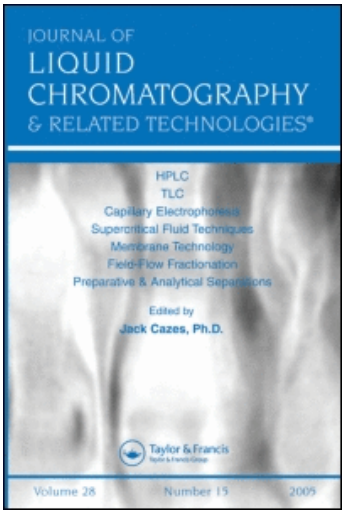

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Simple, Highly Sensitive Micro HPLC Method for the Determination of Coenzyme Q10 and its Major Related Substances

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Simple, Highly Sensitive Micro HPLC Method for the Determination of Coenzyme Q10 and its Major Related Substances

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Abstract: A simiple, rapid, and highly sensitive fast microbore HPLC method with UV detection has been developed for the quantitation of coenzyme Q10 (Co-Q10) and related substances in raw material, pharmaceutical, and cosmetic formulations using a column with hybrid particles. Chromatographic conditions were: 30°C column temperature, 1 μL injection volume at 275 nm. The Co-Q10 analysis was performed within 2.5 minutes with a flow rate of 0.3 mL/min. with a mobile phase consisted of 100% methanol, and the related substances were separated with a gradient elution based on methanol-water. The developed method was validated for selectivity, linearity, precision, accuracy, and robutness. The method was found to be suitable for the quality control of Co-Q10 in pharmaceutical and cosmetic products, as well as the stability indicating studies.

Keywords: Coenzyme Q10, Fast microbore column, Pharmaceutical analysis, Related substances, UV detection

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INTRODUCTION

Coenzyme Q10 (2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone), (Co-Q10) (Figure 1), also known as ubiquinone, is a lipophilic molecule classified as a fat soluble quinone. Co-Q10 is a component of the mitochondrial respiratory chain where it acts controlling the efficiency of oxidative phosphorilation and being essential for the production of cellular energy. Due to its hydrophobicity Co-Q10 is inserted into the phospholipid of the mitochondrial inner membrane. Moreover, Co-Q10 is also considered an antioxidant agent together with other lipophilic antioxidants and plays an intrinsic role in protecting circulating lipoproteins against oxidative damage. Therefore, its concentration may be a useful marker of oxidative stress and antioxidant defense and in those cases of muscular and miocardial diseases in which low levels of Co-Q10 could be found.[1,2]

For these reasons, a method for not only Co-Q10 determination in plasma but also quality control of Co-Q10 in therapy by oral administration and in cosmetic creams is important to develop.

Many analytical methods for Co-Q10 quantitative determination in human plasma have been reported in literature.[3–8] Up to this date, Co-Q10 determinations in biological fluids have been performed by

