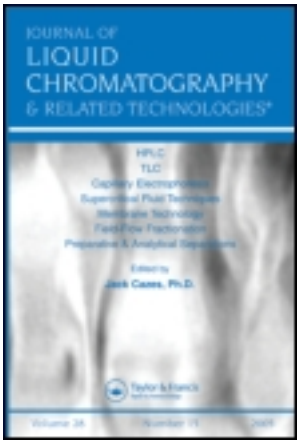

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ljlc20

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Version of record first published: 01 Dec 2011.

To cite this article: Mario Contin , Silvia Lucangioli , Manuela Martinefski , Sabrina Flor & Valeria Tripodi (2011): MINIATURIZED HPLC-UV METHOD FOR ANALYSIS OF COENZYME Q10 IN HUMAN PLASMA, Journal of Liquid Chromatography & Related Technologies, 34:20, 2485-2494

To link to this article: http://dx.doi.org/10.1080/10826076.2011.591028

Journal of Liquid Chromatography & Related Technologies, 34:2485–2494, 2011

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826076.2011.591028

MINIATURIZED HPLC-UV METHOD FOR ANALYSIS OF COENZYME Q10 IN HUMAN PLASMA

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□ *Coenzyme Q10 (CoQ10) is a cofactor in the respiratory chain and a potent endogenous antioxidant. Abnormally low levels of CoQ10 in the circulatory system are often involved with pathological states such as heart failure, mitochondrial and muscular diseases, and cancer. The development of simple, rapid, and highly sensitive methods capable of quantifying low CoQ10 levels in plasma is, therefore, increasingly required.*

In this work, we developed a miniaturized HPLC-UV system for the determination CoQ10 in human plasma using an XTerra microcolumn (50 mm × 2.1 mm i.d., 3.5 μm particle size), methanol:water (98:2, v/v) as mobile phase, 275 nm, flow rate of 0.3 mL/min, 30 °C column temperature, and 2 μL injection volume. The extraction procedure consists of a plasma precipitation with 1-propanol and evaporation under nitrogen to allow a 1.5-fold enrichment. The chromatographic analysis was accomplished in 7 min. As a result, it was possible to quantitate CoQ10 in small sample volumes down to 0.07 μM and detect as low as 0.02 μM in real plasma, with good accuracy and precision, even in pathological conditions. The proposed method was found to be suitable for routine CoQ10 determination in clinical laboratories.

Keywords coenzyme Q10, microcolumn, micro fast HPLC, mitochondrial disease, plasma, UV

Abbreviations CoQ10, coenzyme Q10; EC, electrochemical detection; HPLC, high performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantitation; UV, ultraviolet

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INTRODUCTION

CoQ10 is an essential cofactor integrated in the mitochondrial respiratory chain where it acts as an electron carrier for the production of cellular energy.[1] In addition, it is recognized as a primary regenerating antioxidant playing an intrinsic role against oxidative damage.[2] CoQ10 is found in normal human plasma at very low concentration levels (0.4–1.4 μM).[3,4] It is known that CoQ10 is involved in pathological conditions such as cancer, cardiovascular, mitochondrial and muscular diseases, and, in all of these cases, the CoQ10 is diminished.[5] Interestingly, the treatment with CoQ10 reduces the rate of functional decline in those patients with beneficial therapeutic effects.[6,7]

Several methods for analysis of CoQ10 have been developed because of the interest in CoQ10 as a biomarker for metabolic and oxidative stress abnormalities as well as for the potential monitoring of the oral supplementation of this coenzyme.[1,8]

Plasmatic CoQ10 is currently measured by HPLC[3] with electrochemical detection (HPLC-EC), which seems to be the most commonly used method because of its high sensitivity. However, electrochemical detector is sensitive to high concentrations of lipophilic plasmatic components that may passivate the electrodes and shorten their lifetimes.[9]

Some other authors have reported analytical procedures for CoQ10 determination coupling HPLC to mass spectrometer (HPLC-MS).[9] Reported methods using HPLC-MS are highly sensitive, but they require more expenditure, qualified operators, and sophisticated instrumentation not easily available in clinical routine laboratories.

Although HPLC with UV detection is frequently employed in clinical laboratories, for low concentration levels of some analytes and small volumes injected (<100 μL) the UV detector is, in general, not sensitive enough for quantitation,[9] and it is at least 10 times less sensitive than the electrochemical detector.[10]

One of the current trends of advanced analytical chemistry is the miniaturization of the analytical procedures. Ultrafast separations, consumption of small amounts of both samples and reagents, as well as high sensitivity and easy automatization are some of the most important goals to achieve;[11] therefore, the miniaturization of the HPLC columns allows the performance of CoQ10 analysis with these features.

