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DETERMINATION OF COENZYME Q_{10}, α-TOCOPHEROL AND CHOLESTEROL IN BIOLOGICAL SAMPLES BY COUPLED-COLUMN LIQUID CHROMATOGRAPHY WITH COULOMETRIC AND ULTRAVIOLET DETECTION

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SUMMARY

Coenzyme (Co) Q_{10}, Co $Q_{10}H_2$, α-tocopherol and cholesterol were dissociated from lipoproteins in plasma by treatment with 1-propanol. The supernatant obtained was injected directly for determination of Co Q_{10} and Co $Q_{10}H_2$. Precolumn reduction with borohydride was used for determination of total Co Q_{10} simultaneously with α-tocopherol and cholesterol. Total Co Q_{10} in freeze-dried myocardial biopsies was determined after extraction with 1-propanol and oxidation of Co $Q_{10}H_2$ with ferric chloride. The chromatographic system comprised two reversed-phase columns and a three-electrode coulometric detector and a UV detector coupled in series. A pre-fractionation on the first column protected the coulometric detector from contamination and reduced the time for analysis by eliminating strongly retained solutes. The coulometric electrodes were operated in the oxidation–reduction–oxidation mode, and the last electrode was used for detection of α-tocopherol, Co Q_{10} and Co $Q_{10}H_2$, while cholesterol was detected by UV at 215 nm. The fast isolation procedure made it possible to determine the reduced and oxidized forms of Co Q_{10} in plasma. Quantitative recoveries were obtained for all the analytes studied and normal levels were determined with a coefficient of variation of 2–3%.

INTRODUCTION

Coenzyme (Co) Q_{10} is an essential component of the mitochondrial respiratory chain constituting a redox-link between flavoproteins and cytochromes. The hydroquinone form of Co Q_{10} is a powerful reducing agent and may together with α-tocopherol prevent membrane lipid peroxidation. Human endomyocardial biopsy data have lately indicated a state of Co Q_{10} deficiency in patients with heart

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failure [1] and it has been established that Co Q_{10} has therapeutic effects in advanced heart failure [2]. Co Q_{10} has been determined in blood [3] and myocardial biopsies [4] by liquid chromatography (LC) with UV detection after extensive sample preparation including thin-layer chromatography (TLC). An amperometric detector has been coupled in series with a UV detector to improve the sensitivity for Co $Q_{10}H_2$ [5]. α-Tocopherol has been determined simultaneously with cholesterol [6] and retinol [7] by UV detection with wavelength programming. α-Tocopherol has also been detected simultaneously with ubiquinols by amperometry [8].

A method involving rapid extraction combined with coupled-column chromatography and coulometric detection has been studied in the present work.

α-Tocopherol and Co Q_{10} are transported by lipoproteins in plasma, with α-tocopherol levels in plasma varying with the lipid content [9]. Free cholesterol was included in the present method to obtain a measure of the lipoprotein fraction in plasma.

EXPERIMENTAL

Chemicals and reagents

Co Q_{10} was obtained from Serva (Heidelberg, F.R.G.). Cholesterol and α-tocopherol were obtained from Merck (Darmstadt, F.R.G.). The diethoxy analogue of Q_{10} was synthesized at ACO Läkemedel (Solna, Sweden). Methanol, 1-propanol, sodium perchlorate, perchloric acid and sodium borohydride were all of analytical grade.

Synthesis of internal standard

Co Q_{10} (1 g) was dissolved in 10 ml of hexane and diluted with 40 ml dry ethanol. The substitution of the methoxy groups with ethoxy groups was catalyzed by addition of 1 ml of a solution of potassium hydroxide in ethanol (40 g/l). The reaction was inhibited after 23 min by addition of 1 ml acetic acid. Hexane (100 ml) was added and the organic phase was washed twice with 100 ml water. The organic phase was dried over sodium sulphate and evaporated until an oily residue was obtained. The residue was taken up in 5 ml of hexane and purified by preparative LC. A 0.5-ml volume of the solution was injected onto a LiChrosorb Si-60 column (Merck) packed with 7-μm diameter particles (250 mm $\times$ 25 mm) with a mobile phase containing 40% (v/v) dichloromethane in hexane. The mobile phase was saturated with water prior to use. The diethoxy analogue, mixed monoethoxy derivatives and Co Q_{10} were eluted with capacity factors of 3.7, 5.3 and 7.5, respectively. Seven consecutive separations were made. The fractions containing the diethoxy analogue were evaporated. Crystallisation from ethanol gave 0.2 g of pure diethoxy analogue (yield 30%). The identity was confirmed by mass spectrometry, and the purity was determined both by reversed-phase and by normal-phase LC.