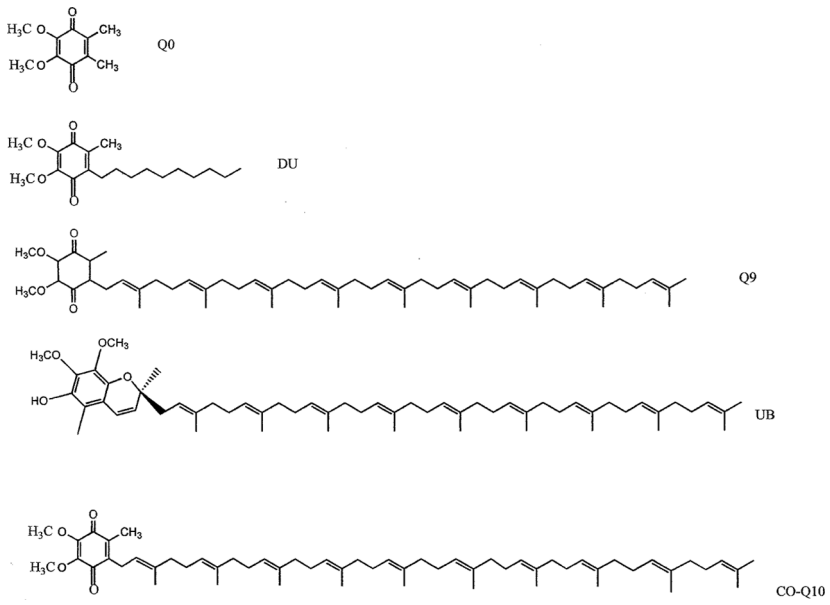

Figure 1. Chemical structure of Co-Q10 and related substances.

liquid-liquid or solid phase extraction before liquid chromatography with electrochemical detection and, in some cases, using UV detection. All these procedures are too time consuming or require the followup of several steps during the operation of the instruments, which make them unsuitable for routine determinations.

Co-Q10 analysis in lipophilic formulations based on soybean oil has been reported.[9] Non-aqueous reversed phase liquid chromatography with a conventional column was used to resolve Co-Q10 and quinones with very close hydrophobicity. However, as far as we know, no HPLC method for analysis of Co-Q10 in pharmaceutical and cosmetic formulations and the separation of its major related substances using a fast and microbore column with UV detection has been reported.

HPLC with UV detection is one of the most common instrumentations employed in laboratory because, in general, it is not necessary to apply sophisticated techniques for assembly.

Moreover, the need for speed, high resolution, sensitivity, and robustness in pharmaceutical and bioanalysis has prompted extensive research in the miniaturization of HPLC columns. Fast HPLC commonly use short columns, 2–5 cm in length with a conventional internal diameter (i.d.) of 4.6 mm and microbore HPLC use columns of 2 or 1 mm i.d. Recently, as a result of employing fast and microbore HPLC systems, analysis time was decreased together with solvent and mobile phase use and dramatic reductions of sample requirements with faster separation compared to those columns of conventional diameter and length.[10–13]

In this work, we propose a new simple method for the analysis of Co-Q10 using a fast and microbore HPLC column combined with a special hybrid particle packing in order to obtain high efficiency in separation with symmetric peaks and better resolution of compounds of different hydrophobicity, and it can be applied to the determination of Co-Q10 in pharmaceutical and cosmetic formulations. In addition, the employment of a gradient system allows separation of Co-Q10 from its related susbtances with different hydrophobicity in a single run.

EXPERIMENTAL

Chemicals and Reagents

Coenzyme Q10 (2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone) (Co-Q10), coenzyme Q9 (2,3-dimethoxy-5-methyl-6-nonaprenyl-1,4-benzoquinone), coenzyme Q0 (2,3-dimetoxy-5-methyl-1,4-benzoquinone) (Co-Q0), decylubiquinone (2,3-dimetoxy-5-methyl-6-decyl-1,4-benzoquinone) (DU) were purchased from Sigma (St. Louis, MO, USA). Ubicromenol (UB) was obtained according to reference [9]. Methanol (HPLC

grade), ethanol (gradient grade), sodium hydroxide, hydrochloric acid and 30% hydrogen peroxide were supplied by E. Merck (Darmstadt, Germany). Ultrapure water was obtained by an EASY pure^{TM} 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.

Equipment

The HPLC system 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).

Chromatographic Conditions

Separation was achieved using a XTerra analytical microcolumn (50 mm × 2.1 mm i.d., 3.5 μm particle size). The column temperature was kept at 30°C. The isocratic mobile phase consisted of 100% methanol and the flow rate was 0.3 mL/min. Gradient conditions based on water as phase A and methanol as phase B (time program = 0 min, 70%A/30%B; 2 min, 60%A/40%B, 4 min, 40%A/60%B, 5 min 30%A/70%B, 6 min 20%A/80%B, 7 min 10%A/90%B, 8 min 0%A/100%B), and flow rate 0.3 mL/min were selected to separate Co-Q10 from its related substances. UV-detection was performed at 275 nm. The injection volume was 1 μL. A run time of 2.5 min was employed for determination of Co-Q10 in pharmaceutical dosage forms and a time of 30 min was necessary to completely separate Co-Q10 from its major related substances.