Fast HPLC employs short columns of 2–5 cm of length with a conventional internal diameter (i.d.) of 4.6 mm while microbore HPLC uses columns of 1–2 mm i.d. Recently, in a previous work,[12] we employed a microbore HPLC column packed with special hybrid particles to develop a rapid method for the quantification of CoQ10 in pharmaceutical and cosmetic formulations. The developed analytical method increases the

sensitivity compared with results obtained by electrochemical detection. In addition, the reported analytical method reduces the required sample volume as well as solvent and mobile phase consumption.

However, to our knowledge, this method has not previously been applied to the analysis of the CoQ10 in biological samples. In this work, we have developed a miniaturized HPLC-UV method for the analysis of CoQ10 in human plasma including pathological samples with low CoQ10 concentrations. The objective of this paper is to describe not only the performance of a simple miniaturized analytical method for determination of CoQ10 in plasma but also to assess a reliable method for diagnostic and monitoring tests in clinical laboratories.

EXPERIMENTAL

Chemicals and Reagents

CoQ10 was purchased from Sigma (St. Louis, MO, USA). Methanol, 1-propanol, and ethanol were supplied by E. Merck (Darmstadt, Germany). All chemicals were HPLC grade. Ultrapure water was obtained by an EASY pure RF equipment (Barnstead, Dubuque, IA, USA). All solutions were filtered through a 0.45 μm nylon membrane (Micron Separations Inc., Westboro, MA, USA) and degassed before use.

Preparation of Standard Solution

Stock solutions of 1 mM CoQ10 were prepared in ethanol protected from light and stored at −20°C for two months. Before analysis the accurate concentration of CoQ10 in the working standard solution was obtained by spectrophotometry at 275 nm, $\varepsilon = 14020$ L/mol. cm. CoQ10 calibration curve at concentration levels of 0.1, 0.35, 1.2, 3.5, 7.0, and 15.0 μM were prepared in the mobile phase from 1 mM CoQ10 stock solutions.

Sample Analysis

All heparinized plasma samples were taken from fasting persons, immediately processed and centrifuged at 2000 g for 10 min at 4°C. Plasma was collected, placed in a capped polypropylene tube, and immediately stored at −70°C until CoQ10 analysis.

Before the analysis, the samples were allowed to thaw at room temperature. First, 600 μL of plasma were supplemented with 800 μL of cold 1-propanol, stirred with vortex for 2 min, and centrifuged at 9000 g during 10 min at 4°C to spin down the protein precipitate and finally the organic

layer was evaporated to dryness under a stream of nitrogen. The dry residue was dissolved in 400 μL of mobile phase and then 2 μL of the treated sample was injected into the equipment. A 1.5-fold enrichment of CoQ10 in the plasma samples was finally obtained prior to the analysis.

Subjects from 9 to 38 years of age were recruited for the quantitation of CoQ10 in plasma: 10 healthy people (5 men and 5 female from 23 to 35 years old), 4 patients with mitochondrial disease treated with CoQ10 (2 male and 2 female from 9 to 19 years old), and 10 patients with mitochondrial diseases without treatment (4 male and 6 female from 12 to 38 years old). Written, informed consent was obtained from all donors before the studies.

Instrumentation and Chromatographic Conditions

The HPLC equipment consisted of a Waters 1525 Binary HPLC pump, 717 plus autosampler, and 2487 Dual λ Absorbance detector (Waters, MI, USA). Chromatograms were processed using Breeze Software (Waters, MI, USA).

Separation was achieved using a XTerra analytical microcolumn (50 mm × 2.1 mm i.d., 3.5 μm particle size) with a guard column XTerra C18 (Waters Corp.). Spherisorb ODS 2 Waters (150 mm × 4.6 mm i.d., 5.0 μm particle size) and microbore Spherisorb S3 ODS 2 Waters (150 mm × 2.1 mm i.d., 3.0 μm particle size) was also used for comparison.

The final chromatographic conditions were: column temperature was maintained at 30°C, the isocratic mobile phase consisted of a mixture of methanol:water (98:2, v/v) and the flow rate was set at 0.3 mL/min. UV-detection was performed at 275 nm with an injection volume of 2 μL. Determination of CoQ10 in plasma samples was accomplished in 7 min.

