity and purity of the Co Q_{10} peak were investigated and the resulting methods were concluded to be suitable for total plasma Co Q_{10} determination. The average level in healthy subjects was 0.80 ± 0.20 mg/l; the minimum detectable Co Q_{10} plasma level was 0.05 and 0.005 mg/l for UV and electrochemical detection, respectively. The methods were applied to many samples and the plasma Co Q_{10} reference values for healthy subjects, athletes, hyperthyroid, hypothyroid and hypercholesterolaemic patients are given.

INTRODUCTION

Coenzyme (Co) Q_{10} (2,3-dimethoxy-5-methyl-6-decaprenylbenzoquinone; Fig. 1), also known as ubiquinone, is classed as a fat-soluble quinone and is an essential component of the mitochondrial respiratory chain, where it acts as an electron shuttle, controlling the efficiency of oxidative phosphorylation [1]. Moreover, it has a function as a membrane stabilizing agent, for avoiding lipid peroxidation and regulating lipid fluidity.

It may occur that the Co Q_{10} mitochondrial content gives a functional limiting effect, particularly in cases of increased respiratory demand [2]. A low Co Q_{10} plasma level may indicate an impaired cellular energetic function. It is important to emphasize how this possibility affects, in the same way, both muscular and myocardial performances. Co Q_{10} administration can counteract and improve functions in patients affected by several kinds of encephalomyopathia due to Co Q_{10} inhibition of biosynthesis or stimulation of degradation [3–5] and in patients affected by heart failure [6].

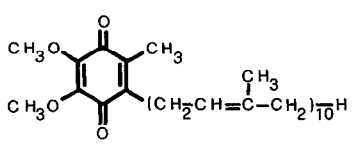

Fig. 1. Coenzyme Q_{10}, oxidized form.

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For all these reasons, plasma Co Q_{10} determination has been included in the routine activity of clinical chemistry laboratories.

Co Q_{10} has been determined in plasma by liquid chromatography (LC) with UV detection after liquid–liquid extraction [7]. Electrochemical detection (ED) gave the possibility of monitoring also the reduced form [8]. For total plasma Co Q_{10} determination, the sample is usually converted into the corresponding reduced form by treatment with sodium tetrahydroborate and subsequently determined by high-performance liquid chromatography (HPLC) with ED [9,10].

All these procedures are too time consuming and unsuitable for routine determinations, particularly when total Co Q_{10} is required, as, from the clinical point of view, this is the most significant value.

We have systematically examined the advantages and limitations of solid-phase extraction (SPE), the ED and automation for the determination of Co Q_{10} by HPLC.

A coulometric detector is able to give a 100% yield of the electrochemical reaction and can detect the oxidized form [10]. The reduced form is unstable at room temperature and quickly becomes oxidized [8,10], so SPE, when performed utilizing a vacuum manifold and subjecting the sample to prolonged air flushing, can cause total conversion of Co Q_{10} into the oxidized form, allowing the determination of total Co Q_{10}.

The column-switching technique improves the automation of the analytical procedures, greatly increasing the laboratory productivity.

An HPLC procedure that is to be applied in clinical chemistry must consider the kind of instrumentation currently available in the laboratory and must allow the method to be run without being obliged to apply a sophisticated technique such as column switching utilizing a UV detector must be sufficient.

In this paper, three very simple and rapid procedures are presented. They have an increasing level of difficulty in utilizing the instrumentation, but can be fully automated, correlate very well and are interchangeable.

EXPERIMENTAL

Chemical and reagents

Co Q_{10} and the internal standard (I.S.), Co Q_9 were kindly supplied by Eisai (Tokyo, Japan). Methanol, *n*-hexane and 2-propanol, all of HPLC grade, and glacial acetic acid, of INSTRA grade, were obtained from J. T. Baker (Deventer, Netherlands). Sodium acetate, of analytical-reagent grade, was purchased from E. Merck (Darmstadt, Germany).

Venous blood was drawn in tubes containing 50 μl of 0.01 *M* tripotassium ethylenediaminetetraacetate.

