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Received September 23, 2008
Revised January 9, 2009
Accepted January 14, 2009

Research Article

A capillary electrophoretic system based on a novel microemulsion for the analysis of coenzyme Q10 in human plasma by electrokinetic chromatography

A new analytical method for determination of coenzyme Q10 (2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone, CoQ10) in human plasma was developed based on CE using a double tensioactive microemulsion. CoQ10 was quantitatively extracted into 1-propanol/hexane and quantified by MEEKC. The microemulsion was prepared by mixing 1.4% w/w sodium bis(2-ethylhexyl) sulfosuccinate, 4% w/w cholic acid, 1% w/w octane, 8.5% w/w butanol, 0.1% w/w PVA and 85% w/w 10 mM Tris buffer at pH 9.0. The optimized electrophoretic conditions included the use of an uncoated silica capillary of 60 cm total length and 75 μm id, an applied voltage of 20 kV, room temperature and 214 nm ultraviolet detection. Selectivity, linearity, LOD, LOQ, precision and accuracy were evaluated as the parameters of validation. Owing to its simplicity and reliability, the proposed method can be an advantageous alternative to the traditional methodology for the quantitation of CoQ10 in human plasma with good accuracy and precision.

Keywords:
CE / Coenzime Q10 / Human plasma / Microemulsion
DOI 10.1002/elps.200800783

1 Introduction

Coenzyme Q10 (2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone, CoQ10) (Fig. 1), a lipid-soluble compound that mainly locates in the mitochondria, plays an important role as an essential electron carrier in the electron transport chain. CoQ10 is known as a powerful antioxidant agent able to protect circulating lipoproteins and cell membranes against oxidative damage [1, 2].

Recent reports have suggested that CoQ10 levels may be lower in certain conditions such as Parkinson disease, alcoholism, thyroid diseases, myocardial diseases, hemodialysis, prematurity, liver diseases, hyperlipidemia, DNA damage and β-thalasemia. For these reasons, the determination of CoQ10 in plasma and the correlation of its levels with the age of patients and states of deficiency are very important to study, since CoQ10 has been recognized as a marker of oxidative stress and antioxidant agent [1, 3].

The amount of CoQ10 found in plasma for healthy people is in the range of 0.4–2 μg/mL [4]. The low concentrations of CoQ10 in plasma, the complexity of this matrix and two molecular properties necessary for CoQ10 function (the high hydrophobicity of the polyprenyl side chain and the easy oxidation of the benzoquinone ring) make the analysis of CoQ10 technically challenging. Many analytical procedures have been reported to quantitate CoQ10 in plasma: HPLC with ultraviolet (UV) or electrochemical detection (ECD) [1, 3, 5–13], HPLC coupled to mass spectrometry [4], and also voltammetric [14], chemioluminiscent [15], fluorimetric [16] and spectrophotometric methods [17]. These procedures usually require pretreatment of the sample before the analysis including precipitation of proteins followed by TLC, SPE and/or liquid–liquid extraction with different solvents [18].

Liquid chromatographic method with ECD (HPLC-ECD) probably seems to be the most common for this purpose because of its high sensitivity; however, this methodology is complicated, time-consuming and it is rather used in the clinical laboratory. CE with its relevant features of performance, such as simplicity, very high resolution in short times of analysis and low cost of operation, has become an alternative methodology to the analysis of different compounds of biological interest [19–23]. In the last few years, EKC systems have emerged as a very attractive separation method in which the selectivity is accomplished by the partition of the analytes between a mobile

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Abbreviations: AOT, sodium bis(2-ethylhexyl) sulfosuccinate; **CA,** cholic acid; **CoQ10,** 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone, coenzyme Q10; **ECD,** electrochemical detection; **UV,** ultraviolet

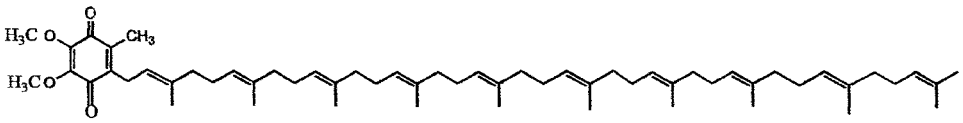

Figure 1. Chemical structure of Coenzime Q10.

phase and a pseudostationary phase together with their electrophoretic mobilities [24–26]. In these systems, the mobile phase is normally an aqueous buffer and the pseudostationary phase may be micelles (MEKC), vesicles (VEKC) or microdroplets (MEEKC) if a microemulsion is implemented.