Apparatus

The liquid chromatograph consisted of a Gilson autosampler (Model 231-401, Gilson, Villiers-le-Bel, France) and two pneumatic six-port valves (Rheodyne,

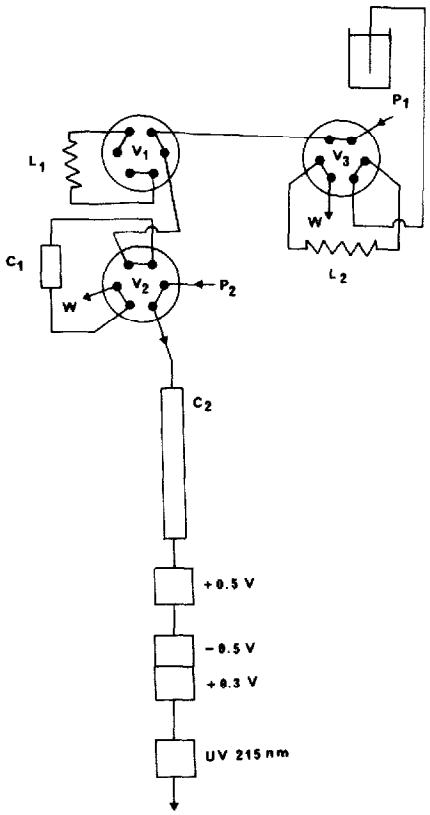

Fig. 1. Column-switching system. Sample loop of automatic injector (L_1) = 0.1 ml. Regeneration gradient loop (L_2) = 2 ml, refilled by gravity flow. W = Waste; P_1 = pump for precolumn; P_2 = pump for analytical column; V_1 and V_2 = six-port pneumatic switching valves; C_1 = precolumn; C_2 = analytical column.

Cotati, CA, U.S.A., Model 7001). The precolumn contained 3-μm Spherisorb ODS-2 (Phase Separations, Queensferry, U.K.) slurry-packed into 20 mm × 2 mm I.D. columns (Upchurch, Oak Harbour, WA, U.S.A.). The analytical column (100 mm × 4.6 mm) was packed with 3-μm Spherisorb ODS-2. A glass column (100 mm × 3 mm) packed with 5-μm Chromspher C_{18} (Chrompack, Middelburg, The Netherlands) was also used for the determination of Co Q_{10} and Co $Q_{10}H_2$. The analytical column was connected to a coulometric detector with three working electrodes housed in cells Model 5021 and 5010 (ESA, Bedford, MA, U.S.A.). The effluent from the coulometric cells was connected to a UV detector operated at 215 nm (SpectroMonitor III, LDC, Berkeley, CA, U.S.A.).

The mobile phases were delivered by an Eldex pump Model A-30-S2 (Eldex, Menlo Park, CA, U.S.A.) and a ConstaMetric I pump (LDC) with pulse dampeners for the precolumn and the analytical columns, respectively. The column-switching events were controlled by a Shimadzu integrator equipped with an I/O port (Model CR3A, Shimadzu, Kyoto, Japan). The system is schematically presented in Fig. 1.

TABLE I

SCHEME OF COLUMN SWITCHING EVENTS

The switching events are given for a flow-rate of 0.5 and 1.3 ml/min for C_1 and C_2, respectively.

Time after injection (min)	Valve operated	Event
0	1	Sample is injected onto C_1
2	2	The analytes are eluted from C_1 to C_2
2.9	2	Elution from C_1 is completed and separation continues on C_2
8	3	C_1 is cleaned with a step gradient of dichloromethane–methanol (50:50, v/v)
10	3	Regeneration of C_1 is completed
10.1	End	Calculation and report
11		Next sample is injected

Myocardial biopsies were ground in a 2-ml glass vessel with ground-in-glass plunger by a Potter-S homogenizer from Braun (Melsungen, F.R.G.).