Quantification and Validation Method

The quantification of CoQ10 was performed using a six point calibration curve ranging from 0.1 to 15.0 μM of CoQ10 in mobile phase. Validation was performed according to international guidelines.[13,14] Specificity of the current method was conducted from a comparison of chromatographic runs between a standard solution of CoQ10 and the plasma sample. The retention time of CoQ10 in standard solutions was used to confirm the presence of CoQ10 in the original sample. Accuracy was evaluated by means of a recovery assay. The recovery assay was carried out by spiking plasma samples with CoQ10 at three different levels by triplicate. The three concentrations corresponded to the high (6 μM), low (0.1 μM), and the middle point level (1.2 μM) of the calibration curve.

The limits of detection (LODs) and limits of quantitation (LOQs) in plasma were determined at signal-to-noise ratio of 3:1 and 10:1, respectively.

RESULTS AND DISCUSSION

Sample Preparation and Chromatographic System

The current method of choice for CoQ10 extraction from plasma is a simple protein precipitation using 1- propanol and direct injection of the extract in the HPLC system with high recoveries of the analyte.[3,15] The sample preparation used in our work is based on that procedure. However, as in pathological conditions CoQ10 concentration in plasma decreases, it would be necessary to concentrate the analyte in the sample by an evaporation procedure. On the other hand, we have obtained better peak shapes if the sample is placed in the mobile phase than in 1-propanol. For this reason, a combination of evaporation to dryness and reconstitution in mobile phase is needed to achieve high recoveries. HPLC-EC is commonly used for determination of CoQ10 either in plasma or in biological tissues. It is chosen for its high selectivity and sensitivity. However, its drawback is that a long time is necessary to obtain a stabilized baseline before analysis. On the other hand, due to the fact that the detector is influenced by high concentrations of some lipophilic components that could be present in the sample, it is probable that these compounds passivate the electrodes and reduce their lifetime.[9] HPLC with UV detection is simple but it possesses lower sensitivity than HPLC-EC methods. Determination of low levels of CoQ10 in biological samples is difficult especially in patients under any pathological condition.

In addition, traditional HPLC columns require long run times for the analysis of high hydrophobic compounds like CoQ10 ($\log P \gg 10$), unless the elution power is increased with isopropanol.

To achieve rapid analysis and increase sensitivity, miniaturization of the column is of a great advantage. The use of short columns (5 cm) with reduced diameters (2.1 mm, i.d.), which were filled with small particle sizes (3.5 μm), allowed the quantitation of CoQ10 in a short time with less consumption of solvents and sample. In addition, the packing option of the column has a pronounced influence on the CoQ10 analysis. The analytical column assayed contains particles with one out of every three silanols replaced, by synthesis, with a methyl group. This hydrophobicity is distributed throughout the entire structure of the particle. The result of this modification is a hybrid particle that can be operated at high speeds, high temperatures, and a wide range of pH. In addition, sharp and symmetrical peaks of high efficiency for hydrophobic compounds such as as CoQ10 were obtained.

A comparison of CoQ10 determination using different sizes and fillings of C18 columns is shown in Figure 1. When traditional columns were used, it was necessary to increase flow rate respect to the use of microcolumns (1.0 mL/min vs. 0.3 mL/min). It was decided to add a small amount of water to the mobile phase (2%) to increase the CoQ10 retention time in order to avoid interferences of the plasma matrix. Table 1 shows the comparison of chromatographic parameters obtained using different columns, CoQ10 amount injected, and flow rate.

Thus, the use of microcolumns, allows not only the decrease of the retention time but also the reduction of the amount injected, thereby, increasing the sensitivity of the method by a factor of 10 times with good efficiency and low solvent consumption (Figure 1).

Quantitation and Validation

Calibration curves were linear over a concentration range of 0.1–15.0 μM. In an analysis of the curve characteristics, it was observed that the correlation coefficient (r^2) was 0.997, the standard deviation of residuals (Sy/x) was 0.17, the standard deviation of intercept ($S_{intercept}$) was 0.13, and the standard deviation of slope (S_{slope}) was 0.04. The chromatographic LOQ value was 0.1 μM and the chromatographic LOD value was