Microemulsions are dispersed systems consisting of nanometer size droplets of an inmiscible liquid, stabilized by surfactant and co-surfactant molecules [27]. Microemulsions can be prepared as water droplets in an oil phase (W/O) or oil droplets in a water phase (O/W). Owing to many potential advantages of microemulsions such as high stability, ability to interact with a wide range of hydrophobic and hydrophilic compounds, transparency and easy preparation, they have been used as a new EKC system with unique selectivity [28–30].

Traditional MEEKC systems employ SDS, a single-chain structure tensioactive, as the surfactant. However, resolution of some hydrophobic compounds using only SDS as the surfactant can be unsuccessful [31]. In a previous paper, we have developed a novel microemulsion system based on sodium bis(2-ethylhexyl) sulfosuccinate (AOT), a double chain, anionic and hydrophobic surfactant, allowing the separation of estrogens with different hydrophobicities in a single run [32]. Bustamante-Rangel *et al.* have used AOT in an MEEKC system to separate α- and δ-tocopherol [33], with log *P* similar to CoQ10 value [34], from other lipophilic vitamins. We have also reported the development of microemulsions with biosurfactants as phosphatidylcholine and bile acids as sodium cholate and/or sodium deoxycholate [35, 36]. The use of a double tensioactive achieved better selectivity, especially for hydrophobic compounds, compared with the traditional MEEKC systems using solely SDS as the surfactant [32].

Determination of CoQ10 in human plasma by MEEKC showed to be an attractive alternative to the established chromatographic methods. To our knowledge, CE analysis of CoQ10 has not previously been published.

In this paper we report the first microemulsion capillary electrophoretic method based on the employment of AOT and bile acid as tensioactives after 1-propanol/hexane extraction procedure for the determination of CoQ10 in human plasma. Our results have also been compared with the traditional EKC systems (MEKC-SDS and MEEKC-SDS), MEEKC system with AOT as the unique tensioactive (MEEKC-AOT) and a standard HPLC method with UV detection [7].

2 Materials and methods

2.1 Instrumentation

All CE separations were performed using a capillary ion analyzer (Waters, Milford, MA, USA) and data were processed by an Empower Pro software (Waters). An uncoated fused-silica capillary of 60 cm length (53 cm to the detector) and 75 μm id (MicroSolv Technology, Eatontown, NJ, USA) was employed.

A diode array spectrophotometer (Agilent 8452) was used to determine the accurate concentration of CoQ10 in standard solutions.

2.2 Reagents

CoQ10, AOT, cholic acid (CA), PVA and all standards tested for potential interferences: estradiol, estriol, estrone, progesterone, cortisol, testosterone, thyroxine, cholesterol, triglycerides, DL-α-phosphatidylcholine dipalmitoyl (synthetic, 99%), α-tocopherol, vitamin K1 were purchased from Sigma (St. Louis, MO, USA). Epicuron 200 (lecitine 95%) was a gift from Degussa Health & Nutrition (Freising, Germany). Tris(hydroxymethyl)-aminomethan, *n*-octane, 1-butanol, 1-propanol and ethanol (HPLC grade) were supplied by E. Merck (Darmstadt, Germany). *N*-hexane was purchased from Carlo Erba. Ultrapure water was obtained from an EASY pure™ RF equipment (Barnstead, USA). All solutions were filtered through a 0.45 μm nylon membrane (Micron Separations, USA) and degassed before use.

