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Determination of Coenzyme Q in Human Plasma

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Summary

Coenzyme Q_{10} (CoQ_{10}) levels in human plasma were determined by high-performance liquid chromatography (HPLC) with UV detection. CoQ_{10} was dissociated from lipoproteins by methanol and subsequently cleaned-up on silica gel and octadecyl silica solid-phase extraction cartridges. HPLC separation was performed on a C_{18} reversed-phase column. The methanol-hexane mobile phase provided a greater possibility of separation procedure adjustment allowing the shortest possible elution time without loss of resolution than a two-alcohol mobile phase. Quantitation was based on the peak heights using a standard addition method. The lower limit of detection was 8 ng on-column, corresponding to 90 μg ubiquinone per litre of plasma in an actual sample. Thirty-one randomly selected plasma samples from apparently healthy, 18 to 56-year-old individuals (males and females) were analyzed for total CoQ_{10}. The average level in these subjects was 0.47±0.18 mg/l with the range of 0.26–1.03 mg/l. The method was also applied to the determination of ubiquinone plasma level changes in one healthy volunteer over a period of one month and after oral intake of CoQ_{10}.

Key words

Coenzyme Q_{10} – HPLC – Quantitative determination – Plasma level

Introduction

Coenzyme Q (CoQ; 2,3-dimethoxy-5-methyl-6-multiprenyl-1,4-benzoquinone) is a lipophilic, redox active component present in all animal cellular membranes, where it is involved in redox reactions of diverse cellular functions. CoQ is best known as an obligatory component of the electron transport chain in mitochondria (Crane *et al.* 1957). It is a vital electron and proton carrier which supports ATP synthesis in the mitochondrial inner membrane (Cramer and Knaff 1990, Trumpower 1982). Its first action in energy transduction is electron accumulation from the dehydrogenases for substrates oxidized by the mitochondrial cristae membranes. Secondly, it encompasses a vectorial proton movement to establish a proton gradient across the membrane that can be coupled to ATP production (Mitchell 1991).

Besides its activities in the electron transport chain, ubiquinols have also been implicated as antioxidants protecting cellular membranes and plasma lipoproteins from free radical (e.g. O_2^-) damage (Beyer 1992, 1994). Indeed, ubiquinols strongly inhibited lipid peroxidation in model systems (Frei *et al.* 1990, Yamamoto *et al.* 1990) and biological membranes *in vitro* (Forsmark *et al.* 1991, Matsura *et al.* 1992) as well as *in vivo* (Sugino *et al.* 1987, 1989).

In man, extracellular CoQ is incorporated and transported primarily within low density lipoproteins (LDL) where it is present predominantly in the reduced form $CoQ_{10}H_2$ (Stocker *et al.* 1991). While the biological function of circulating $CoQ_{10}H_2$ is not established at present, this hydroquinone is a highly efficient antioxidant in LDL under conditions of oxidative stress.

The protective activity of $CoQ_{10}H_2$ for LDL lipid peroxidation can affect the oxidative LDL modification that might play a role in the early developmental stages of atherosclerosis (Steinbrecher *et al.* 1990). It has also been demonstrated that low CoQ_{10} plasma levels are associated with increased coronary risk factor in cardiac patients (Hanaki *et al.* 1991). The linear relationship between the concentration of plasma CoQ_{10} and free cholesterol content in healthy men and in various patient groups has been well documented (Karlsson *et al.* 1990, Johansen *et al.* 1991). Moreover, prolonged treatment with statins, which leads to decreased plasma cholesterol through inhibition of 3-hydroxy-3-methylglutaryl-CoA dehydrogenase, has recently also been shown to decrease plasma CoQ_{10} levels as well (Ghurlanda *et al.* 1993). A low plasma coenzyme Q_{10} content might reflect lower biosynthesis activity of the joint mevalonate pathway. In addition, LDL/ubiquinone ratio is also suggested to be a risk factor for atherogenesis (Hanaki *et al.* 1993). For these reasons, plasma CoQ_{10} determination should be included in routine procedures of clinical chemistry laboratories.