Preparation of Standard Solutions

Standard Solution of Co-Q10

A stock solution of Co-Q10 containing 1.0 mg/mL was prepared in ethanol. The standard solution of 0.6 μg/mL was obtained by appropriate dilution with methanol:water (95:5).

Standard Solution of Co-Q10 and Related Substances

Working solutions of Co-Q10, Co-Q9, UB, DU, and Co-Q0 containing 1 mg/mL of each one were prepared in ethanol. Standard solutions of

Co-Q10 (240 μg/mL), Co-Q9 (50 μg/mL), ubicromenol (80 μg/mL), DU (20 μg/mL), and Co-Q0 (2 μg/mL), respectively, were prepared by appropriate dilution with methanol:water (95:5).

Sample Preparation

The contents of Co-Q10 in pharmaceutical formulation, tablets (10 mg/dose) and cosmetic cream (50 mg/100 g) were assayed. Twenty tablets were weighed, finely powdered, and an amount equivalent to one tablet was accurately weighed. An amount of 2.5 g of cream was accurately weighed. In both cases, 50 mL of ethanol was added and sonicated for 10 min. An adequate portion was centrifuged at 4000 rpm for 10 min. and an aliquot taken from the supernatant was appropriate diluted with methanol:water (95:5).

Stress Conditions

The stock solutions of Co-Q10 (2 mg/mL) in ethanol were taken to different stress conditions. Oxidation: 1 mL of Co-Q10 stock solution was added to 1 mL of a 3% hydrogen peroxide solution. Acidic: 10 mL Co-Q10 stock solution and 10 mL 0.5 M hydrochloric acid were refluxed during 1 h. Alkaline: similar conditions to the method described under ''acidic'', using 0.5 M sodium hydroxide. Light: 1 mL Co-Q10 stock solution was exposed to white light during one week. All samples were diluted with methanol:water (95:5) to obtain 300 μg/mL of Co-Q10 prior to analysis. After degradation, samples were analyzed using isocratic and gradient chromatographic systems.

RESULTS AND DISCUSSION

Fast Microbore HPLC-UV Method Development

Quantitation of Co-Q10 in Pharmaceutical Products

Commonly, the methods for determination of Co-Q10 in plasma or biological tissues were HPLC with electrochemical detector (ECD) and they are chosen for their high selectivity and sensitivity. However, a long time is necessary to obtain a stabilized baseline before analysis, and due to the ECD is sensitive to high concentrations of some lipophilic components that could be present in the sample, it probably could passivate the electrodes and shorten their lifetime.[14]

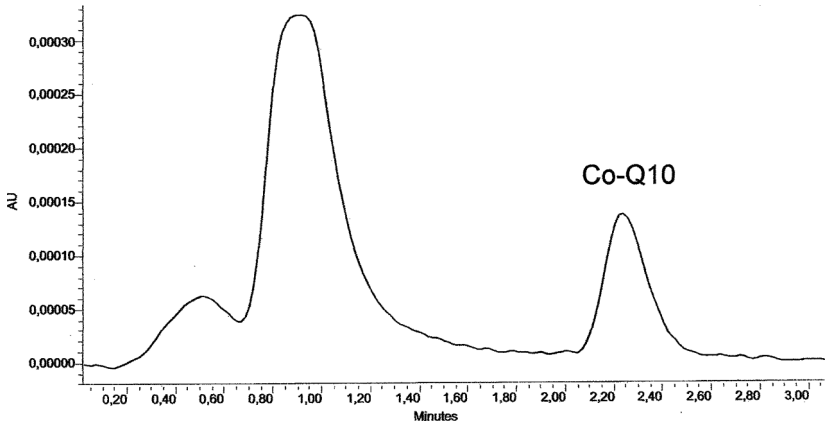

Figure 2. Representative chromatogram of Coenzyme Q10 (0.6 μg/mL). Experimental conditions are given in the text.