2.3 Electrophoretic system

The separations were performed using an oil-in-water microemulsion system consisting of 1.4% w/w AOT, 4% w/w CA, 1% w/w octane, 8.5% w/w 1-butanol, 0.1% w/w PVA and 85% w/w 10 mM Tris buffer at pH 9, as the background electrolyte. The electrophoretic conditions were hydrostatic injection (10 cm height) for 20 s, a positive voltage of 20 kV and UV detection at 214 nm at room temperature. A traditional MEEKC system (MEEKC-SDS) consisting of 1.44% w/w SDS, 0.81% w/w octane, 6.61% w/w 1-butanol and 91.14% w/w 20 mM sodium phosphate buffer from pH 7 to 9 was used. MEEKC-AOT system was prepared by mixing 1.4% w/w AOT, 1% w/w octane, 8.5% w/w 1-butanol and 89.1% w/w 10 mM Tris buffer at pH 9. An MEKC system with SDS dissolved in 20 mM sodium phosphate buffer at pH 9 (MEKC-SDS) was also prepared in the electrophoretic system for comparison of methods.

2.4 Conditioning of the capillary

The capillary was rinsed at the beginning of each day with 0.1 M potassium hydroxide for 5 min, washed with water for

10 min and then filled with the microemulsion for 20 min. Between runs, the capillary was conditioned during 1 min with 0.1 M potassium hydroxide, 2 min with water and 3 min with the background electrolyte. To assure the highest intra- and inter-day precision, at the end of the day, the capillary was flushed with air, rinsed with ethanol, flushed with air again, then with 0.1 M potassium hydroxide for 5 min and finally with water for 10 min.

2.5 Microemulsion preparation

Amounts of AOT, octane and 1-butanol cited in Section 2.3 were mechanically stirred until dissolution. CA, PVA and Tris buffer were prepared aside and each one was slowly added to AOT solution in order to finally obtain a clear system. The microemulsion obtained was let to stand for 10 min at room temperature before use.

2.6 Stock and standard solutions

Stock solution of CoQ10 containing 0.5 mg/mL was prepared in ethanol, protected from light and stored at −20°C till 2 months. The working standard solution (20 μg/mL) was obtained from the stock solution by dilution with 10 mM Tris buffer at pH 9 containing 15% ethanol. Before analysis the accurate concentration of CoQ10 in the working standard solution was obtained by spectrophotometry at 275 nm, $\varepsilon = 14\,020$ L/mol cm. The working standard solution was used to check the conditions of the electrophoretic system.

2.7 Sample preparation

All heparined 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 −80°C.

Before the analysis the samples were allowed to thaw at room temperature. Firstly, 600 μL of plasma were supplemented with 1200 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 1.0 mL of *n*-hexane was added. The mixture was vortexed for 5 min and centrifuged at 9000*g* for 10 min at 4°C. Next, the clear hexane layer was transferred to another tube and the procedure was repeated twice. The hexane layers were combined and evaporated to dryness under a stream of nitrogen. The dry residue was dissolved in 60 μL of 10 mM Tris buffer at pH 9 containing 15% ethanol and then injected into the capillary electrophoretic equipment. A tenfold enrichment of CoQ10 in the plasma samples was finally obtained.

2.8 Quantification and validation methods

The quantification of CoQ10 was performed using calibration curves. Calibration curves were obtained using a pool plasma sample without detectable amounts of CoQ10 and processed as described above. The dry residue was dissolved in Tris buffer with 15% ethanol spiked with CoQ10 standard to a final concentration of 2, 5, 10, 20 and 40 μg/mL, which correspond to 0.2, 0.5, 1.0, 2.0 and 4.0 μg/mL of CoQ10 in plasma, respectively.

Validation was performed according to the international guidelines [37, 38] (http://www.ich.org). 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. These three concentrations correspond to the upper limit, lower limit and the middle point of the calibration curve.