Various analytical techniques have been employed for ubiquinone analysis in samples of biological origin. For quantitative CoQ_{10} determination in the plasma, the most generally used method is liquid chromatography after liquid-liquid extraction with UV or electrochemical detection (Takada *et al.* 1982, Zhiri and Belichard 1994). Electrochemical detection provides the possibility of more sensitive monitoring of the reduced form (Lang *et al.* 1986, Grossi *et al.* 1992). For total plasma CoQ_{10} determination, the sample is usually converted into the corresponding reduced form and subsequently analyzed by HPLC with electrochemical detection (Okamoto *et al.* 1988, Edlund 1988).

In the present paper, we have described a modified procedure for the quantitative determination of total plasma ubiquinone using HPLC on a reversed-phase column with UV detection. The proposed method is discussed from the analytical point of view. Its usefulness is illustrated in a group of healthy subjects who were examined in order to estimate ubiquinone plasma levels in the regional population. Since CoQ_{10} is popular at present as an active compound of various pharmaceutical preparations we have also monitored the plasma level changes after oral supplementation with a large dose of this "vitamin".

Materials and Methods

Chemicals and reagents: the CoQ_{10} standard was from Sigma (St. Louis, USA), methanol, n-hexane and 2-propanol, all of HPLC or analytical grade were obtained from Lachema (Brno, Czech Republic). A stock standard solution (100 mg/l) was prepared in n-hexane and stored at -20 °C. For separation, the mobile phases consisting of n-hexane in methanol and a 2-propanol/methanol mixture were employed at a flow rate of 0.5 ml/min. In order to prevent degradation of CoQ_{10} by dissolved oxygen during column separation, the mobile phase was deaerated by bubbling with argon gas. Commercial coenzyme Q_{10} (10 mg in a capsule) from Bio-Garten (Austria) was used for oral application.

Instrumentation: The liquid chromatograph consisted of a pump, LCP 4000 (Ecom, Prague), a reversed-phase analytical column, Sepharon C18 150 x 3 mm (5 μm) with a guard column 50 x 3 mm (10 μm) (Tessek, Prague) and a LC-55B UV detector (Perkin-Elmer, USA). For manual injection, a sampler LCI-02 was used. Solid-phase extraction cartridges containing silica or octadecyl-bonded silica (60 m) were obtained from Tessek.

Samples: a) Plasma samples (3 ml) from healthy fasted male and female subjects (18–56 years old) were obtained from the Blood Transfusion Department, Faculty Hospital, Brno-Bohunice (no later than one day after blood collection). b) For the CoQ_{10} supplementing experiment, samples of 8 ml of venous blood were withdrawn daily in the morning within a week by venipuncture from a fasted healthy volunteer. The heparinized blood was immediately spun down at 1500 g for 15 min at room temperature. All analyzed plasma samples were kept at -20 °C in polypropylene tubes. On the day assigned for analysis, the samples were thawed in a water bath at room temperature. Plasma (1 ml) in triplicate was pipetted into 10 ml glass centrifuge tubes one of which was supplemented with 0.5 μg of CoQ_{10} standard. Samples were then subjected to liquid-liquid and subsequent solid-phase extraction (see Results and Discussion section). The aliquots of both standards and plasma extracts were injected onto the chromatographic column. Separated CoQ_{10} in its oxidized form was detected at 275 nm and a detector response was registered on a chart recorder. Quantitation was based on peak heights by using the standard addition method.

The recovery of the standard was calculated by subtracting the amounts found in the plasma with and without the standard added.

Results and Discussion

The method described here includes qualitative and quantitative analysis and applications. In order to obtain maximum speed of analysis, the separation conditions were adjusted to allow the shortest possible elution time without loss of resolution. For this purpose, two eluants were tested using the standard solution (20 mg/l) in hexane. The first mobile phase consisting of 2-propanol-methanol (1:4 v/v) was found unsuitable for the determination because of excessively long separation time. Its shortening was practically impossible as pressure on the column was increasing enormously. The second mobile phase test was performed with eluant composition of the hexane-methanol (1:9 v/v) according to Andersson (1992). The run time was substantially shorter (about 25 min) and could be reduced by increasing hexane content in the mixture. The final mobile phase with 15 % hexane in methanol was found to be suitable for CoQ_{10} routine determination, giving a retention time 8.5 min (Fig. 1A).