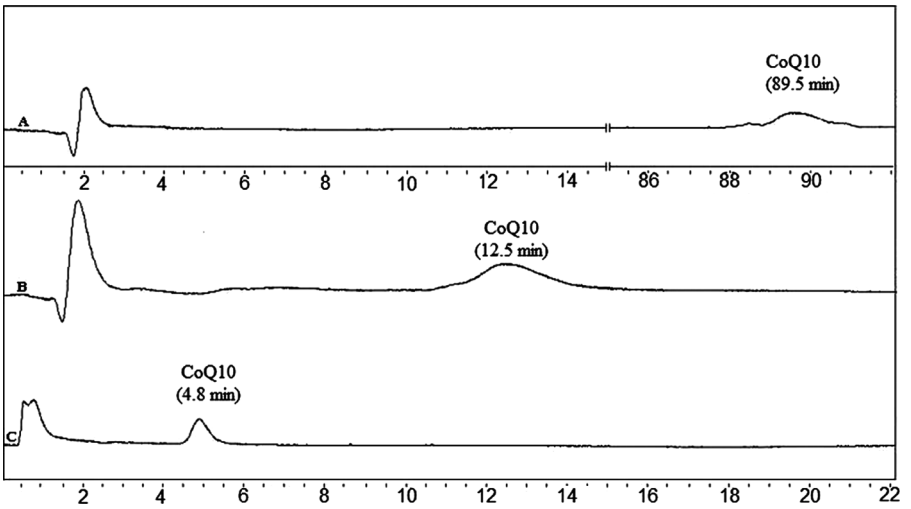

FIGURE 1 Comparison of analysis of CoQ10 standard using different HPLC columns. Retention time in parentheses. (A) Traditional C18-column (15.0 cm × 4.6 mm i.d.). Mobile phase: methanol:water (98:2, v/v), flow rate: 1.0 mL/min. CoQ10 standard: 2.0 μM. (B) Microbore C18-column (15.0 cm × 2.1 mm i.d.). Mobile phase: methanol:water (98:2, v/v), flow rate: 0.3 mL/min. CoQ10 standard: 2.0 μM. (C) XTerra C18 microcolumn (50 mm × 2.1 mm i.d.). Mobile phase: methanol:water (98:2, v/v), flow rate: 0.3 mL/min. CoQ10 standard: 1.0 μM.

TABLE 1 Comparison of Chromatographic Parameters in the CoQ10 Analysis Using Different HPLC Columns Chromatographic Parameter

Column	Amount injected (ng)	Mobile phase (methanol: water, v/v)	Flow rate (mL/min)	Retention factor (ḱ)[a]	Tailing[a]	N[a,b]
C_{18} 150 mm × 4.6 mm, 5 μm particle size	172.2	98:2	1.0	39.8	1.10	33124
C_{18} 150 mm × 2.1 mm, 3 μm particle size	17.2	98:2	0.3	7.3	1.15	256
C_{18} 50 mm × 2.1 mm, 3.5 μm particle size	1.7	98:2	0.3	4.8	1.04	1422

[a]Calculated according to USP 32 (16).
[b]Number of theoretical plates.

0.03 μM. As a result of the 1.5-fold sample concentration, it was possible to quantitate amounts of the analyte in sample down to 0.07 μM and detect as low as 0.02 μM of CoQ10 in a real plasma sample.

The intra- and inter-day precision of the results obtained for analysis of CoQ10 in plasma was tested by analyzing three concentration levels of the calibration curve at low (0.1 μM), middle (1.2 μM), and high level (6.0 μM) of the analyte.

TABLE 2 Parameters of Validation of the Analytical Method for Determination of CoQ10 in Plasma

Parameter			
Linear range (μM)	0.1–15.0		
Calibration curve	$Y = 5.61\ x + 0.84$ ($Sy/x = 0.17$, $S_{intercept} = 0.13$, $S_{slope} = 0.05$)		
$r^{2\ a}$	0.997		
LOD (μM)[b]	0.03		
LOQ (μM)[b]	0.1		
	Spiked levels		
	Low (0.1 μM)	Middle (1.2 μM)	Upper (6.0 μM)
Precision (RSD)[c]			
Intra-day (n = 3)	5.7	4.0	5.2
Inter-day (n = 9)	6.3	4.3	5.6
Accuracy[d]			
Recovery %	89.0 (3.6)	95.3 (3.1)	94.0 (4.0)

[a]The mean calibration curves were obtained on three different days with triplicate at five different levels.
[b]LOD and LOQ values correspond to a detection and quantitation of 0.02 μM and 0.07 μM of CoQ10 without concentration, respectively.
[c]RSD values of normalized areas are the averages from three concentration levels of the calibration curves.
[d]Recovery mean values obtained from three individual samples on three different days at three concentration levels. RSD values in parenthesis.

Accuracy was evaluated from recovery studies of CoQ10 in plasma samples spiked at three concentration levels. The CoQ10 recoveries attained in biological samples ranged between 89.0% and 95.3% with acceptable relative standard deviation (RSD) values (Table 2).