LOD and LOQ were calculated on the basis of the signal-to-noise evaluation. The LOQ was taken as the minimum amount of the analyte that provides precise measurements. The LOQ in plasma was defined as the sample concentration giving a peak height ten times the level of the baseline noise. The LOD was defined as the sample concentration generating a peak of height three times the level of the baseline noise.

The selectivity was tested by analyzing potential interferences and the procedure described above was followed.

2.9 Analysis of samples

Quantitation of CoQ10 in plasma taken from five healthy people (two females and three males, 23–35 years old) was analyzed and the results were compared with those obtained by the HPLC standard method.

3 Results and discussion

3.1 Surfactant types and concentration

Although traditional MEKC and MEEKC with SDS systems can resolve many of hydrophobic compounds, CoQ10, an extremely hydrophobic molecule (log *P*>10) [39] cannot be resolved from the plasma components with SDS as the only surfactant (Figs. 2A–C). Similarly, a microemulsion based on solely AOT could not resolve CoQ10 from the other components in a plasma sample (Figs. 2D and E). However, a microemulsion based on a double surfactant, such as AOT and CA in pair, allowed, for the first time, the separation of CoQ10 from the components of a normal plasma with good resolution by CE analysis (Figs. 2F and G). The optimal concentration of AOT was 1.4% w/w and 4% CA for complete resolution and adequate stability of the microemulsion. Longer migration time of the analytes was obtained at higher concentrations of AOT and CA but resolution was poorer at CA concentrations lower than 4%.

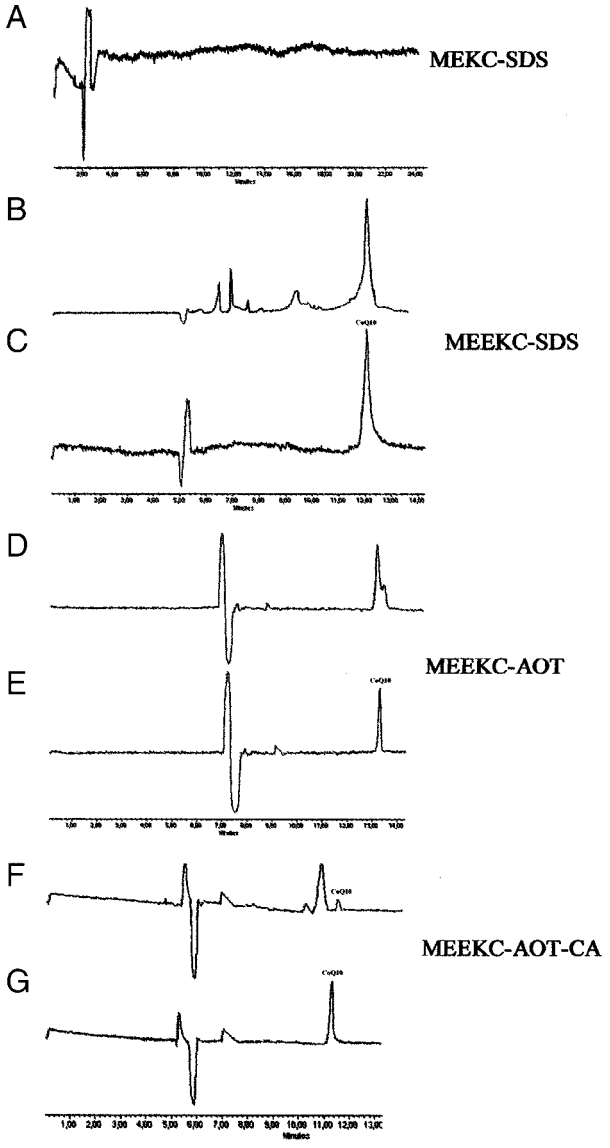

Figure 2. Electropherograms of (A) CoQ10 standard by MEKC-SDS, (B) plasma blank spiked with CoQ10 standard by MEEKC-SDS, (C) CoQ10 standard by MEEKC-SDS, (D) plasma blank spiked with CoQ10 standard by MEEKC-AOT, (E) CoQ10 standard by MEEKC-AOT, (F) plasma blank spiked with CoQ10 standard by MEEKC-AOT-CA and (G) CoQ10 standard by MEEKC-AOT-CA.