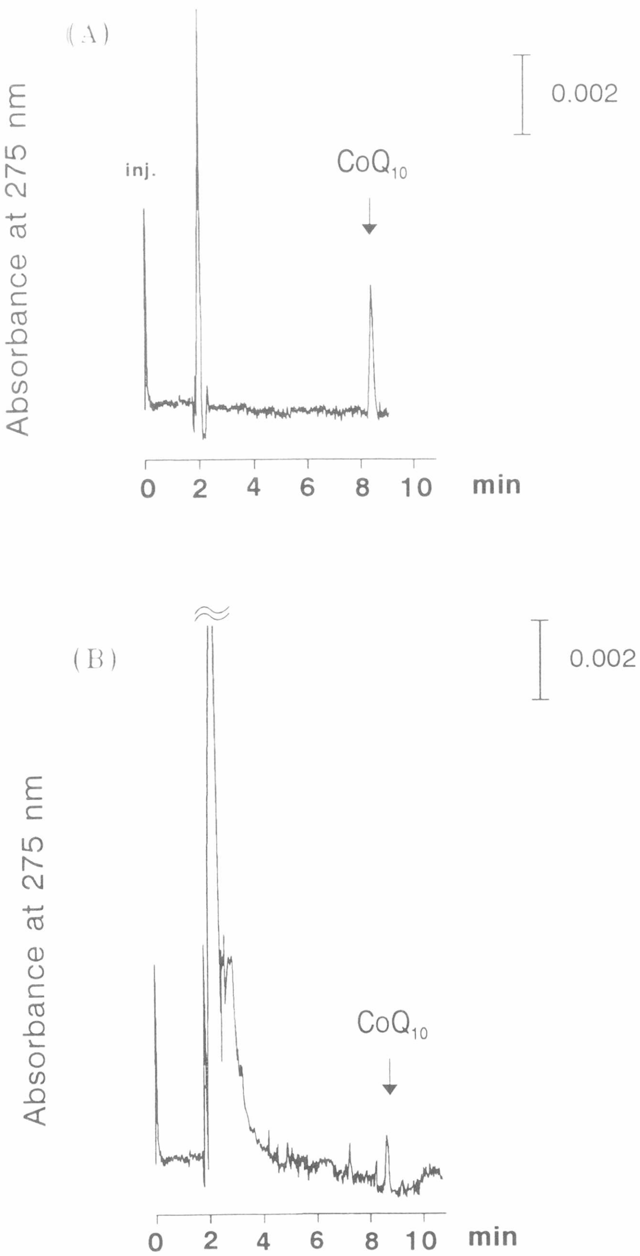

Fig. 1
HPLC chromatograms of coenzyme Q_{10} (0.1 μg) standard (A) and the plasma sample extract (B). 20 μl of the extract in 2-propanol was injected into the C18 column and separation was performed with mobile phase methanol – hexane 85 : 15 (v/v) at a flow rate 0.5 ml/min. UV detector was set at 0.02 a.u.f.s.

Quantitative determination of ubiquinone in a crude extract of plasma lipids according to Takada *et al.* (1982) was found unsatisfactory. The main problems concerned the large solvent front, other peaks interfering with that of CoQ_{10}, the sample injected was not clean and caused short column lifetime by strongly retained substances. An additional purification step was necessary. The crude lipid extract was subjected to subsequent clean-up on silica gel and C_{18} solid-phase extraction cartridges according to Grossi *et al.* (1992), slightly modified in eluant volumes (see Fig. 2). Both the silica and the C_{18} cartridges could easily be regenerated by hexane and water, respectively, after the elution step. The final extract obtained was clean enough for determination of CoQ_{10} as is demonstrated on a typical chromatographic profile of a plasma sample in Fig. 1B. No late-eluting peaks interfering with that of CoQ_{10} was detected. To account for changes in detection sensitivity, the working standard was injected frequently between samples.