SPE extraction cartridges containing silica (100 mg) (Analytichem Bond Elut Si) or octadecyl-bonded silica (50 mg) (Analytichem Bond Elut C_{18}) and the column-switching disposable precolumn containing octadecyl-bonded silica (50 mg; 40 μm), 20 mm × 2 mm I.D. (Analytichem AASP Cassette C_{18}), were obtained from Varian Sample Preparation Products (Harbor City, CA, USA).

The analytical columns were protected with a Model 7315 on-line filter Rheodyne (Cotati, CA, USA).

The reversed-phase (RP) analytical column, Ultrasphere XL C_{18}, 3 μm (70 mm × 4.6 mm I.D.) with a C_{18} guard column, 3 μm (5 mm × 4.6 mm I.D.) were suppled by Beckman (San Ramon, CA, USA). The normal-phase (NP) analytical column, Si 60 Supersphere, 4 μm (50 mm × 4 mm I.D.) was from E. Merck.

Instrumentation

The SPE clean-ups were performed using a Model SPE-21 vacuum manifold (J. T. Baker). The Model 2510 HPLC pump and the Model 2050 UV detector (set at 275 nm, 0.02 a.u.f.s.) were obtained from Varian (Walnut Creek, CA, USA). For manual injection, a Model 7125 valve (Rheodyne) fitted with a 50-μl loop was used.

For ED a multi-electrode Coulochem 5100 A electrochemical detector fitted with a Model 5021 conditioning cell and a Model 5011 analytical cell, all from Environmental Sciences Assoc. (ESA) (Bedford, MA, USA), was employed. To oxidize Co Q_{10} before HPLC separation, a Model 5020 guard cell (ESA) was added.

For applying the column-switching technique, a Model 222 autosampler, a Model 401 diluter, an Anachem interface, all from Gilson (Villiers-le-Bel, France), an AASP solid-phase autosampler and a ten-port valve, all from Varian, were utilized, jointly with the HPLC pump and the electrochemical detector.

Preparation of standards

Stock solutions (100 mg/l) of Co Q_{10} and Co Q_9 (I.S.) were prepared in *n*-hexane and stored at 4°C.

To obtain standard solutions, Co Q_{10}-free plasma was fortified with Co Q_{10} to give concentrations of 0, 0.625, 1.25, 2.50 and 5.0 mg/l. These standards were prepared fresh daily.

HPLC mobile phases

For normal-phase separation, degassed *n*-hexane was used as the mobile phase at a flow-rate of 1 ml/min; for reversed-phase (RP) separation with UV detection, 2-propanol–methanol (1:4) was employed at a flow-rate of 2 ml/min; for RP separation with ED and for column-switching HPLC, 50 m*M* sodium acetate in glacial acetic acid–2-propanol–methanol (24:450:1435) was used at a flow-rate of 2 ml/min.

Electrode connections

The cells were assembled as follows: analytical column→ Model 5021 conditioning cell (set at −0.60 V) → Model 5011 analytical cell (set at detector 1 −0.15 V, detector 2 +0.45 V, response 4, gain 10 × 30). Only for the Co $Q_{10}H_2$ study an additional Model 5020 guard cell (set at +0.50 V) was installed between the injection valve and the analytical column.

Column-switching HPLC conditions

The column-switching instrumentation was assembled as reported previously [11,12].

Samples

Samples of 5 ml of venous blood were taken in the morning by venepuncture from the forearm of subjects who had fasted for at least 8 h. The samples were immediately centrifuged at 2000 *g* for 15 min at room temperature. The plasma was separated and kept at −20°C in polypropylene tubes. On the day assigned for analysis the samples were thawed for 2 h at room temperature.

Method A

Plasma (1 ml) was subjected to liquid–liquid extraction according to Takada *et al.* [7] and subsequently to RP-HPLC with UV detection.