3.2 Oil, co-surfactant and additives

Several oils and co-surfactants were evaluated during the development of the microemulsion system. Octane was the most adequate component of the system able to produce the oil droplet and the most stable microemulsion.

Butanol showed to be the most adequate co-surfactant to stabilize the microemulsion but high concentrations of this component not only produced higher migration times but also disturbed the peak shapes.

Addition of PVA as an additive was also evaluated. The best peak shape and stability of baseline were observed at 0.1% PVA but at higher PVA concentrations the UV signal was reduced.

3.3 Buffer type, concentration and pH

Buffer type was evaluated using phosphate buffer between 10–30 mM and Tris buffer (5–20 mM) in the range of pH from 7 to 9. The results showed that the employement of Tris buffer allowed good capacity buffer, better peak shape and resolution from the unknown peaks together with higher stabilization of the microemulsion system in comparison with phosphate buffer. The optimum concentration of Tris buffer was found to be 10 mM. The influence of pH was evaluated in the range from pH 7 to 10, and the best pH value to produce baseline separation of the analytes was achieved at pH 9.0.

3.4 Sample diluent and injection times

Dilutions of CoQ10 standard and sample solutions of CoQ10 were prepared in 10 mM Tris buffer and addition of different percentages of ethanol was proven. It was observed that 15% of ethanol showed an appropriate solubilization of the analyte and good peak shapes.

Injection times from 10 to 30 s were evaluated. The best time for injection in order to obtain good peak shape and resolution was 20 s.

3.5 Voltage and temperature

Although temperature variations did not change resolution of CoQ10 from the unknown peaks, at low temperatures the migration times of the analytes became longer. From these results, room temperature was chosen for analysis.

A capillary of 60 cm length and 75 μm id at 20 kV of voltage was employed because good resolution together with adequate intensity of current could be achieved during the run.

3.6 Sample preparation

CoQ10, a lipophilic molecule, is always associated with lipoproteins and other lipophilic compounds at low concentration levels in plasma. For this reason a sample pretreatment and preconcentration steps are needed in order to reach good selectivity and sensitivity of the method.

Pretreatment of sample was found necessary because some components of the plasma interfere in the quantitation of CoQ10 and also reduce the capillary lifetime. Traditional sample preparation methods for determination of CoQ10 in plasma were generally based on protein precipitation in alcohol before liquid–liquid extraction. Other authors mentioned SPE as an alternative method for the clean-up of samples [5].

Pretreatment with C18 and silica columns were separately and sequently assayed in the same procedure. However, these methods needed more manipulation and cost and did not improve the CoQ10 recovery respect to liquid–liquid extraction. Different combinations of sample pretreatment procedures were tested and the results of CoQ10 recoveries are given in Table 1.

We found that protein precipitation with cold 1-propanol followed by *n*-hexane extraction was the best method of prepurification. To increase sensitivity and to improve the sample preparation before injection it was necessary to evaporate hexane to dryness and finally to dissolve the residue in 60 μL of 10 mM Tris buffer-15% ethanol. A tenfold concentration was achieved following the above-mentioned procedure.

We also tested different conditions for redissolution of the residue. Reconstitution in microemulsion, buffer and buffer with different percentages of ethanol added (5, 10, 15 and 20%) was assayed. The better peak shape was obtained when the residue was redissolved in buffer mixed with 15% of ethanol.

3.7 Validation

After optimization of the method, the validation procedure was accomplished according to the bioanalytical environment.

Selectivity, linearity, LOD and LOQ, precision and accuracy were chosen as the parameters for the validation of the procedure developed.