Chromatographic system and column switching

A short precolumn was used to eliminate both polar compounds (reagents) and strongly retained solutes. The mobile phase for the precolumn consisted of 10% (v/v) water in methanol during sample injection. The flow-rate was set to 0.5 ml/min by P_1. The fraction containing the analytes of interest was transferred from the precolumn to the main column by a mobile phase containing 15% (v/v) 1-propanol in methanol with 33 m*M* perchloric acid and 57 m*M* sodium perchlorate. The separation was continued on the main column with the same mobile phase, delivered by P_2 at 1.3 ml/min, while the precolumn was washed with 2 ml of dichloromethane–methanol (50:50, v/v). The dichloromethane–methanol solution flowed continuously through L_2 by gravity flow and the dichloromethane gradient was created by switching V_2. These switching events are listed in Table I.

The 1-propanol concentration in the mobile phase for C_2 was increased from 15 to 25% (v/v) when the total Co Q_{10} was determined in the oxidized form.

Detection system

The detection system was comprised of three coulometric working electrodes and one UV detector coupled in series. α-Tocopherol and Co $Q_{10}H_2$ were oxidized at the first electrode, reduced at the second and oxidized again at the third electrode. Co Q_{10} was reduced and oxidized at the second and third electrode, respectively. The coulometric signal of the third electrode was used for detection of α-tocopherol, Co $Q_{10}H_2$ and Co Q_{10}, while cholesterol passed the electrochemical cells unaffected and was detected by UV absorption at 215 nm.

Preparation of plasma samples

All sample handling was performed in shaded light to avoid photochemical decomposition of Co Q_{10}. Venous blood samples were collected in heparinized

Venoject tubes (Leuven, Belgium). The blood was centrifuged and the plasma was frozen in polypropylene tubes at −70°C within 30 min after sampling. The frozen samples were thawed in a water bath at ambient temperature and 0.3 ml plasma was pipetted into a polycarbonate tube. A 1-ml volume of 1-propanol containing 2.8 μg/ml internal standard (diethoxy analogue of Co Q_{10}) was added and mixed. The samples were left to stand for 3 min, followed by re-mixing and centrifugation at 2300 *g* for 5 min. A 100-μl volume of the supernatant was injected within 45 min after thawing for determination of Co Q_{10} and Co $Q_{10}H_2$. A 0.5-ml volume of the supernatant was transferred to a new tube containing about 2 mg of sodium borohydride for determination of α-tocopherol, cholesterol and total Co Q_{10}. The samples were ready for injection after 5 min and were stable for about 24 h.

Preparation of myocardial biopsies

The specimens weighing 0.4–4 mg were immediately frozen and subsequently freeze-dried. The freeze-dried tissue (0.1–1 mg) was weighed immediately after it was taken out of the vacuum and then ground in 0.4 ml of 1-propanol containing 0.56 μg/ml internal standard. A homogenous suspension of cell fragments was obtained after 40–50 strokes at 600 rpm. The homogenate was centrifuged for 5 min and the supernatant was transferred to a new tube containing 0.1 ml of 6 m*M* ferric chloride in 0.24 *M* hydrochloric acid. The oxidized sample (100 μl) was injected for analysis of total Co Q_{10}.

RESULTS AND DISCUSSION

Sample handling and extraction

Co Q_{10} is a very lipophilic crystalline compound with good solubility in hydrocarbons. It is slightly soluble in ethanol, almost insoluble in methanol and insoluble in water. Co Q_{10}–Co $Q_{10}H_2$ is a reversible redox couple with a redox potential ($E_{\frac{1}{2}}$) of 0.1 V at pH 7.4 [10]. The redox equilibrium involves two protons and is pH-dependent ($\Delta E_{\frac{1}{2}}/\Delta$ pH = −0.059). Thus, Co Q_{10} H_2 is readily oxidized by oxygen or ferric ions. Powerful reducing agents like sodium borohydride or dithionite are needed to obtain quantitative reduction of Co Q_{10}. Co Q_{10} is found in all cells containing mitochondria. The homologue with ten isoprene units is found in man, while Co Q_9 is dominant in the rat [5]. The concentration of Co Q_{10} in erythrocytes is about 3% of the plasma levels [16]. Co Q_{10} is transported in the systemic circulation by lipoproteins together with other compounds, like triglycerides, cholesterol esters and cholesterol which are also insoluble in water.