Method B: plasma Co Q_{10} determination with UV detection

In a 100 × 16 mm polypropylene tube, 1 ml of plasma was deproteinized with 1 ml of methanol, then 0.1 ml of I.S. (Co Q_9, 25 mg/l in *n*-hexane) and 3.9 ml of *n*-hexane were added. The tube was vortex mixed and centrifuged at 2000 *g* for 10 min. A 3-ml portion of the *n*-hexane phase was transferred into another tube, 4 ml of *n*-hexane were added to the aqueous phase and the extraction was repeated. A 7-ml volume of the *n*-hexane phase was passed through a 100-mg silica SPE cartridge, previously activated with 2 ml of *n*-hexane; the cartridge was then washed with 2 ml of *n*-hexane and dried under a 380-mmHg vacuum for 1 min. Co Qs were eluted with 2 × 0.5 ml of methanol and the collected methanol was then purified on a 50 mg C_{18} SPE cartridge, previously activated with 2 ml of methanol and equilibrated with 2 ml of water. After a washing step with 1.5 ml of methanol, Co Qs were eluted with 2 × 0.15 ml of 2-propanol and 50 μl of the eluted fraction were injected into the RP-HPLC column for separation and UV detection.

Method C: plasma Co Q_{10} determination with electrochemical detection

In a 100 × 16 mm I.D. polypropylene tube, 0.1 ml of plasma was deproteinized with 0.2 ml of methanol, then 10 μl of I.S. (Co Q_9 25 mg/l in *n*-hexane) and 1 ml of *n*-hexane were added. The tube was vortex mixed, 1.5 ml of *n*-hexane were added and the tube was vortex mixed again and centrifuged at 2000 *g* for 10 min. A 2-ml volume of the *n*-hexane phase was passed through a 100-mg silica SPE cartridge and all the steps were performed as above but, unlike in method B, the C_{18} cartridge was eluted with 2 × 0.2 ml of 2-propanol and 50 μl of the eluted fraction were injected into the RP-HPLC column for separation and ED.

Method D: plasma Co Q_{10} determination with column-switching HPLC

Plasma (0.1 ml) was extracted and purified on a silica cartridge as in method C. The 1-ml methanol fraction eluted from silica was collected in a 75 × 12 mm I.D. polypropylene tube and placed on a Gilson 222 autosampler. The disposable precolumn utilized was C_{18}, 40 μm, 50 mg (20 mm × 2 mm I.D.) and, using the dilute syringe as a pump, was

activated with 0.5 ml of methanol, equilibrated with 0.5 ml of water, loaded with 0.2 ml of the sample and washed with 1.5 ml of methanol. The AASP ten-port valve then connected the precolumn to the analytical column for 0.5 min, the Co Qs were transferred to the analytical column by 1.0 ml of the HPLC mobile phase, the separation was performed on the C_{18} analytical column and the effluent was monitored by ED. As soon as the ten-port valve had returned to the load position, the precolumn was replaced with a new one. The 5-ml loop was washed with 25 ml of 2-propanol and the clean-up of the next sample then took place.

RESULTS AND DISCUSSION

Co Q_{10} is a lipophilic molecule, freely soluble in hydrocarbons, insoluble in water, almost insoluble in methanol and soluble in hot ethanol, 2-propanol and 1-propanol.

The liquid–liquid extraction method (A) was taken as a reference method to compare the improvements obtained by applying the SPE sample clean-up. In Fig. 2, a typical chromatographic profile obtained applying method A is shown.

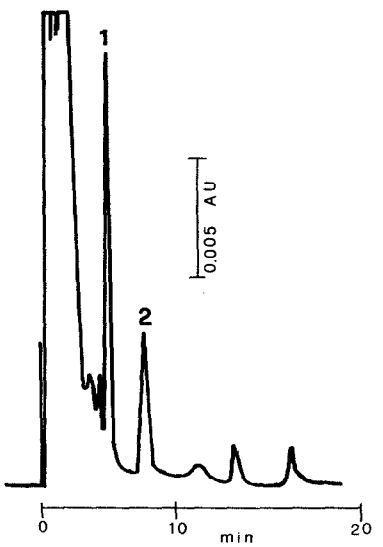

Fig. 2. Analytical profile obtained on applying method A to a plasma containing (1) Co Q_9 (I.S.), 2.50 mg/l, and (2) Co Q_{10}, 0.92 mg/l. Column, C_{18}, 3 μm (75 mm × 4.6 mm I.D.) (Beckman); mobile phase, 2-propanol–methanol (1:4); flow-rate, 2 ml/min; detection, UV, 275 nm, 0.02 a.u.f.s.; chart speed, 5 mm/min.