Certain evidences exist about the presence of low endogenous levels of CoQ9 in human plasma that cause interference.[3] We quantitated CoQ10 in the plasma sample by the standard addition method with the incorporation of CoQ9 in the real samples. We spiked plasma samples with a final concentration of 3.0 μM of CoQ9. Finally, the plasma was extracted as described previously and analyzed. The CoQ10:CoQ9 peak area ratio in the sample was calculated from interpolation in the calibration curve constructed with spiked amounts of CoQ10 in the range between 0.1 and 15.0 μM to fixed amounts of 3.0 μM of CoQ9. Comparing concentration levels of CoQ10 in the analyzed samples we cannot find any differences using both quantitative methods. It was also showed that, at least in the studied specimens, there is no evidence of endogenous detectable amounts of CoQ9 using this method. As a consequence, we decided to use calibration curves for quantitation in further analyses.

APPLICATION TO REAL SAMPLES

CoQ10 range is between 0.4 and 1.4 μM in healthy subjects,[3,4] and it is expected to observe lower values in pathological conditions. For this reason, it is important to develop an analytical procedure capable of quantitating the lowest concentrations as far as it is possible. In our case, it was possible to determine levels of CoQ10 in plasma samples as low as 0.07 μM.

Our results showed that plasmatic CoQ10 in healthy subjects were in agreement with those reported in literature. The results of CoQ10 obtained in those people ($n = 10$) together with its quantitation in patients with mitochondrial disease are shown in Table 3.

In Figure 2, the chromatograms of CoQ10 in a patient with mitochondrial disease and in a CoQ10 standard are shown for comparison.

TABLE 3 Mean Values of CoQ10 in Plasma Samples from Healthy Subjects and Patients

Samples	CoQ10 in plasma (μM ± SD[a])
Healthy subjects ($n = 10$)	0.63 ± 0.35
Patients with mitochondrial disease treated with CoQ10 ($n = 4$)	1.3 ± 1.0
Patients with mitochondrial disease without treatment ($n = 10$)	0.25 ± 0.13

[a]Standard deviation.

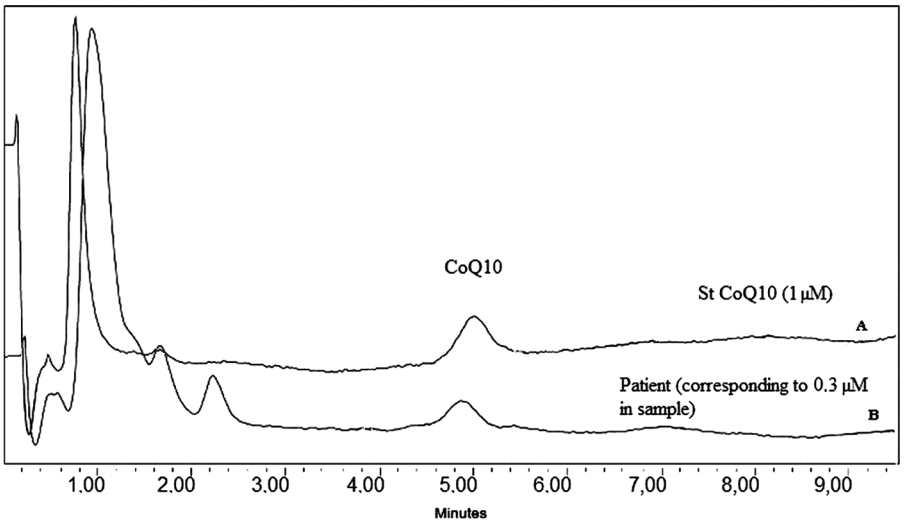

FIGURE 2 A: Chromatogram of CoQ10 standard (1.0 µM); B: Chromatogram of CoQ10 in plasma of a patient with mitochondrial disease without treatment (0.2 µM). Chromatographic conditions: XTerra C18 microcolumn (50 mm × 2.1 mm i.d., 3.5 µm particle size). Mobile phase: methanol:water (98:2, v/v), flow rate: 0.3 mL/min., UV detection at 275 nm, injection volume: 2 µL.

CONCLUSIONS

A simple and highly sensitive method using fast microbore HPLC with UV detection has been developed for the CoQ10 quantitation in human plasma. The proposed method allows shorter analysis time, less organic solvent consumption, and low LOD and LOQ values close to those reported by HPLC-EC method with the employment of affordable instrumentation. Additionally, the method requires small sample volumes and makes internal standard quantitation unnecessary. This method is suitable for routine CoQ10 determination, especially in clinical chemistry laboratories.

ACKNOWLEDGMENTS

The authors wish to thank to Dr. Cecilia Dobrecky for assistance.

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