To achieve rapid analysis with high sensitivity, miniaturization of the column is a good alternative. The use of a short column with reduced diameters such as 5 cm and 2.1 mm (i.d.) allows the quantitation of Co-Q10 in a short time with less consumption of solvent and sample. In addition, the UV detection makes the analysis simpler (Figure 2). The isocratic chromatographic system using 100% methanol as mobile phase was found to be suitable, and good results in terms of chromatographic parameters such as column efficiency (N: 1032/column), retention factors (k′: 3.5), and tailing factor (T: 1.05) were obtained. These parameters were calculated according to USP 29.[15]

Related Substances

Co-Q10 related substances such as coenzyme Q9 (Co-Q9), ubicromenol (UB), decylubiquinone (DU), and coenzyme Q0 (Co-Qo) are compounds with different hydrophobicity, but some of them are more hydrophilic compounds than Co-Q10. The choice of the column has a pronounced influence on the separation of Co-Q10 and its related substances due to their different chemical structures and hydrophobicity (Figure 1). The analytical column assayed contains particles in which one out of every three silanols is replaced with a methyl group during the synthesis. This hidrophobicity is distributed throughout the entere structure of the particle. The result is a hybrid particle that can be operated at high speeds, high temperatures, wide pH range, and permits obtaining exceptionally sharp and simetrical peaks with high efficiency for different hydrophobicity compounds. Moreover, this type of column allows generating faster gradients with respect to conventional columns, to

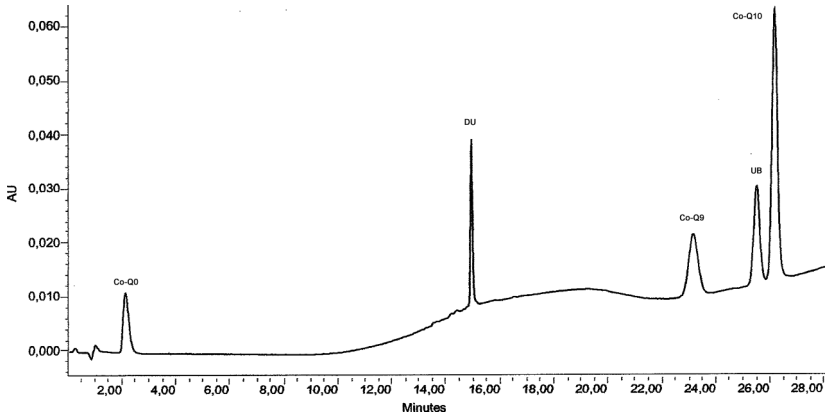

Figure 3. Chromatogram of Co-Q0 (2 μg/mL), DU (20 μg/mL), Co-Q9 (50 μg/mL), UB (80 μg/mL), and Co-Q10 (240 μg/mL). Experimental conditions are given in the text.

achieve a baseline separation of Co-Q10 and its related compounds within 30 minutes of run time (Figure 3). Chromatographic parameters are given in Table 1.

Validation

The validation of the developed LC method was accomplished following the International Conference on Harmonization (ICH) guidelines with respect to specificity, precision, linearity, limits of detection (LOD) and quantification (LOQ), accuracy, and robustness.[16]

Specificity

The stability indicating capability of the assay was examined by accelerated stress conditions of Co-Q10 (acid, alkaline, oxidation, and light) and

Table 1. Chromatographic parameters of Co-Q10 related compounds

Parameter[a]	Co-Q0	DU	Co-Q9	UB
k′	1.5	16.0	25.4	28.0
N	928	33705	35344	66564
T	1.05	1.04	1.07	1.01
Rs	24[b]	18[c]	16[d]	2[e]

[a]According to USP 29 (15); [b]Between Co-Q0 and DU; [c]between DU and Co-Q9; [d]between Co-Q9 and UB; [e]between UB and Co-Q10.

