

Analysis of coenzyme Q₁₀ in human plasma by high performance liquid chromatography

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(Received July 24, 2009; Accepted November 9, 2009)

고성능액체크로마토그래피를 이용한 혈장 내 코엔자임 큐텐 분석

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(2009. 7. 24. 접수, 2009. 11. 9. 승인)

요 약: 코엔자임 큐텐은 비타민과 유사한 물질로 체내 중 미토콘드리아에 주로 분포 되어 있으며 항산화 작용을 나타낸다고 알려져 있다. 본 연구는 27-44세의 건강한 성인 남·녀 24명을 대상으로 하였고 혈장 내 코엔자임 큐텐의 농도를 정량 평가하기 위하여 UV 검출기가 장착된 고성능액체 크로마토그래피를 사용 하였다. 혈장 내 코엔자임 큐텐을 분리하기 위해서 메탄올로 지방단백질을 제거하였고 노말헥산을 이용하여 액체-액체 추출법으로 코엔자임 큐텐을 용해 시켰다. 그 후 원심분리를 실시하여 노말헥산과 제단 백된 물질을 층분리 시키고 상층액을 다른 유리 시험관에 옮겨 담았다. 옮겨진 노말헥산 층은 질소 가스를 이용하여 증발·농축을 시켰으며 남은 잔사에 순수한 에탄올로 재용해 시켜 검사 장비 내로 주입 하였다. 이 때 사용된 컬럼은 C18 역상 컬럼이었고 275 nm의 파장이 이용되었으며 메탄올과 에탄올을 85:15 로 섞은 이동상을 1.7 mL/min의 유속으로 흘렸다. 본 검사 방법의 정량한계(limit of quantitation : LOQ, N/S=10)는 0.02 mg/L로 평가 되었고 0.1-2.0 mg/L의 농도 범위에서 검량선을 작성 하였다. 대상군 24명의 혈장 중 코엔자임 큐텐의 농도는 0.41-0.98 mg/L의 범위에서 분포 하고 있었으며 평균 농도는 0.62±0.13 mg/L 였다. 본 연구의 결과 본 검사 방법은 특수성을 가지고 있었으며 혈장 내 CoQ₁₀을 분석하는데 충분한 정량한계를 가지고 있었기에 임상에 적용이 가능하고 연구 기관에서 사용하기에 적합하다고 사료 된다.

Abstract: Coenzyme Q₁₀(CoQ₁₀), a vitamin E-like substance, represents a components of the complex antioxidant system of the human organism. CoQ₁₀ levels in human plasma were determined by high performance liquid chromatography (HPLC) with UV detection. It was dissociated from lipoproteins by methanol and extracted into n-hexane with liquid-liquid extraction procedure, after centrifugation, the supernatant was dried under nitrogen gas stream. The residue was dissolved in the absolute ethanol. Determination of CoQ₁₀ was performed on a C₁₈ reversed-phase analytical column with ultraviolet detection at 275 nm and the mobile phase containing 15% (v/v) ethanol in methanol at a flow rate of 1.7 mL/min. The low limit of quantitation was 0.02 mg/L

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(S/N=10), the linearity between the concentration and peak height is from 0.1 to 2.0 mg/L. Twenty-four randomly selected plasma samples from apparently healthy, 27 to 44 year old individuals (males and females) were analyzed for total CoQ₁₀. The average level in these subjects was 0.62±0.13 mg/L with the range of 0.41-0.98 mg/L. This method has a specific and a sufficient limit of quantitation (LOQ) for analysis of CoQ₁₀ in human plasma in both a clinical study and research at laboratories.

Key words: coenzyme Q₁₀, antioxidant, micronutrients

1. Introduction

Coenzyme Q₁₀(CoQ₁₀) is a lipid soluble compound that occurs in the mitochondria of animal and human cells and is synthesized by hydroxybenzoic acid which reacts with isopentenylpyrophosphate under an enzyme system in the line of mitochondria.¹ It is a vital electron and a proton carrier which supports ATP synthesis in the mitochondrial inner membrane.²⁻³ Its first action in energy transduction is an 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.⁴

CoQ₁₀ is beneficial for preventing cellular damage during myocardial ischemia and reperfusion and has been used orally to treat various cardiovascular disorders that include angina pectoris, hypertension, arrhythmia, and congestive heart failure. A decreased ratio of plasma CoQ₁₀ to low density lipoprotein(LDL) cholesterol could be associated with an increased risk of atherosclerosis.⁵ CoQ₁₀ has been suggested to have both anti-cancer and immune system enhancing properties when CoQ₁₀ tested in animals.⁶