This method was found to be unsuitable for routine determinations owing to the following problems: a high solvent front; some later-eluting peaks causing a long run time; an interfering peak close to that of the I.S.; the injected sample was not clean and caused frit clogging; the precolumn lifetime was short owing to strongly retained substances; there was a large volume of *n*-hexane to be evaporated and the extraction was too time consuming; and automatic sampler injection was impossible owing to the presence of precipitate.

To improve the clean-up, we tried to load the plasma directly on to RP cartridges, but unfortunately the plasma proteins, to which Co Q_{10} is bonded, were not retained and the recovery was zero.

In Table I, the Co Q_{10} breakthrough volumes obtained on various SPE cartridges are reported. For elutate monitoring, RP-HPLC with UV detection and NP-HPLC [UV detector set at 275 nm, k' (Co Q_{10}) = 3.3] were used. The silica cartridge showed a high breakthrough volume in *n*-hexane and was suitable for sample concentration from large solvent volumes, but unsuitable for sample clean-up, because any solvent that was able to wash out interfering substances had also eluted the analyte.

It must be emphasized that methanol, which was almost unable to solubilize crystalline Co Q_{10}, could solubilize and elute it when Co Q_{10} was loaded on silica, probably because molecule–stationary phase interactions are weaker than molecule–molecule interactions, typical of the crystalline state. Co

TABLE I

Co Q_{10} BREAKTHROUGH VOLUME (ml) ON DIFFERENT BOND-ELUT EXTRACTION CARTRIDGES

Solvent	Cartridge		
	Si 100 mg	NH_2 100 mg	C 18 50 mg
n-Hexane	≫14	12	<1
Light petroleum (b.p. 40–60°C)	≫12		
Chloroform	<1	<1	<1
Methanol	0.2	0.2	3
2-Propanol	0.3	0.3	0.15
1-Propanol	0.3	0.3	<0.3

TABLE II

RECOVERY OF Co Q_{10} APPLYING *n*-HEXANE FROM LIQUID–LIQUID EXTRACTION TO SILICA CARTRIDGE

Sample (ml)		Solvents (ml)				Recovery after elution with 1 ml of methanol (%)
Plasma[a]	Water[a]	Methanol	Ethanol	Acetonitrile	*n*-Hexane	
	1				4	98
	1		1		4	1.5
	1			1	4	3
	1	1			4	97
1	1[b]				4	1
1		1			4	80
1		1			4 + 4	98

[a] Spiked with Co Q_{10} to give 100 mg/l.
[b] Unspiked.

Q_{10} solution in methanol remained stable for several days at room temperature.

In conclusion, the silica cartridge, is useful for replacing the original solution of Co Q_{10} in a large volume of *n*-hexane with a Co Q_{10} solution in a small volume of methanol, whereas the C_{18} cartridge is suitable for clean-up and concentration steps.

Utilizing the C_{18} cartridge, it is possible to load a methanol solution of Co Q_{10} and perform the subsequent washing step with methanol; moreover, of the elution cartridge with 2-propanol represents a quantitative concentration step.

In contrast to what was reported by some authors [10], 1-propanol was not a good solvent for the Co Q_{10} loading step on the C_{18} cartridge.

A reliable clean-up flow-scheme is plasma Co Q_{10} liquid–liquid extraction in *n*-hexane, solvent replacement with methanol performed on a silica cartridge and final clean-up performed on a C_{18} cartridge. First the plasma has to be deproteinized to break the Co Q_{10}–protein bonds; *n*-hexane alone is unable to perform this step, whereas methanol, ethanol and acetonitrile are effective.

Table II shows the recoveries obtained by coupling liquid–liquid extraction with silica cartridge treatment.

Ethanol and acetonitrile were partially miscible with *n*-hexane, so the subsequent loading step on silica was adversely influenced.

In Fig. 3 a typical chromatographic profile obtained on applying method B is shown, and it is clear that no late-eluting peak is detected and the solvent front is very low.