Isolation procedures for Co Q_{10} in plasma usually involve extraction with mixtures of hexane and methanol or ethanol [8,10,11]. The organic phase is evaporated, and the residue is dissolved in a suitable solvent for injection on LC columns or prepurification by TLC prior to LC.

Isolation procedures for Co $Q_{10}H_2$ or Co Q_{10} should be fast and should contain as few steps as possible to avoid oxidation. Powerful antioxidants cannot be used since they will reduce the oxidized form and too high Co $Q_{10}H_2$ concentrations

TABLE II

EXTRACTION RECOVERIES USING DIFFERENT SOLVENTS

Compound	Solvent	Volume (ml)		Recovery (mean ± S.D.) (%)	*n*
		Plasma	Solvent		
Co Q_{10}	Ethanol	0.3	1	24	2
Co Q_{10}	Acetone	0.5	1	36	2
Co Q_{10}	Acetone	0.3	1	40	2
Co Q_{10}	2-Propanol	0.5	1	46	2
Co Q_{10}	1-Propanol	0.5	1	88	2
Co Q_{10}	1-Propanol	0.4	1	100.9	7
Co Q_{10}	1-Propanol	0.3	1	101 ± 3.7	8
Internal standard	1-Propanol	0.3	1	100.5 ± 2.1	12
Cholesterol	1-Propanol	0.3	1	104 ± 4.6	8
α-Tocopherol	1-Propanol	0.3	1	100 ± 1.7	8

will be obtained. Total Co Q_{10} can be determined by precolumn oxidation or reduction.

Automated systems with coupled columns suitable for direct injection of plasma have recently arisen as an alternative to manual purification and compounds sensitive to oxidation have been determined with good precision [12–14]. However, lipophilic compounds that are present in lipoprotein particles like very low-density (VLDLs), low-density (LDLs) and high-density lipoproteins (HDLs) and chylomicrons are not retained on extraction columns [15]. Precolumn treatment with pentylamine and methanol for 2 h has been used to promote the extraction by disruption of the lipoproteins [15]. The addition of surface-active agents like dodecylsulphates has been used to improve the recoveries of Co Q_{10} from plasma by liquid–liquid extraction [17].

In this work attempts were made to extract Co Q_{10} from plasma on short columns packed with ODS-silica. Co Q_{10} in plasma was not retained on ODS columns and precolumn addition of a dodecylsulphate had no effect. Different organic solvents which are miscible with water in all proportions were then used to extract cholesterol, α-tocopherol and Co Q_{10} from plasma. The recoveries improved with increasing lipophility and volume of solvent (see Table II). Quantitative recoveries with acceptable dilution (0.3/1.3) of the sample was obtained with 1-propanol. 1-Propanol is the most lipophilic alcohol that is miscible with water. It was a slightly more effective extraction medium than 2-propanol and much more effective than acetonitrile and lower alcohols.

The proteins were precipitated during extraction and were separated by centrifugation. The clear supernatant was injected directly for analysis of Co Q_{10} and Co $Q_{10}H_2$. Co $Q_{10}H_2$ was stable for about 45 min and then gradually oxidized to Co Q_{10}. The internal standard was partially reduced in the extracted samples when stored for longer periods (16 h). Co $Q_{10}H_2$ was stable in plasma stored at $-70°C$ for at least five months (see Table III), but storage at $-20°C$ resulted in oxidation. Selective precipitation of VLDLs plus LDLs by heparin and Mn^{2+}