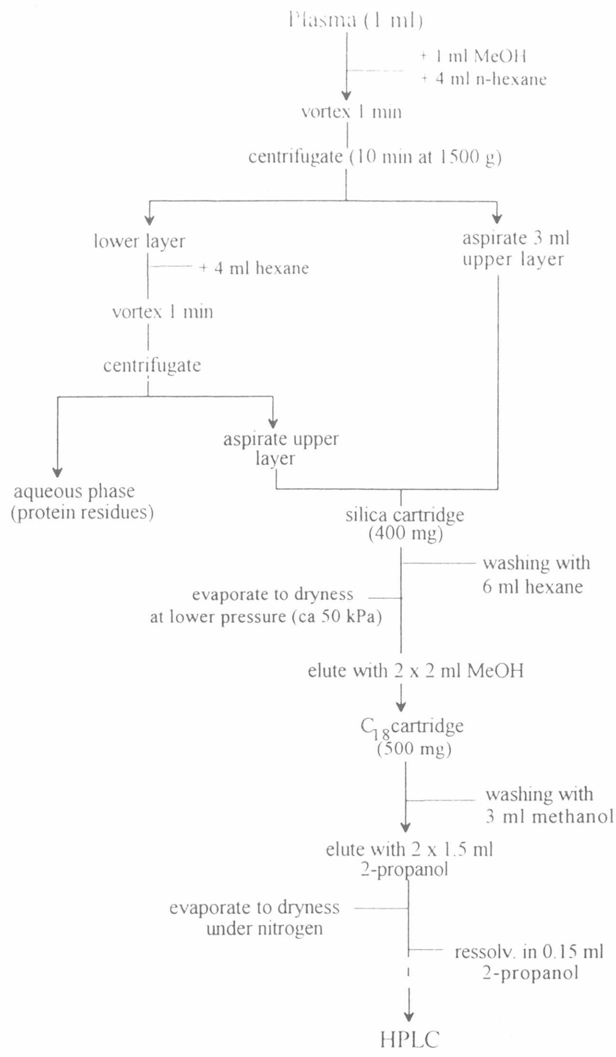

Fig. 2
Schema for the extraction procedure of coenzyme Q_{10} from the plasma.

In order to verify the linearity of UV detection, the calibration curve was constructed. A linear relationship between the concentration and peak height was confirmed within the range of up to 20 mg/l (Fig. 3). The lower limit of detection at signal-to-noise ratio of 3 : 1 (peak to peak) was approx. 8 ng on-column injected CoQ_{10}. This is equivalent to 90 μg of actual concentration of total CoQ_{10} (oxidized plus reduced) per litre of plasma when the sample was treated according to the procedure described (for recovery see below). The minimum detectable level is not too far from that of 50 μg/l obtained by the method with UV detection reported by Grossi *et al.* (1992).

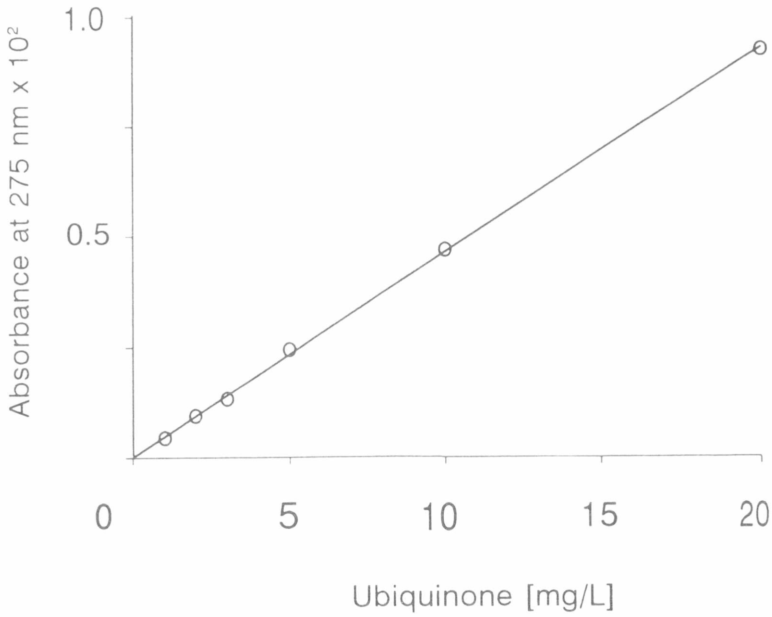

Fig. 3
Calibration curve for coenzyme Q_{10}. Standards were dissolved in hexane and 20 μl were injected on the column. The detector response (absorbance, A) can be expressed by the following equation: $A = ac + b$, where $a = (4.6 \pm 0.1)\ 10^{-4}\ l\ mg^{-1}$; $b = (1.5 \pm 11.1)\ 10^{-4}$; $r = 0.997$.