The theoretical plate number, calculated for the Co Q_{10} peak, was $N = 1629$. This low efficiency is due to the high eluting power of 2-propanol in the analytical HPLC system; in fact, injecting Co Q_{10} solubilized in methanol, we obtained $N = 3559$. Reduction of the injection volume to 10 μl eliminated

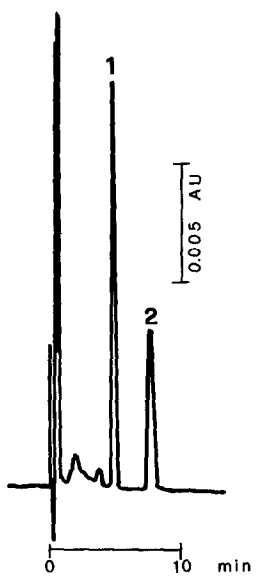

Fig. 3. Analytical profile obtained on applying method B to a plasma containing (1) Co Q_9 (I.S.), 2.50 mg/l, and (2) Co Q_{10}, 0.92 mg/l. Conditions as in Fig. 2.

TABLE III

SPE EXTRACTION OPTIMIZATION STUDY

Co Q_{10} recovery from silica cartridge by elution with methnaol (%)		Co Q_{10} released from C_{18} cartridge when the breakthrough volume is exceeded (loading + washing step)		Co Q_{10} recovery from C_{18} cartridge by elution with 2-propanol	
Volume of methanol employed (ml)	Co Q_{10} recovery (%)	Volume of methanol (ml)	Co Q_{10} released (%)	Volume of 2-propanol employed (ml)	Co Q_{10} recovery (%)
0.25	83.8	3.0	0.0	0.25	93.5
0.50	99.3	3.5	0.2	0.30	97.7
0.75	99.9	4.0	0.9	0.40	98.5
1.00	100.0	4.5	2.5	0.50	99.0
		5.0	4.0		
		5.5	5.6		

TABLE IV

PLASMA Co Q_{10} RECOVERIES (% ± R.S.D., n = 10) WITH DIFFERENT METHODS

Plasma Co Q_{10} (mg/l)	Method			
	A	B	C	D
1.21	92.1 ± 6.5	97.7 ± 3.9	98.5 ± 2.2	98.3 ± 2.5
0.32	87.3 ± 7.1	93.4 ± 4.2	96.2 ± 3.4	95.1 ± 3.7

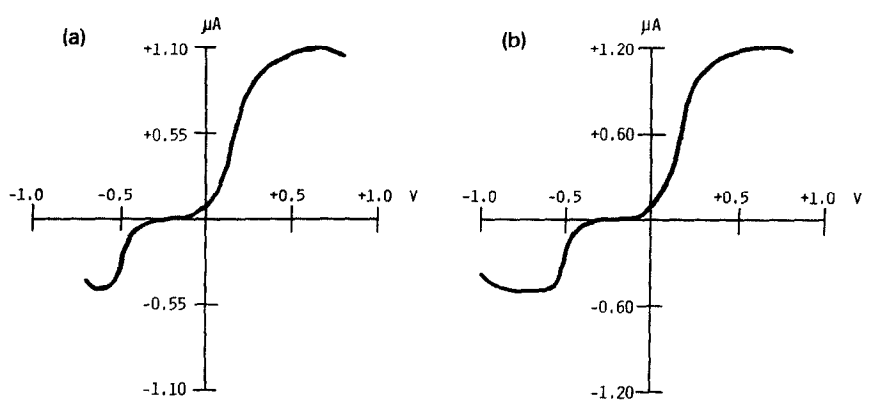

Fig. 4. Hydrodynamic voltammograms obtained for (a) Co Q_9 and (b) Co Q_{10}. Abscissa, applied voltage; ordinate, detector response (μA).