Selectivity of the method was verified by evaluating different potential endogenous interferences as estradiol, estriol, estrone, progesterone, cortisol, testosterone, thyroxine, cholesterol, triglycerides, lecitine and α-tocopherol. In addition, other lipophilic compounds, as synthetic DL-α-phosphatidylcholine dipalmitoyl and vitamin K1, were also employed. Selectivity of the method was demonstrated by calculation of mobilities and retention factors of the potential interferences according to Lucangioli *et al.* [35, 36] (Table 2). The mobilities of EOF and the microdroplet were measured using ethanol and vitamin K1 as markers, respectively. Vitamin K1 was chosen as the microdroplet marker because it was the analyte fully retained in assays [40]. None of them produced interferences during the analysis of CoQ10 (Table 2 and Fig. 3). These k'-values demonstrated that MEEKC-AOT-CA system is selective for lipophilic compounds.

Linearity was studied at five different concentration levels according to point 2.8. The average correlation coefficient was 0.9945. The mean calibration curves (inter-day precision) were obtained in three different days with triplicates at five different levels. LOQ and LOD were calculated on the bases of a peak height 10 and three times the level of the baseline noise, respectively (Table 3). The precision of CoQ10 assay in plasma was tested by analyzing at three different concentration levels of the analyte in the calibration curve: low (1.5 μg/mL), middle (3.0 μg/mL) and upper level (10.0 μg/mL).

Table 1. Sample preparation methods using different solvents

Solvent (s)	Recovery (%)
1-Propanol	57.0
Methanol	15.0
2-Propanol	60.0
Ethanol	20.0
1-Propanol+SPE (C18)	41.5
Methanol+hexane	20.0
Methanol+hexane+SPE (silica)	68.0
Methanol+hexane+SPE (silica and C18)	63.4
Ethanol+chloroform	42.0
Ethanol+chloroform+hexane	60.0
1-Propanol+hexane	97.9

Table 2. Electrophoretic mobilities and retention factors (k') for CoQ10 and potential interferences in a plasma matrix[a)]

Compound	μ_{eff}[b)]	k'
Estriol	−17.03	1.7
Testosterone	−17.33	1.8
Cortisol	−17.63	1.9
Estradiol	−18.20	2.1
Progesterone	−18.47	2.2
Estrone	−18.74	2.3
Cholesterol	−19.01	2.4
Triglycerides	−19.78	2.7
Lecitine	−20.73	3.3
Thyroxine	−20.95	3.4
CoQ10	−21.61	4.0
DL-α-Phosphatidylcholine dipalmitoyl	−22.23	4.6
α-Tocopherol	−23.01	5.7

a) Mobilities are expressed in $10^{-9}\,m^2\,V^{-1}\,s^{-1}$.
b) $\mu_{eff} = \mu_{meas} - \mu_{eof}$; μ_{eff} of vitamin K1: −27.03.

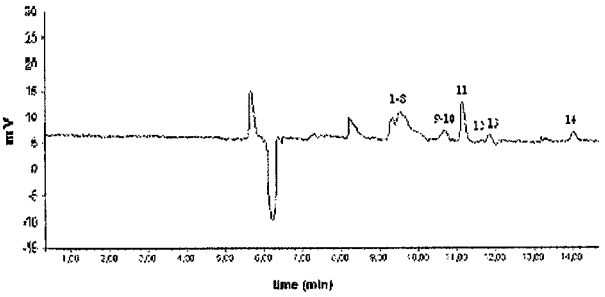

Figure 3. Electropherogram of CoQ10 together with the potential interferences in a plasma matrix: (1) estriol, (2) testosterone, (3) cortisol, (4) estradiol, (5) progesterone, (6) estrone, (7) cholesterol, (8) triglycerides, (9) lecitine, (10) thyroxine, (11) CoQ10, (12) DL-α-phosphatidylcholine dipalmitoyl, (13) α-tocopherol and (14) vitamin K1.

Accuracy was evaluated from recovery studies of CoQ10 in plasma samples spiked at three concentration levels. The CoQ10 recoveries attained in biological samples were between 96.3 and 99.0% with acceptable RSD values (Table 3).