Various analytical techniques have been employed for CoQ₁₀ analysis in samples of a biological origin. For a quantitative CoQ₁₀ determination in the plasma, the most generally used method is spectrophotometer and high performance liquid chromatography (HPLC) with different methods of detection:ultraviolet (UV), electrochemical (ECD) or mass spectrometry (MS).⁷⁻¹⁰ However, HPLC-UV method has a lower limit of quantitation (LOQ) than spectrophotometer (LOQ=0.07 mg/L). Also, it has a sufficient LOQ for

analysis of CoQ₁₀ in human plasma and lower cost than any other HPLC methods.¹⁰ Liquid-liquid extraction method is a common extraction method which has high recovery rate and a low cost, even though a preparatory process is more complicated than a solid phase extraction (SPE) column method.^{7-8,11}

In the present paper, our method modified procedure of Peter (2001).¹¹ Procedure of Peter used 1-propanol for liquid-liquid extraction but we used n-hexane which is a common organic solution for liquid-liquid extraction. While procedure of Peter used ECD for the detection of CoQ₁₀ in human plasma but we used UV which has a low cost than ECD method and a sufficient LOQ for analysis of CoQ₁₀ in human plasma. In addition, whereas process of Peter used a reversed-phase Microsorb-MV column (150×4.6 mm, 5 μm) which has a short run time (RT) and not a good resolution (R=0.88), we used reversed-phase Hypersil ODS column (250×4.6, 10 μm). As a result, we got a better resolution (R=2.16). Its usefulness is illustrated in a group of healthy subjects who were examined in order to estimate CoQ₁₀ plasma levels in the regional population.

2. Materials and Methods

2.1. Materials

The Coenzyme Q₁₀(CoQ₁₀, standard) and coenzyme Q₉(CoQ₉, internal standard) was purchased from Sigma (St. Louis, USA), methanol, ethanol, n-hexane, all of HPLC or analytical grade were obtained from Merck (Darmstadt, Germany). All the containers were wrapped with aluminium foil to prevent from light exposure.

2.2. Sample

Twenty-four healthy male and female volunteers (27-44 years old) were selected from Seoul clinical laboratories. Venous blood was collected in a Vacutainer[®] Tube (Becton Dickinson) containing heparin as anticoagulant and mixed by gentle inversion 5-10 min. The plasma was separated by centrifugation (2000 g for 10 min) in the dark and stored in the frozen condition (-20 °C) until subsequent assay.

2.3. Instrumentation

The HPLC system consisted of HP1100 pump, HP 1100 variable absorbance detector, HP1100 column heater, HP1100 autosampler and integration software was used HP chemstation (Hewlett Packard Inc., Germany). Column (Hypersil ODS, 10 μ m, 250 \times 4.6 mm) was purchased from Agilent Technologies (U.S.A).

A mobile phase was methanol:ethanol (85:15, v/v). Flow rate was 1.7 mL/min and the column temperature was set at to 30 °C. UV detector was set at 275 nm (Table 1).

Table 1. Operating condition of HPLC for CoQ₁₀ analysis

Items	Conditions
HPLC	HP 1100 series. Germany
Column	Hypersil ODS, (250 \times 4.6 mm, 10 μ m)
Mobile phase	Methanol : Ethanol = 85 : 15(v/v)
Flow rate	1.7 mL/min
Detector	275 nm, UV detector (HP 1100. Germany)
Injection volume	30 μ L

2.4. Preparation of plasma samples

Frozen plasma samples were thawed at room temperature in the dark before analysis. Extractions were performed in 10 mL amber glass test tubes with stoppers. An aliquot of 80 μ L internal standard (CoQ₉, 2 mg/dL in absolute ethanol) was added into 1.0 mL of plasma and was mixed for 10 sec by a vortex mixer, and then the sample was deproteinized with 1.5 mL of methanol. The treated sample was statically placed in the dark for 10 min. Four mL of n-hexane was transferred to 10 mL amber glass test tubes and was dried at 25 °C in the water bath under nitrogen gas stream. The residue was dissolved in the absolute ethanol 200 μ L and was injected onto HPLC system.