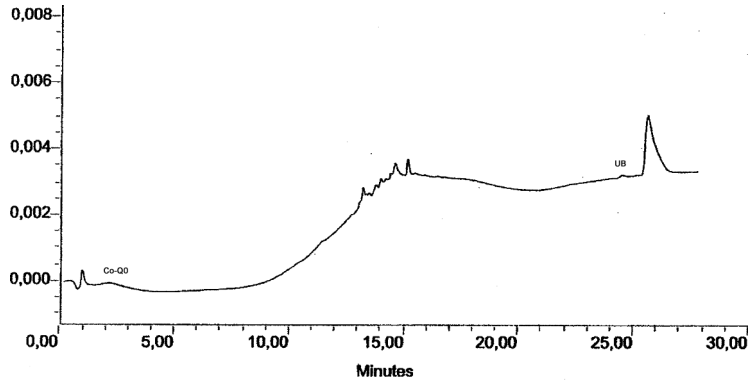

Figure 4. Chromatogram of Co-Q10 under acidic hydrolysis. Experimental conditions are given in the text.

specificity was also evaluated by comparing chromatograms of excipient blanks of each pharmaceutical dosage form. Degradation was observed when Co-Q10 was subjected to acidic, alkaline, and light, and the presence of unknown peaks in the chromatogram was evaluated (Figure 4). Exposure to oxidation conditions, however, resulted in the missing of the peak of Co-Q10.

These experiments indicate that the proposed method is convenient for screening of Co-Q10 related compounds with different hydrophobicity in raw material (Figure 5) and it can also be used in stability

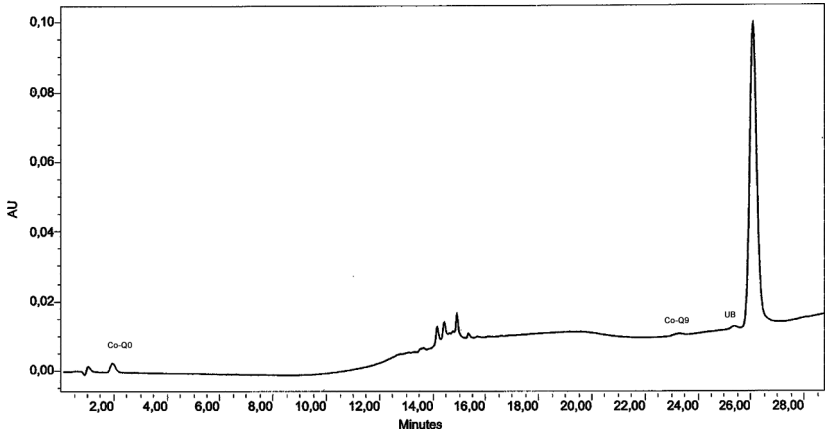

Figure 5. Chromatogram of Co-Q10 raw material (Co-Q10, 400 μg/mL and Co-Q0 1% w/w, Co-Q9 0.3% w/w, and UB 0.4% w/w).

Table 2. Figures of merit for analysis of Co-Q10 related compounds

Parameter	Co-Q0	DU	Co-Q9	UB
Precision %RDS				
Intra-day (n = 6)				
Migration time	1.1	0.5	0.8	1.0
Peak area	1.0	1.2	0.9	1.1
Inter-day (n = 18)				
Migration time	1.5	0.9	1.0	1.1
Peak area	1.5	1.5	1.0	1.1
Linear range [μg mL^{-1}]	0.2–50	1.0–100	0.8–100	1.0–100
r	0.9996	0.9997	0.9996	0.9995
LOD [μg mL^{-1}]	0.06	0.3	0.2	0.3
LOQ [μg mL^{-1}]	0.2	1.0	0.8	1.0

indicating studies. Figures of merit of the analysis of Co-Q10-related quinones are given in Table 2.