3.8 Analysis of plasma samples and comparison with traditional HPLC method

CoQ10 values quantified in plasma samples using the developed MEEKC system were in agreement with those reported by the traditional HPLC system [7] and published reference values [1, 17, 18]. The results obtained are given in Table 4.

An electropherogram of a pool plasma without detectable amounts of CoQ10 used as blank (Fig. 4A) and an electropherogram of a plasma sample from a healthy subject (Fig. 4B) are shown for comparison.

Table 3. Parameters of validation of the analytical method for determination of CoQ10 in plasma

Parameter			
Linear range (μg/mL)	2–40		
Calibration curve	$y = 0.7x + 4.4$		
$r^{a)}$	0.9945		
LOD (μg/mL)[b)]	1		
LOQ (μg/mL)[b)]	3		
Precision (RSD)[c)]			
Spiked levels	Low	Middle	Upper
Intra-day ($n = 3$)	4.5	1.7	1.4
Inter-day ($n = 9$)	5.6	2.1	1.6
Accuracy[d)]			
Spiked levels	Low	Middle	Upper
Recovery%	98.5 (3.3)	96.3 (3.0)	99.0 (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 0.1 and 0.3 μg/mL of CoQ10 without concen tration, 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 are given in parenthesis.

Table 4. Comparison of the mean values of CoQ10 in plasma samples from healthy subjects determined by MEEKC and HPLC methods

Mean CoQ10 value in plasma samples ($n = 5$)	MEEKC (μg/mL)	HPLC (μg/mL)	$r^{a)}$
	1.11 ± 0.47	0.99 ± 0.50	0.9938

a) Correlation coefficient between results performed by MEEKC and HPLC methods.

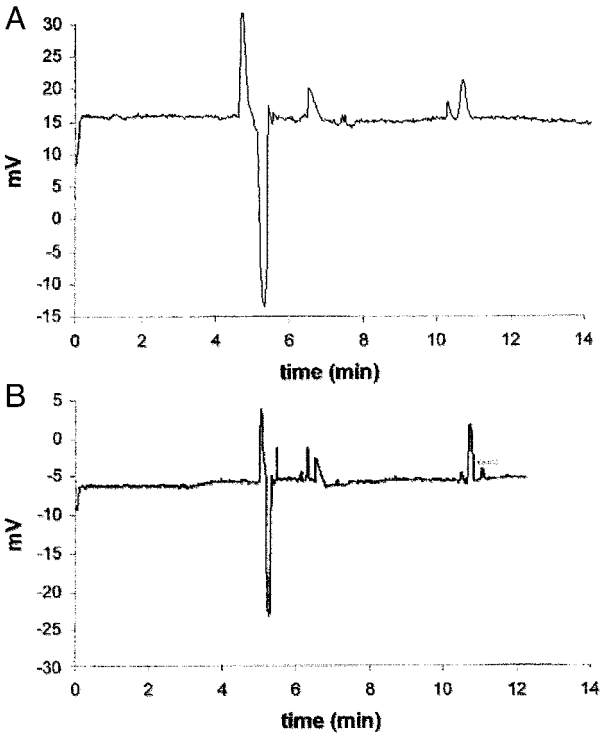

Figure 4. (A) Electropherogram of a pool plasma sample without detectable amounts of CoQ10 (blank). Experimental conditions are described in the text. (B) Electropherogram of a plasma sample of a healthy subject (0.56 μg/mL of CoQ10). Experimental conditions are described in the text.

4 Concluding remarks

A new microemulsion system based on the employment of two combined surfactant agents such as AOT and CA resulted to be a simple and suitable analytical method for the separation of CoQ10 from the plasma components. A novel capillary electrophoretic method is proposed for rapid quantitation of CoQ10 in plasma samples with good precision and accuracy. Determinations of CoQ10 are foreseen as practical applications in medical studies and clinical laboratory in the next future.

The authors thank Dr. Jorge Muse for assistance.

The authors have declared no conflict of interest.

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