An aliquot of 80 μ L internal standard (CoQ₉, 2 mg/dL in absolute ethanol) and 10 μ L of CoQ₁₀ stock solution (0.00, 0.01, 0.05, 0.10, 0.20 mg/mL in absolute ethanol, respectively) were added to 1.0 mL of fresh plasma to prepare the calibration curves. Calibration curve was constructed in the concentration range of 0.1-2.0 mg/L. The peak-area ratio increments of CoQ₁₀/CoQ₉ were plotted as a function of the concentration of CoQ₁₀. Where the peak-area ratio increment was the difference value of “peak-area ratio of total CoQ₁₀/CoQ₉” minus “peak-area ratio of endogenous CoQ₁₀/CoQ₉”.

3. Results

Typical HPLC-UV chromatograms showed that

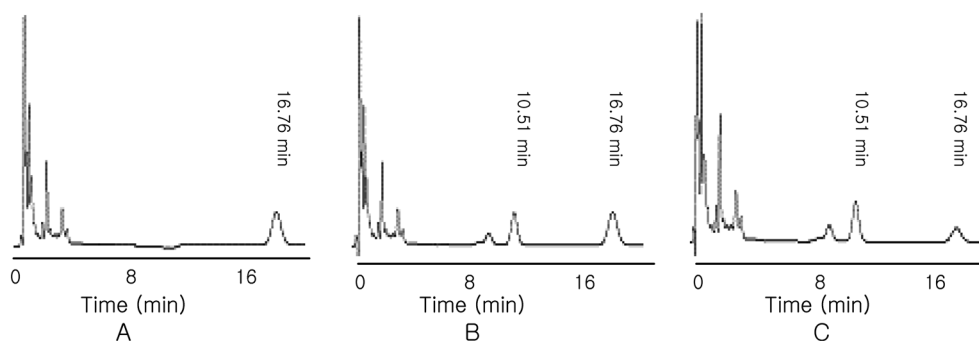


Fig. 1. Typical chromatograms obtained from (A) Fresh plasma (Endogenous CoQ₁₀ peak t_{R2} = 16.76 min), (B) Fresh plasma supplemented with internal standard CoQ₉ (CoQ₉ peak t_{R1} = 10.51 min, CoQ₁₀ peak t_{R2} = 16.76 min), and (C) the subject plasma supplemented with internal standard CoQ₉ (CoQ₉ peak t_{R1} = 10.51 min, CoQ₁₀ peak t_{R2} = 16.76 min).

the endogenous CoQ₁₀ peak in fresh plasma appeared around at 16.76 min (Fig. 1A) and the retention time of internal standard CoQ₉ was about at 10.51 min (Fig. 1B). Chromatograms in Fig. 1A and B were obtained from fresh plasma without or with supplement of CoQ₉. Chromatogram in Fig. 1C was obtained from subject plasma that was supplemented with internal standard CoQ₉.

The method was applied to some real samples and in Table 2 the data which obtained for a group of normal subjects are presented. The average level in < 30 subjects was 0.61±0.12 mg/L with the range of 0.46-0.81 mg/L and 30-39 subjects was 0.60±0.11 mg/L with the range of 0.41-0.78 mg/L and ≥40 subjects was 0.70±0.25 mg/L with the range of 0.54-0.98 mg/L. The average level in male subjects was 0.65±0.28 mg/L with the range of 0.44-0.98 mg/L and in female subjects was 0.61±0.09 mg/mL with the range of 0.41-0.81 mg/L.

The minimum concentration of CoQ₁₀ that could be quantitated in plasma was 0.02 mg/L. This was determined to noise ratio of approximately 10 based on signal. Calibration curve was $Y=1.8476C+0.0142$, Y=the peak-area ratio increment and C=sample concentration ($r=0.9999$, Fig. 2). The absolute recoveries of CoQ₁₀ ranged 99.12±4.00% to 100.93±2.51% as showing Table 3, and the precision of intra-day and

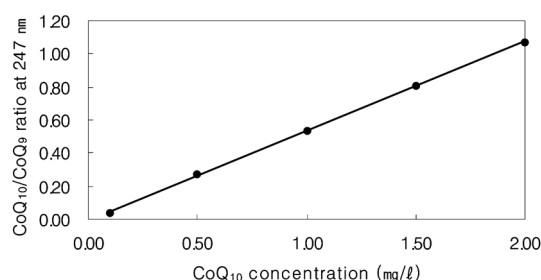


Fig. 2. Calibration curve for CoQ₁₀ standards were dissolved in hexane and 30 were injected on the column.

