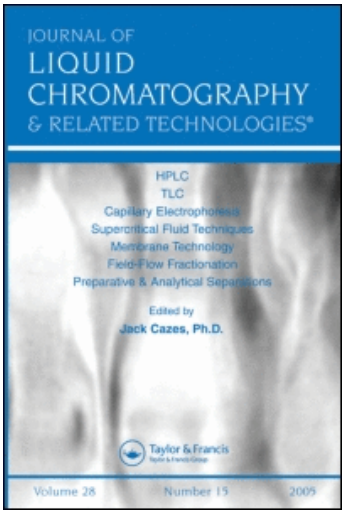

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REVERSED-PHASE LIQUID CHROMATOGRAPHIC ANALYSIS OF COENZYME Q10 AND STABILITY STUDY IN HUMAN PLASMA

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ABSTRACT

The objectives of this study were to design and validate a method for the assay of coenzyme Q10 (CoQ10) in human plasma and to evaluate the stability of CoQ10 in several conditions currently observed for routine determinations. CoQ10 was extracted from plasma with n-hexane after dissociation from lipoproteins with methanol. Ethoxy-CoQ10 was used as internal standard. CoQ10 was isolated and then quantitated by high performance liquid chromatography (HPLC) using a binary gradient. Linearity (r = 0.9999), recovery (97 %) and intra- and inter-run precision (1.7 and 2.0 % respectively) appeared to be satisfactory. The stability of CoQ10 in crude plasma was tested under various conditions (i.e. six hours at room temperature, freezing and thawing, storage for up to 24 weeks at - 20 °C), the stability of CoQ10 in n-hexane extracts was also tested (24 hours in autosampler rack at room temperature). CoQ10 content was found to be unaffected by any of the tested conditions.

INTRODUCTION

Coenzyme Q10 (CoQ10) is a lipid-soluble benzoquinone derivative having multiple functions within the cell (1), particularly as electron carrier within the respiratory chain of the mitochondria. This function has been proposed as the basis for energy transduction in heart cells (2). At physiological concentrations CoQ10 is also recognized as an effective lipid-soluble antioxidant (3). CoQ10 has been measured in plasma (6,7) and blood (8) by HPLC. In recent years increased

attention has been focused on the determination of plasma CoQ10 levels in humans. In particular, it has been demonstrated that low plasma CoQ10 levels were associated with an increased coronary risk factor in cardiac patients (4). Moreover a chronic treatment with statins, that are decreasing plasma cholesterol through inhibition of 3-hydroxy-3-methylglutaryl coenzyme A, has recently been shown as markedly decreasing plasma CoQ10 levels (5).

MATERIALS AND METHODS

Human plasma

Human plasma samples were obtained from two donors (Centre de tranfusion sanguine Dijon, France). One sample was used for method validation, the other sample being used for the CoQ10 stability study.

Chemicals and reagents

CoQ10 was obtained from Sigma. Ethoxy-CoQ10 was synthesized according to EDLUND (9) but purified using a flash chromatography technique instead of a preparative HPLC. Their chemical structures are shown in Figure 1. Bovine serum albumin was purchased from Sigma. All solvents were of HPLC grade: methanol, ethanol and isopropanol were supplied by Carlo Erba, n-hexane was obtained from SDS. Milli-Q™ grade water (Millipore) was used.

Chromatographic conditions

The chromatograph we used consisted of a 126 model binary HPLC pump (Beckman), a 460 autosampler (Kontron) and a 168 UV-VIS diode array detector (Beckman) operating at 275 nm. It was fitted with a Chromasil C_{18} column (150 x 4.6 mm, particle size 5 µm) maintained at 35 °C with a BAS LC 22A oven. Pump and detector control as well as UV signal acquisition and integration were

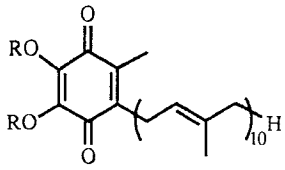

Figure 1. Structures of CoQ10 (R = CH_3) and Ethoxy-CoQ10 (R = C_2H_5).

performed by a Beckman Gold chromatography software running on a IBM PS/2 computer.

The solvents used for separation were A (methanol/isopropanol 95/5 v/v) and B (ethanol/isopropanol 95/5 v/v). The flow-rate was 1 ml/min with isocratic elution from 0 to 2 min (90% A and 10% B), linear gradient elution from 2 to 10 min (10 to 35 % B), isocratic elution from 10 to 20 min (35 % B) and return to initial conditions at 20 min for 5 min.

Plasma extraction procedure

Plasma sample (0.5 ml) was pipetted into a 10 ml amber glass centrifuge tube and then 2 µg of IS (100 µl of a 20 µg/ml solution in n-hexane), 2 ml of methanol and 5 ml of n-hexane were added. The tube was shaken horizontally at high speed for 10 min and centrifuged at 2500 x g for 5 min to separate the two phases. Most of the n-hexane layer was collected in a 10 ml conical glass tube. Five ml of n-hexane was then added to the first tube and the extraction was repeated. The n-hexane layers were combined and evaporated to dryness under nitrogen at 35 °C. The dry extract was re-dissolved in 50 µl of n-hexane and transferred into conical autosampler vials. 10 µl was injected into the column.

Calibration

Calibration standards were prepared by spiking 0.5 ml of a bovine serum albumin aqueous solution (40 g/l) with appropriate amounts of CoQ10 to obtain

0.25 - 0.50 - 1 and 2.5 µg/ml. The standards were processed as described above for plasma samples. Peak-height ratios following the IS method were used to quantify CoQ10 in the samples.