Linearity, LOD, and LOQ

Calibration curves at six concentration levels (0.15, 0.30, 0.60, 1.00, 1.50, and 2.00 μg/mL) of Co-Q10 were analysed in duplicate in three separate runs. The LOD (S/R = 3) and LOQ (S/R = 10) were 19 ng/mL and 65 ng/mL, respectively, comparable to conventional HPLC methods with electrochemical detection[5,14] (Table 3).

Precision and Accuracy

Precision was evaluated for intraday (n = 6) and interday assays (n = 18) and it was expressed as %RSD for retention times and areas (Table 3).

Accuracy was evaluated from recovery studies of samples of the Co-Q10 from their matrix. Placebo samples prepared with all the excipients contained in each one of the different pharmaceutical formulations at concentration levels of 80, 100, and 120% (w/w) of the nomimal values were spiked with the Co-Q10. Three replicate preparations of each level were assayed. Percentages of recovery values in the range between 98.9% and 101.5% were obtained (Table 3).

Robustness

In order to study the robustness, a Plackett-Burman design was employed. Seven parameters were selected: column temperature, flow rate, injection volume, detector wavelength, percentage of water in the

Table 3. Parameters of validation of method of analysis of Co-Q10

Parameter			
Linear range [μg mL^{-1}]	0.065 – 2.0 (y = 0.08 + 2.0X)		
r	0.9996		
LOD [ng mL^{-1}]	19		
LOQ [ng mL^{-1}]	65		
Precision %RDS			
Intra-day (n = 6)			
Migration time	0.3		
Peak area	1.0		
Inter-day (n = 18)			
Migration time	0.5		
Peak area	1.5		
Accuracy[a]			
Spiked levels	80%	100%	120%
Tablets	99.8 (0.5)	101.5 (0.3)	100.3 (0.7)
Cosmetic cream	100.1 (1.5)	98.9 (1.3)	99.7 (1.7)

[a]Recovery mean values obtained from three individual samples on three different days.
RSD values in parenthesis.

diluent solvent, time of use of the column, and time for sonication of the sample. The effect of each variable was investigated at two levels as indicated in Table 4. Each series in the design consisted of six replicate injections of Co-Q10 standard solution and three replicate injections of samples of pharmaceutical formulations following Youden and Steiner's experimental design is presented in Table 5.[17]

Table 4. Variables and their levels for robustness test

Selected variables[a]	Units	Abbreviation	High level	Low level
Column temperature	ºC	A,a	32	28
Flow	mL min^{-1}	B,b	0.32	0.28
Injection volume	μL	C,c	1.2	0.8
Time of use of the column	–	D,d	new	old
Time of sonicated of the sample	min	E,e	11	9
Wavelenghth	nm	F,f	277	273
Injection solvent (content of water)	%	G,g	10	2

[a]Upper and lower case letter represent high and low level of the variable, respectively.

Table 5. Robustness test design

	Experiment Number							
Variables	1	2	3	4	5	6	7	8
	A	A	A	A	a	a	a	a
	B	B	b	b	B	B	b	b
	C	c	C	c	C	c	C	c
	D	D	d	d	d	d	D	D
	E	e	E	e	e	E	e	E
	F	f	f	F	F	f	f	F
	G	g	g	G	g	G	G	g
Observed result	s	t	u	v	w	x	y	z

Experimental design according to reference 17.

The effects of variations in chromatographic parameters were evaluated using %RSD between replicate injections, k′, mean theorical plates (N), tailing factors (T), and content of Co-Q10 in the real samples (Table 6). Data and analyses obtained confirm the robustness of the analytical method for the experimental variations in the parameters included in the study.

Quantitative Analysis of Co-Q10 in Different Products

Quantitation of the Co-Q10 in pharmaceutical and cosmetic formulations was performed under the experimental conditions described above (Figure 6). The results obtained are in good agreement with the labeled values (Table 7).