Table 4. Precision of intra-assay and inter-assay by the analytical method ($n=5$)

Added (mg/L)	Intra-day		Inter-day	
	Found (mg/L)	C.V (%)*	Found (mg/L)	C.V (%)*
0.100	0.099 ± 0.03	2.05	0.101 ± 0.01	2.45
1.000	1.009 ± 0.03	3.63	1.019 ± 0.03	3.86
2.000	1.998 ± 0.08	4.72	1.976 ± 0.03	3.00

*C.V (%) = Coefficient of variation

inter-day were shown in Table 4. The coefficient of variation (C.V, %) values ranged from 2.05% to 4.72% for intra-day assay, and from 2.45% to 3.86% inter-day assay.

4. Discussion

CoQ₁₀ is almost insoluble in water and is soluble in hot ethanol and is freely soluble in n-hexane. The determination method was established by improving the extraction recoveries. The use of methanol to precipitate protein in plasma and n-hexane to extract the CoQ₁₀ and CoQ₉ improved the extraction efficiency.¹² CoQ₁₀ and the internal standard CoQ₉ are unstable under the light thus all the samples were wrapped with aluminium foil and treatments were carried out under the illumination of yellow light or in the dark. In addition, CoQ₁₀ is also unstable at the high temperature. It is found that there are peak of decomposed CoQ₁₀ in water bath at 40 °C but stable at 25 °C or at 30 °C under nitrogen gas stream for drying. Temperature of an analytical column influences on a chromatogram peak. From a column which set at

Table 2. CoQ₁₀ plasma level in healthy subjects (range 27-44 years) and in male and female (the same group of analyzed)

Age(years)	CoQ ₁₀ Con. (mg/L)	Mean ± SD	n
< 30	0.46 - 0.81	0.61 ± 0.12	7
30-39	0.41 - 0.78	0.60 ± 0.11	14
≥ 40	0.54 - 0.98	0.70 ± 0.25	3
Male	0.44 - 0.98	0.65 ± 0.28	4
Female	0.41 - 0.81	0.61 ± 0.09	20

Table 3. Recovery of the CoQ₁₀ by the analytical Method ($n=5$)

Added (mg/L)	Found (mg/L)	Recovery (%)
0.100	0.099 ± 0.03	99.12 ± 4.00
1.000	1.009 ± 0.03	100.93 ± 2.51
2.000	1.998 ± 0.08	99.91 ± 3.46

30 °C, we obtained an optical resolution rate and a clear peak for CoQ₁₀ and CoQ₁₀.¹²

CoQ₁₀ concentration has generally a decreasing tendency as people pass their twenties but in our study, it doesn't seem to follow the previous pattern.¹³ For that reason, we considered several certain factors which can affect the result such as a failure of diet control as a main reason, a different life-style and eating habits. However, CoQ₁₀ concentration was significantly higher than patients with cardiomyopathies of unclear etiology and cardiovascular diseases (0.27±0.02 and 0.23±0.02 mg/L, respectively).¹⁴

CoQ₁₀ level of normal subjects were somewhat lower or higher than those found by other authors. For example, P. Kaplan (1995) obtained CoQ₁₀ levels over a range of 0.26-1.02 mg/L in healthy men.¹⁵ There are also very marked differences between the examined groups of "normal" individuals so we called either sedentary subjects or endurance athletes. Jonansen (1991) showed 0.69 mg/L CoQ₁₀ with the range of 0.36-0.80 mg/L in healthy men, Karlsson (1991) estimated 0.60 mg/L in normal subjects, 0.4 mg/L in endurance athletes and 0.90 mg/L for sedentary subjects.^{16,17} It is also necessary to note that the differences in CoQ₁₀ plasma levels mentioned above may be caused by the different life-style or nutritional habits of the analyzed groups reported as compared to those of this regional population.

In conclusion, we proposed a simple, a rapid and an isocratic HPLC method for determination of CoQ₁₀ in human plasma. An extraction process using n-hexane as solvent allows a rapid and simple sample extraction and minimizes oxidation of CoQ₁₀ during samples processing. A chromatogram run takes only 20 min and the sample preparation does not exceed 100 min. From the presented results, this optimized a method which provides excellent sensitivity, precision and accuracy for relatively high-throughput assessment of CoQ₁₀ in human plasma. This method is suitable for research and it can be easily adapted for clinical testing purposes. Studies are in progress to establish reference intervals and to evaluate the clinical significance of plasma concentration of CoQ₁₀ in several patient populations.

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