CoQ10 stability

CoQ10 stability in crude plasma

The plasma sample used for the stability study was analysed five times on day one and the mean value obtained was used as reference for all subsequent measurements in plasma, all carried out in quintuplicate. The same day (day one), CoQ10 stability was first assessed after a six hour storage period on the bench at room temperature. The influence of two consecutive freezing-thawing cycles on CoQ10 stability was also investigated on day one. Separate aliquots were frozen at - 20 °C and their CoQ10 content was measured at the end of the following storage periods at -20 °C: 4, 8, 12, 16, 20 and 24 weeks.

CoQ10 stability in n-hexane extracts

This was assessed on the plasma aliquots stored for 4 weeks at -20 °C. The n-hexane extracts were first injected immediately following extraction as described above. The same samples were then re-injected after a 24 hour storage period at room temperature in HPLC vials in the autosampler rack. In order to prevent the evaporation of n-hexane, the HPLC vials were recapped after the first injection. The reference sample for this experiment was that injected immediately after extraction.

For each condition tested (plasma and n-hexane extracts), the mean CoQ10 content was compared to the reference value and stability was considered acceptable if the relative variation did not exceed ± 15 %.

RESULTS AND DISCUSSION

Chromatograms

Figure 2 shows typical chromatograms of a 1 µg/ml standard extract (2A) and of plasma sample extract (2B). CoQ10 and IS eluted at 15 and 17 min respectively.

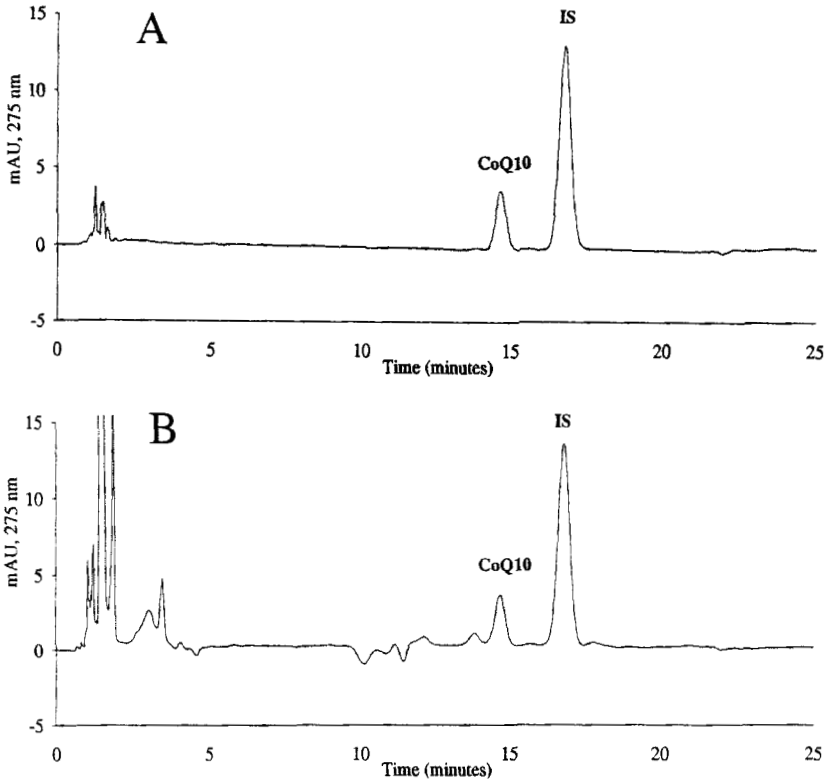

Figure 2. Chromatograms of 1 µg/ml standard extract (A) and plasma sample extract (B).
Conditions as described under Materials and Methods.

TABLE 1
Recovery of CoQ10 from Human Plasma

CoQ10 added µg/ml	Expected value µg/ml	Measured value µg/ml	Recovery %	n
0	-	0.99	-	6
0.25	1.24	1.21	97.2	2
0.50	1.49	1.45	97.0	2
1.00	1.99	1.95	97.7	2

TABLE 2
Intra and Inter-run Precision

Assay	Mean µg/ml	Standard deviation µg/ml	CV %	n
Intra-run	0.99	0.02	1.7	6
Inter-run	0.92	0.02	2.0	6

Method validation

Linearity

The method appears to be linear over the calibration concentration range (r = 0.9999).

Recovery

We evaluated the accuracy of the method by measuring the recovery of authentic CoQ10 added to human plasma samples (Table 1). Recoveries ranged from 97.0 % to 97.7 % (mean 97.3 %).

TABLE 3

CoQ10 Stability (all measurements n=5)

Testing conditions		Mean µg/ml	Standard deviation µg/ml	% relative variation
Plasma				
Reference		1.30	0.01	-
6 hours at room temperature		1.22	0.02	- 6.6
Freezing-thawing	one cycle	1.29	0.02	-1.0
	two cycles	1.40	0.06	+ 7.1
Storage -20 °C	4 weeks	1.37	0.09	+ 5.3
	8 weeks	1.32	0.04	+ 1.1
	12 weeks	1.33	0.04	+ 2.1
	6 weeks	1.34	0.04	+2.5
	20 weeks	1.28	0.04	- 1.9
	24 weeks	1.22	0.02	- 6.8
n-hexane extracts				
24 hours in autosampler vials *		1.42	0.08	+ 3.8

* reference value: plasma stored 4 weeks at -20 °C.

Precision

The coefficients of variation (CVs) observed for intra- and inter-run assays were ≤ 2.0 % (Table 2).

CoQ10 stability

The results for CoQ10 stability are shown in Table 3. Since relative variations ranged between - 6.8 and + 7.1 %, plasma CoQ10 content was considered unaffected under the studied conditions.

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