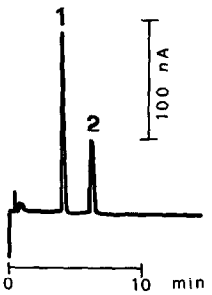

Fig. 5. Analytical profile obtained on applying method C to a plasma containing (1) Co Q_9 (I.S.), 2.50 mg/l, and (2) Co Q_{10}, 0.92 mg/l. Column, C_{18}, 3 μm (75 mm × 4.6 mm I.D.) (Beckman); mobile phase, 50 m*M* sodium acetate in glacial acetic acid–2-propanol–methanol (24:450:1435); flow-rate, 2 ml/min; electrochemical detector, conditioning cell, −0.60 V; detector 1, −0.15 V; detector 2, +0.45 V; gain, 10 × 30; integrator attenuation, 10; chart speed, 5 mm/min.

this difference and a high efficiency was obtained for both the solvents, but the sensitivity was inadequate for monitoring low Co Q_{10} plasma levels.

In Table III, the data obtained for the SPE extraction optimization study are reported.

In Table IV, Co Q_{10} recoveries and relative standard deviations (R.S.D.s) for method B are given. The I.S. recovery was 95.1 ± 3.8% and no peak was found in unspiked plasma at the retention time of the I.S. The calibration graph was linear ($y = 31.2x$, where x = Co Q_{10} concentration in plasma in mg/l and y = Co Q_{10} peak height in mm), also without using the I.S. peak height for calculations, and the minimum detectable Co Q_{10} level in plasma was 0.05 mg/l.

ELECTROCHEMICAL DETECTION

Sodium acetate and acetic acid were added to the mobile phase employed for ED to ensure the conductivity necessary for electrochemical reactions.

In Fig. 4, the hydrodynamic voltammograms for Co Q_9 and Co Q_{10} are reported. They were obtained by repeated injections into the HPLC system of a 4.2 mg/l solution of standards in 2-propanol, increasing the detector potential by 0.05 V in each subsequent run.

Our electrochemical cells have a coulometric (100%) yield and can be considered as electrochemical reactors; consequently, the detector response being higher in oxidation, the best results were obtained by reducing Co Q_{10} in the conditioning cell (−0.60 V) and monitoring with the analytical cell operating in the oxidation mode (+0.45 V).

In Fig. 5, a typical analytical profile obtained by applying method C to a plasma sample is shown. No solvent peak appears; the only peaks detected were those of the I.S. and Co Q_{10}. The sensitivity was very high, the detector was set at medium gain and was useless to operate with a higher plasma volume.

In Table IV, the Co Q_{10} recoveries and R.S.D.s for method C are given. The time necessary to complete a run was 7 min and the calibration graph was linear ($y = 15.5x$, where x = Co Q_{10} concentration in plasma in mg/l and y = Co Q_{10} peak height in mm), also without using the I.S. peak height for calculations, and the minimum detectable Co Q_{10} level in plasma was 0.01 mg/l.

The identity and purity of the Co Q_{10} and Co Q_9 peaks were confirmed for methods B and C: the Co Q_{10} UV spectrum was characterized by an absorbance maximum at 275 nm and two half-waves at 292 and 259 nm, while the hydrodynamic voltammogram (Fig. 4) showed a maximum of response at +0.60 V and a half-wave at +0.18 V. Pure stan-

TABLE V

RESULTS OF PURITY AND IDENTITY STUDIES (± R.S.D., n = 5) FOR Co Q_{10} AND Co Q_9 PEAKS

HPLC detection	Ratio of peak heights obtained at	Co Q_{10} peak		Co Q_9 peak	
		Standard	Sample	Standard	Sample
UV	292/275 nm	0.52 ± 0.02	0.51 ± 0.02	0.59 ± 0.02	0.57 ± 0.02
UV	259/275 nm	0.52 ± 0.02	0.53 ± 0.02	0.51 ± 0.02	0.50 ± 0.02
ED	+0.15/+0.45 V	0.438 ± 0.010	0.444 ± 0.011	0.454 ± 0.010	0.458 ± 0.010
ED	+0.60/+0.45 V	1.041 ± 0.025	1.064 ± 0.025	1.086 ± 0.026	1.075 ± 0.027

TABLE VI

COLUMN-SWITCHING HPLC OPTIMIZATION

Precolumn	Optimized valve reset time (min)	Breakthrough volume (ml of methanol)	
		Passed through the precolumn	Total
C_2	0.1	<0.2	0.825
C_8	0.1	0.3	0.925
C_{18}	0.5	1.475	2.100

dards and real plasma samples (n = 5) were extracted with method B and injected three times into the HPLC system, with the UV detector set at 275, 292 and 259 nm. Then extraction method C was applied with the electrochemical detector 2 set at +0.45, +0.15 and +0.60 V.