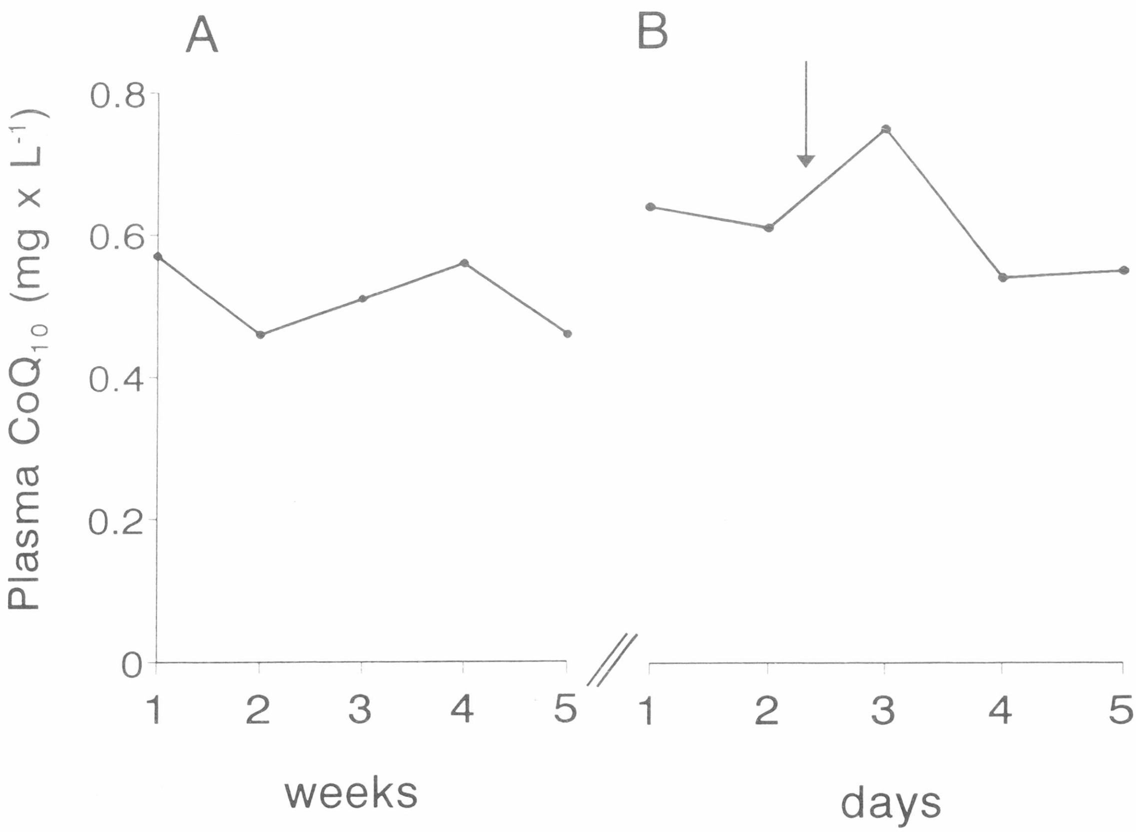

Fig. 4
Coenzyme Q_{10} plasma levels in a healthy volunteer over a period of one month (A) and after oral supplementation with a single dose of CoQ_{10} (B). Arrow indicates the intake of 130 mg 6 h before blood withdrawal.