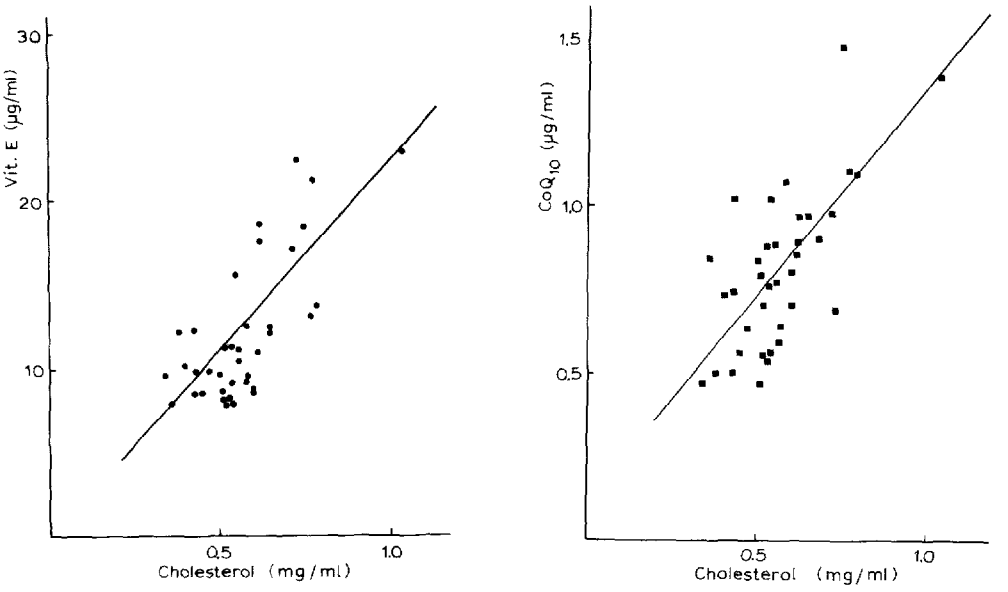

Fig. 2. Correlation between serum cholesterol and α-tocopherol in 38 healthy subjects. $y = 24.1\,x - 1.76$; $r = 0.704$; $P < 0.0001$.

Fig. 3. Correlation between serum, cholesterol and total Co Q_{10} in 38 healthy subjects. $y = 1.246\,x + 0.109$; $r = 0.709$; $P < 0.0001$.

TABLE III

COMPARISON BETWEEN PRECOLUMN OXIDATION AND REDUCTION FOR DETERMINATION OF TOTAL Co Q_{10}

Sample No.	Total Co Q_{10} concentration after precolumn (μg/ml)			Percentage oxidized Co Q_{10} when stored at $-70°C$		
	Oxidation	Reduction	Difference	0 weeks	20 weeks	Difference
1	0.97	0.89	0.08	7.6	6.6	−1.0
2	1.05	1.02	0.03	8.4	7.7	−0.7
3	0.86	0.83	0.03	6.8	7.3	+0.5
4	1.03	0.98	0.05	8.2	10	+1.8
5	0.79	0.73	0.06	7.7	7.2	−0.5
6	1.40	1.48	−0.08	6.4	7.1	+0.7
7	0.50	0.50	0	11.0	11	0
Mean			0.024	8.01	8.13	−0.1
S.D.			0.053	1.50	1.68	1.06

[18,19] in samples from five individuals showed that $79 \pm 5\%$ of Co Q_{10} was present in this fraction and the remainder in the HDL fraction.

A correlation between the serum concentrations of Co Q_{10}, α-tocopherol and free cholesterol was found (see Figs. 2 and 3) with correlation coefficients of 0.71. Thus the Co Q_{10} concentration in serum lipids may give a better reflection of

tissue levels than absolute concentrations. We used free cholesterol levels as a measure of plasma lipids since this compound was readily detected in the method for Co Q_{10} by adding a UV detector. The mean Co Q_{10} serum level and Co Q_{10}/cholesterol ratio were 0.818 ± 0.245 μg/ml and $1.45 \pm 0.339 \cdot 10^{-3}$, respectively. The mean α-tocopherol serum level and α-tocopherol/cholesterol ratio were 12.0 ± 4.3 μg/ml and $21.1 \pm 5.2 \cdot 10^{-3}$, respectively (Figs. 2 and 3).

Myocardial biopsies were freeze-dried as described by Vadhanavikit et al. [4], and ground in 1-propanol. Quantitative recovery of Co Q_{10} was verified by regrinding the residue from the first extraction in propanol without the addition of internal standard (I.S.).

The amounts of Co Q_{10} and the internal standard found in the second extract corresponded to the remaining solvent volume from the first extraction, and the peak-height ratio between Co Q_{10} and I.S. did not change after regrinding.