Table 6. Deviations for each result obtained using Younder and Steiner's statistical method

	%RSD	N	k′	T	Content Co-Q10
DA	−0.30	−21.5	1.01	0.02	−0.08
DB	0.35	10.8	0.29	−0.07	−0.19
DC	−0.30	47.8	1.03	−0.06	−0.03
DD	0.00	20.9	−1.19	−0.08	0.03
DE	0.25	−24.4	0.62	0.03	−0.09
DF	0.10	5.5	−0.55	−0.04	−0.05
DG	−0.25	10.3	−0.62	−0.08	0.07
Ec[a]	1.00	82.5	2.94	0.17	0.27

[a]Critical statistic ($Ec = tc_{(p=0.05)} \sqrt{2}$ SD).

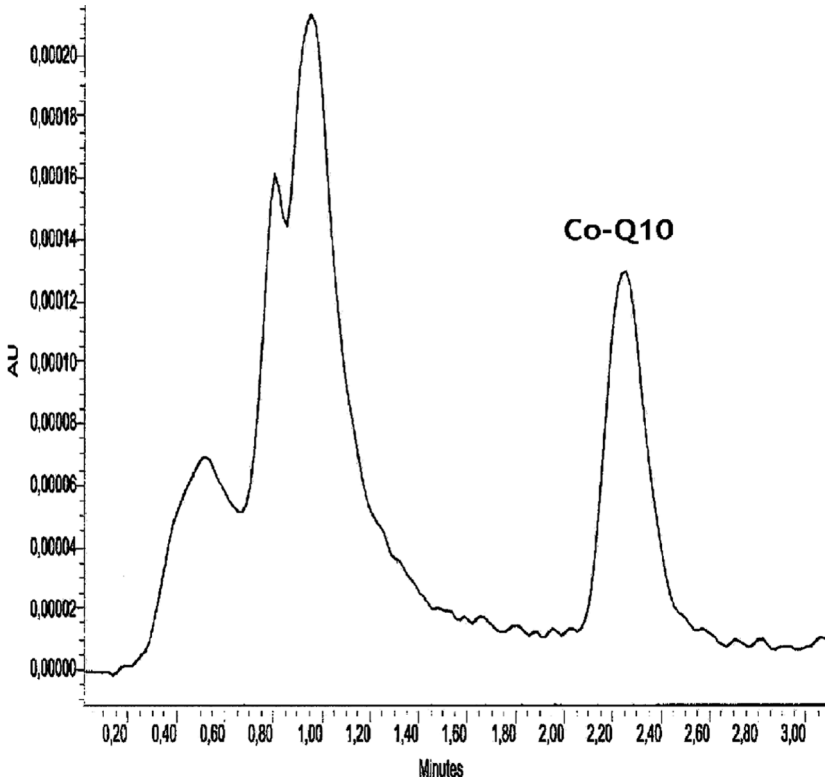

Figure 6. Chromatogram of Co-Q10 in tablet formulation. Experimental conditions given in the text.

Table 7. Analysis of Co-Q10 in pharmaceutical and cosmetic formulations

	Label	Found[a]
Tablet (mg/ dose)	10.0	10.7 (1.3)
Cosmetic cream (g/ 100 g)	0.050	0.045 (1.8)

[a]Results are expressed as mean values (n = 3), RSD values in parenthesis.

CONCLUSIONS

A simple, rapid, and sensitive fast microbore HPLC method with UV detection has been developed for the quantitation of Co-Q10 in real

samples using a column with hybrid particles. Moreover, with the same technology using a fast gradient system, the resolution of its major related substances was obtained. The content of Co-Q10 in pharmaceutical dosage form and cosmetic formulations was found to be appropriate for routine analysis. The advantages of the proposed method in comparison to the published method are shorter analysis time, less organic solvent used, the separation of related substances with very different hidrophobicity in a single run, and the lower LOD and LOQ obtained comparable to those reported by ECD, making this system suitable for the determination of Co-Q10 in biological fluids, as well as the stability indicating studies.

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