Standard addition method was routinely used for quantitation of CoQ_{10} in plasma (0.5 μg CoQ_{10} per ml of plasma, see Materials and Methods). Many other authors mostly use internal (Edlund 1988, Zhiri and Belichard 1994, Grossi *et al.* 1992) or external (Lang *et al.* 1986) standardization. An internal standard, however, can be affected by different extraction behaviour and does not guarantee improvement in the assay precision. In contrast to the latter, the standard addition method is advantageous, when the recovery of CoQ_{10} varies between the samples, but it is reproducible with the same sample (Filser *et al.* 1989). In our case, the recovery value was found to be 64±6 % (mean ± relative standard deviation, 9 parallel analyses of a plasma samples mixture). The recovery is similar to that obtained by Lang *et al.* (1986) in plasma sample (71 %), but is lower than those reported by some other authors using the internal standard (Grossi *et al.* 1992, Edlund 1988).

Table 1
CoQ plasma levels in healthy subjects (range 18–56 years) and in male and female (the same group of analyzed)

Age (years)	CoQ_{10}[a]	n
<30	0.42±0.16	12
30–49	0.48±0.22	15
≥50	0.47±0.10	4
Male	0.49±0.21	16
Female	0.44±0.15	15

[a] *CoQ plasma contents are expressed in mg per l, ± S.D.*

The method was applied to some real samples and in Table 1 the data obtained for a group of normal subjects are presented. The ubiquinone values were somewhat lower than those found by other authors. "Physiological" values, however, were not given, as CoQ_{10} plasma levels can vary over a wide range within the group of healthy individuals. Grossi *et al.* (1992), for example, obtained CoQ_{10} levels over a range of 0.2–1.21 mg/l in healthy men. There are also very marked differences between the examined groups of "normal" individuals, called either sedentary subjects or endurance athletes. Johansen *et al.* (1991) shows 0.69 mg/l CoQ_{10} with the range of 0.36–0.8 in healthy men, Karlsson *et al.* (1991) estimated 0.6 mg/l in normal subjects, 0.4 mg/l in endurance athletes and 0.9 mg/l for sedentary subjects.

It is also necessary to note that the differences in CoQ_{10} plasma levels mentioned above may be caused by the different life-style or nutritional habits of the analyzed groups reported (Italy, Sweden) as compared to those of this regional population.

In the present report no differences were found in the CoQ_{10} plasma content either between the age categories or between males and females. The concentration range was quite considerable (0.26–1.03 mg/l) in all the analyzed individuals. Whether this was related to the pharmacological treatment or the nutritional status cannot be evaluated at this stage.

In the other application, CoQ_{10} plasma levels in one healthy individual were measured within a period of one month. Fig. 4A shows the values for five plasma specimens taken consequently at weekly intervals. Mean ubiquinone plasma levels were assessed in each sample analysis in three parallel controls. They varied slightly within the range of 0.46 and 0.57 mg/l.

In recent years, a number of studies were undertaken to examine the CoQ_{10} distribution into tissues after short-term (days) or long-term (months) oral administrations (Schardt *et al.* 1986, Mortensen 1993, Karlsson *et al.* 1991). Usually 100 mg oral CoQ_{10} intake a day for several weeks significantly increased the skeletal muscle and blood ubiquinone content in selected groups of patients and healthy men depending distinctly on initial blood values (Karlsson *et al.* 1991). In our investigation, the same healthy man received a single oral dose of CoQ_{10} (130 mg) to ascertain whether his plasma level would be influenced. Fig. 4B shows that the CoQ_{10} content in blood withdrawn 6 h after intake was only slightly increased. The subsequent ubiquinone plasma levels decreased again and were comparable to those in the previous experiment.

Extraction followed by HPLC analysis is to be preferred strongly over direct spectrophotometric measurement of ubiquinone, which requires a sensitive spectrophotometer and large amounts of the sample. In our HPLC procedure, we tried to combine the advantages of additional sample purification (removal of interfering substances from the crude extract), UV detection (availability of necessary equipment monitoring oxidized, i.e. more stable form of CoQ_{10}) and standard addition method (equal extraction behaviour of the standard). The proposed method has been found to be suitable for determination and investigation of ubiquinone in human plasma. A chromatographic run takes only 10 min, and the sample (in series) preparation does not exceed 90 min, including centrifugation.

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