Chromatographic system

Co Q_{10} and related compounds can be separated by reversed-phase chromatography with mobile phases containing methanol or ethanol and a less polar modifier to control the retention. An acidic buffer was used to obtain the conductivity and protons required for coulometric reduction. A mixture of methanol and 1-propanol was preferred to ethanol because of its lower viscosity. α-Tocopherol,

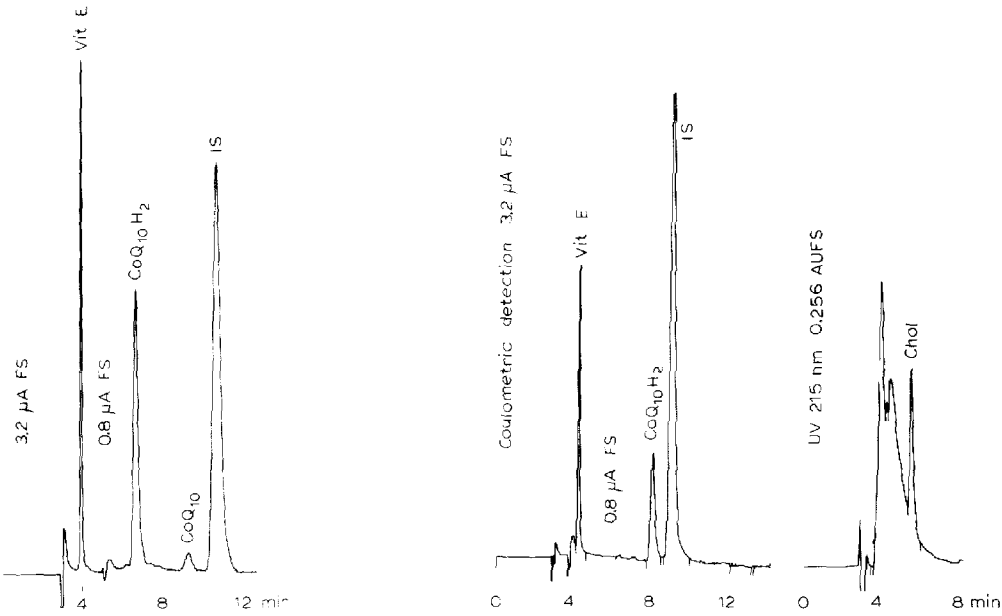

Fig. 4. Chromatogram obtained with a plasma sample. Injection volume, 100 μl. Co $Q_{10}H_2$, 2.94 μg/ml; Co Q_{10}, 0.32 μg/ml. Mobile phase, 25% (v/v) 1-propanol in methanol with acidic perchlorate buffer. Analytical column (2), 5-μm Chromspher C_{18}, (100 mm×3 mm glass column). Column-switching events were adjusted according to the mobile phase flow-rate (0.75 ml/min).

Fig. 5. Chromatogram obtained with a plasma extract treated with borohydride. Injection volume, 100 μl. Conditions as described in the text. α-Tocopherol (Vit E), 10.5 μg/ml; total Co Q_{10}, 1.42 μg/ml; cholesterol, 0.65 mg/ml.

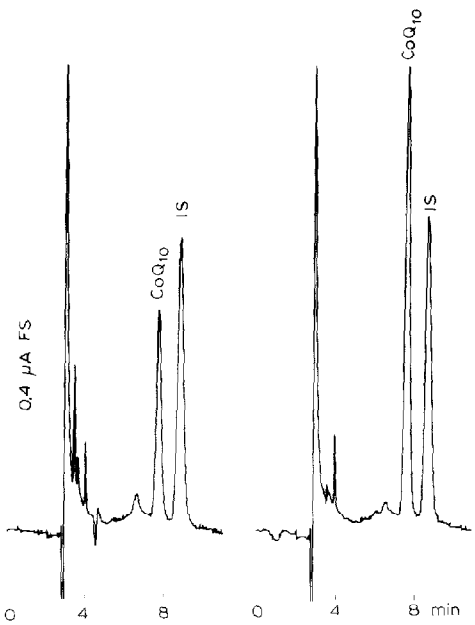

Fig. 6. Chromatograms obtained with a standard solution (left) and a myocardial biopsy specimen (0.418 mg dry weight) containing 0.569 μg/mg total Co Q_{10} (right). Injection volume, 100 μl. Conditions as described in the text.