The results are given in Table V. Comparison of the values obtained for the Co Q_{10} peak for the standard with those obtained for the samples showed out that they were very close, indicating that the peaks referred to the same substance and there were no co-eluted substances in the same peak.

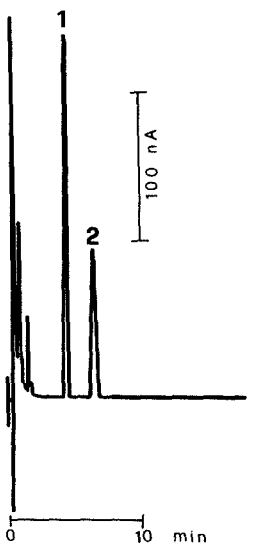

Fig. 6. Analytical profile obtained on applying method D to a plasma containing (1) Co Q_9 (I.S.), 2.50 mg/l, and (2) Co Q_{10}, 0.92 mg/l. Column-switching precolumn, C_{18}, 40–60 μm (20 mm × 2 mm I.D.). Analytical column, mobile phase and detector as in Fig. 5. Integrator attenuation, 512; chart speed, 5 mm/min.

Column switching

In Table VI, the results obtained by employing different precolumns are shown. As the dead volume between the injection port of the Model 222 autosampler and the precolumn was 0.625 ml, the amount of methanol employed in the final washing step was always increased by this volume. In previous column-switching methods for determining other analytes [11,12], we used a final washing step with water before switching the valve. For Co Q_{10} this step must be avoided, because water causes precipitation of the analyte in the precolumn and the HPLC mobile phase volume, necessary for precolumn elution, would be increased excessively.

In Fig. 6 a typical chromatogram, obtained from plasma samples applying method D, is shown and in Table IV Co Q_{10} recoveries and R.S.D.s from plasma are given. The I.S. recovery was 95.2 ± 3.7% and no peak was found in unspiked plasma at the retention time of the I.S.

The calibration graph was linear (y = 46.0x, where x = Co Q_{10} concentration in plasma in mg/l and y = Co Q_{10} peak height in mm) and the minimum detectable Co Q_{10} level in plasma was 0.005 mg/l.

As, from the second sample on, the preparation of the sample takes place simultaneously with HPLC separation of the previous sample, the total analysis time is 8 min, even though the preparation time is 4 min and the HPLC separation time is 8 min.

Total plasma Co Q_{10} determination

The clinical requirement is to determine total plasma Co Q_{10}, the single oxidized or reduced fraction being irrelevant [2] owing to Co Q_{10} functioning as a homogeneous pool [13].

As reported, ubiquinols are rapidly oxidized even at −20°C [8,10]; powerful antioxidants cannot be used, as they would reduce the oxidized form [10]; during storage the percentage of the reduced form with respect to the total decreases, but the total content remains unchanged [9]. Powerful reducing agents, such as sodium tetrahydroborate or dithionite, are needed to effect the quantitative reduction of Co Q_{10}, even though tetrahydroborate absorbed

on the injector and/or the analytical column can reduce the oxidized form in next runs [10]. Moreover, the Co $Q_{10}H_2$ level in 2-propanol or ethanol gradually decreases [7,9]. A glass column is necessary for original Co $Q_{10}H_2$ determination [10].

For all these reasons, for total Co Q_{10} determination (*i.e.*, Co Q_{10} plus Co $Q_{10}H_2$), we avoided both the direct original Co $Q_{10}H_2$ determination and the utilization of reducing agents and preferred to employ an electrochemical reactor to convert the injected Co $Q_{10}H_2$ into the oxidized form.

After liquid–liquid extraction of plasma using method A, samples were injected into the RP-HPLC system equipped with an electrochemical detector and Co $Q_{10}H_2$ was oxidized in the guard cell installed between the injection valve and the analytical column. As a 100% yield of the oxidation reaction in the guard cell was obtained, we could determine the total amount of Co Q_{10} in plasma samples (n = 12). The same samples were injected without the guard cell and only the oxidized fraction was determined. No difference was observed in the values obtained, indicating that only oxidized Co Q_{10} was present in our samples.

For determining the retention time of Co $Q_{10}H_2$, a solution containing the I.S. + Co Q_{10} was injected into the HPLC system with the guard cell set at −0.60 V, in this way reducing Co Q before HPLC separation. The retention times were Co Q_9H_2 2.46, Co $Q_{10}H_2$ 3.63, Co Q_9 4.40 and Co Q_{10} 6.64 min. Applying method C, no peak with a retention time around 3.6 min was detected and none was detected before the oxidized I.S.

As method A also extracts the reduced from [7], these results confirm that in our samples Co Q_{10} was present only in the oxidized form. The same electrochemical reactor was applied by injecting plasma samples stored and extracted with methods B, C and D (n = 12) and fresh samples (n = 12), extracted immediately after the sampling, and the same results were obtained. This confirms that our extraction procedures fully oxidize the Co $Q_{10}H_2$ and the proposed methods are suitable for total Co Q_{10} determination.

In Table VII, the results of the correlation study among the proposed methods are reported. The methods were applied to many real samples and in Table VIII the data obtained for a group of healthy subjects and some patients not submitted to any pharmacological treatment are given.

TABLE VII

CORRELATION STUDY AMONG THE METHODS

Plasma samples, n = 34. Co Q_{10} range: 0.20–1.21 mg/l.

Y = method	X = method	Correlation coefficient, r
B	A	0.985
C	A	0.993
D	A	0.994

In endurance athletes a low plasma Co Q_{10} level has been found, which correlates with the high aerobic power that they developed [14–16]. A similar study on patients with thyroid diseases showed the connection between thyroid hormones and Co Q_{10}, as reported by other authors [17].

Finally, a study on hypercholesterolaemic patients, under pharmacological treatment with HMGCoA-reductase inhibitors, was useful for investigating the effect of these drugs on cholesterol and Co Q_{10}, which follow the same mevalonate pathway [18]. Our data correlated well with those obtained by other authors, both for healthy subjects [19] and various groups of patients [14–18].

CONCLUSIONS

Utilizing the proposed methods, cleaner and more replicable chromatographic profiles were obtained, in addition to simplifying the extraction and analytical procedures considerably. The reduction

TABLE VIII

SUMMARY OR RESULTS OF APPLICATION TO SAMPLES FROM PATIENTS

Subjects[a]	Plasma Co Q_{10} (mg/l)[b]	n
Healthy subjects, 20–60 years (m. and f.)	0.80 ± 0.20	50
Endurance athletes (m.)	0.58 ± 0.17	40
Hyperthyroid patients (f.)	0.27 ± 0.13	21
Hypothyroid patients (f.)	0.62 ± 0.11	11
Hypercholesterolaemic patients (m. and f.)	1.15 ± 0.15	30

[a] m. = Male; f. = female.

[b] Mean value ± 2 S.D.

in the volumes of *n*-hexane employed allowed the elimination of glass-tubes with blind nipples and simplified the mixing, vortex mixing being adequate.

The utilization of silica SPE cartridges for solvent replacement, instead of the traditional evaporation under a stream of nitrogen, produced a substantial shortening of the preparation time, a decrease in the analytical errors (less product was lost on the tube walls) and a cost reduction (saving of nitrogen); moreover, it allowed efficient solubilization in methanol, essential for the subsequent clean-up on a C_{18} cartridge.

The very good correlation among the methods enables them to be utilized without distinction: the choice depends on the availability of the instruments in the laboratory. The reduction in the analytical time generates a 2.2-fold increase in the productivity of the instruments. For preparing 40 samples utilizing method A it would take 4 h over two days, whereas applying our methods 1 h 20 min is sufficient and the preparation can be done in a real time of less than 2 h, including centrifugations.

If an automatic sampler is available, 160 samples per day can be analysed, with an instrumental utilization of 24 h and an operator availability of 8 h per day. Further advantages of the methods are that the total Co Q_{10} is determined directly and the data obtained routinely in various laboratories are comparable and suitable for statistical studies.

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