cholesterol and Co $Q_{10}H_2$ were less retained than Co Q_{10} (see Fig. 4) and the simultaneous analysis of total Co Q_{10}, α-tocopherol and cholesterol was performed with precolumn reduction of Co Q_{10} (Fig. 5). The concentration of 1-propanol in methanol was 15% for the determination of total Co Q_{10} in the reduced form (Co $Q_{10}H_2$) and 25% for Co Q_{10} (Fig. 6). A single-column system was used initially with plasma extracts, but the coulometric detector was overloaded by plasma components, which resulted in large solvent fronts and a gradual decrease of the coulometric yield. A precolumn switching system was used to solve these problems.

Large sample volumes can be injected only if the sample itself is a weak eluent compared with the mobile phase. Plasma extracts contained 77% propanol, which limited the sample volume to 100 μl for analysis of α-tocopherol. Weakly retained sample components were eluted with 10% water in methanol. An enrichment factor of 10 was obtained for Co Q_{10} and 0.5 ml of plasma extract could be injected without excessive band broadening. Old columns gave rise to on-column oxidation of Co $Q_{10}H_2$ on some occasions. This effect resulted in a raised baseline between Co $Q_{10}H_2$ and Co Q_{10}, indicating that the oxidation proceeded during the whole passage through the column and not just at the inlet. Old columns could be deactivated by pumping 100 ml of 0.1 *M* phosphoric acid through the column to wash out metal ions. The injector and the precolumn were washed with a small volume of acidic ferric chloride when the analysis was changed from total Co Q_{10} to Co Q_{10} and Co $Q_{10}H_2$ to avoid injector and/or on-column reduction by borohydride absorbed on the column.

Coulometric detection

The noise of a coulometric detector is strongly dependent on the background current. Many metal ions and oxygen are reduced at the same potential as Co Q_{10}, and high background currents (20–30 μA) were obtained when Co Q_{10} was reduced by coulometry. An acidic buffer was used as a proton source for the reduction ($\Delta E_{\frac{1}{2}}/\Delta$ pH = −0.059). A low background current (0–0.1 μA) was obtained for the oxidation of Co $Q_{10}H_2$.

The coulometric detector was comprised of one single and one dual coulometric cell. The cells were coupled in series and operated in the oxidation–reduction–oxidation mode. Thus, only reversible redox couples were detected at the last electrode. Both Co Q_{10} and Co $Q_{10}H_2$ were detected at the last electrode at the highest sensitivity. Lipids that were reversibly adsorbed on the coulometric electrode, like cholesterol, gave a reduction of the background current (negative peak) when eluted from the column. The precolumn separation eliminated strongly retained solutes and improved the baseline stability.

Accuracy and precision

Recoveries were quantitative for the analytes studied (Table II) and total Co Q_{10} could be determined in the oxidized form without internal standard. The internal standard was needed when the samples were reduced with borohydride and effervescent samples were injected. The internal standard was also useful for checking the redox status of the samples. When Co $Q_{10}H_2$ was oxidized in plasma extracts, the internal standard was partially reduced, as could readily be seen in the chromatogram. We could not detect any reduced internal standard or decrease of Co $Q_{10}H_2$ when plasma samples were injected within 45 min after thawing. Total Co Q_{10}, α-tocopherol and cholesterol were determined with a coefficient of variation of 2%, Co Q_{10} levels in plasma were low (0.05–0.15 μg/ml) and were determined with a coefficient of variation of 10% with 100-μl samples. The precision for Co Q_{10} in plasma can be improved by injection of larger sample volumes, but α-tocopherol will then break through on the precolumn.

CONCLUSIONS

Rapid extraction of Co Q_{10} from plasma with 1-propanol made it possible to determine both the reduced and oxidized forms. A precolumn separation step was necessary to protect the coulometric cell from overloading and from the irreversible absorption of sample components. High selectivity and sensitivity was obtained with a three-electrode coulometric detector operated in the oxidation–reduction–oxidation mode. It is suggested that serum Co Q_{10} levels should be related to serum lipids and free cholesterol may be used as a representative of the lipid fraction in plasma. The method for total Co Q_{10}, α-tocopherol and free cholesterol has a high sample capacity and 50–100 samples per day can be analyzed when an automatic injector is used.

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