Short communication

Analysis of coenzyme Q_{10} in lymphocytes by HPLC–MS/MS

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ABSTRACT

Coenzyme Q_{10} (CoQ_{10}) deficiency syndromes are potentially treatable disorders. Skeletal muscle is the most widely accepted tissue for their study, but sampling is an invasive procedure. Cultured skin fibroblasts seem to improve the biochemical diagnosis, but their growth requires a certain period of time. Our aim was to set up a minimally invasive, fast and reliable analytical procedure to measure CoQ_{10} in lymphocytes, to prevent any delay in diagnosing primary CoQ_{10} deficiency. HPLC–MS/MS analysis of CoQ_{10} showed high sensitivity and specificity. The reference range was established in apparently healthy volunteers ($n = 33$); the mean of CoQ_{10} in lymphocytes was 107 nmol/g protein (95% confidence interval: 105–120) and 2.0 nmol/UCS (95% confidence interval: 2.06–2.46). Therefore, the range was narrower when normalized to units of citrate synthase (UCS) than when normalized to grams of protein. The method was linear from 0.01 to 1 μM with a good precision and sensitivity (limit of quantification 0.01 μM). Intra-assay and inter-assay coefficients of variation were lower than 13%. Recovery was higher than 95%. In our hands, lymphocytes seem to be a reliable matrix as they reflect intracellular content of CoQ_{10}. In addition, they can be obtained by a minimally invasive procedure (venipuncture).

1. Introduction

Coenzyme Q_{10} (CoQ_{10}) is an essential protein for electron transfer in the mitochondrial respiratory chain among other important functions. It is present in all cellular membranes of eukaryotic organisms, and it is synthesized and broken down inside the cell [1,2]. CoQ_{10} is the predominant ubiquinone species in humans.

CoQ_{10} deficiency syndromes are potentially treatable disorders [3]. Immediate diagnosis leads to better prevention and correction of the clinical symptoms. Current methods for the determination of CoQ_{10} content are based on reversed phase high performance liquid chromatography (HPLC) with ultraviolet detection [4,5], electrochemical detection [6–8] or tandem mass spectrometry detection (MS/MS) [9,10]. Currently, it is under discussion which is the best biological tissue to detect CoQ_{10} defects. Skeletal muscle is the most widely accepted tissue, but performing muscle biopsy is an invasive procedure. Cultured skin fibroblasts seem to improve the biochemical diagnosis [3], as they are cultured in standard conditions obviating changes due to diet or therapy. Nevertheless, their growth requires a certain period of time. On the other hand, plasma is not appropriate to analyze CoQ_{10} due to clear dietary influences. Lymphocytes might be a good alternative as they are easy to obtain and may reflect the intracellular content of CoQ_{10} better than plasma levels [10,11], but its use has been limited to some studies [5,10].

Our aim was to set up a minimally invasive, fast and reliable analytical procedure to measure CoQ_{10} in lymphocytes in order to prevent any delay in the diagnosis of its deficiency.

2. Materials and methods

2.1. Reagents

Histopaque 1119 and histopaque 1077, methylamine, coenzyme Q_9 (CoQ_9), CoQ_{10}, saccharose, EDTA, heparine and Tris–HCL were obtained from Sigma–Aldrich, Madrid (Spain). All other solvents and chemicals were of analytical grade and were obtained from a variety of sources.

2.2. Instrumentation

A HPLC (Alliance HT 2795) was equipped with a 2.1 mm × 50 mm Symmetry C18 HPLC column (3.5 μm particle size; Waters). Tandem mass spectrometry (MS/MS) analysis was performed using a Micromass Quattro micro™ API with Masslynx™ (V3.2) software (Waters/Micromass, Manchester, UK).

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2.3. Blood mononuclear cells (lymphocytes) preparation

5–10 mL of venous blood was collected into a lithium heparin tube. Lymphocytes isolation was performed within 24 h after collecting the sample. Briefly, 5 mL of blood sample containing 0.25 μL of Na_2EDTA 5 mmol/L was transferred into a 15 mL conical plastic tube to which 4.5 mL PBS $1\times$ was added. The mixture was transferred into a 10 mL conical tube containing a discontinuous gradient consisting of 1.5 mL histopaque 1119 and 1.5 mL histopaque 1077. It was centrifuged at $700 \times g$ during 40 min at room temperature and lymphocyte fraction was transferred to another 15 mL conic tube, to which 12 mL of 0.9% NaCl containing 5 mmol/L EDTA was added. This solution was mixed and centrifuged at $700 \times g$ for 10 min. To lysate the red cells, the pellets were resuspended in 1 mL 0.9% NaCl, 3 mL of water and, after 90 s 1 mL of 3.6% NaCl was added. The lysis process was repeated twice. The pellet was washed with 4 mL 0.9% NaCl containing 5 mmol/L EDTA, centrifuged at $700 \times g$ 10 min and suspended in 500 μL SETH solution (250 mM saccharose, 2 mM EDTA, 10 mM, Tris–HCL and 50 U/mL heparin), sonicated $3\times$ for 5 s on ice and stored at $-80\,^\circ$C. The CoQ_{10}, citrate synthase (CS) and protein analyses were performed in those isolated lymphocyte lysates (ILL).

2.4. Analytical method

2.4.1. Sample preparation

10 μL of CoQ_9 (1 μM, as internal standard, IS), and 800 μL of methanol were added to 100 μL of ILL or plasma, the mixture was vortexed and centrifuged at $15,700 \times g$ 10 min; 700 μL of the extract was evaporated to dryness under nitrogen at room temperature, it was dissolved in 100 μL of methanol and 50 μL were injected into the HPLC–MS/MS. The stability of previously processed samples, kept at $-20\,^\circ$C, was evaluated during one month without any change. The results were expressed in nmol CoQ_{10}/g protein or nmol CoQ_{10}/UCS. CS activity was determined as described by Faloona and Srere [12]. Protein concentrations in lymphocytes were measured by the classical method of Lowry [13], using bovine serum albumin as standard.

2.4.2. MS/MS optimization and HPLC–MS/MS analysis

To select the appropriate detection conditions and to optimize the mass spectrometer parameters 10 μM of both CoQ_9 and CoQ_{10} were monitored in the positive ion mode. To obtain the precursor and product ions, different CV or CE in full scan or in daughter scan mode were respectively tested. Nitrogen (at flow rate of 50 L/h) and argon (adjusted to obtain a vacuum of 3×10^{-3} bar) were used as nebulizing and collision gas, respectively. Dwell time for each transition was 200 ms and the run-time was 16 min. Analytical conditions of the HPLC–MS/MS were those described by Teshima and Kondo [9], with some modifications. Briefly, the mobile phase was composed of 50% methanol with methylamine 5 mM, 45% 2-propanol and 5% water acidified with formic acid (0.5 mL/L), at a flow rate of 0.2 mL/min and isocratic conditions. CoQ_{10} concentration was quantified relative to the IS by an external calibration curve.

2.4.3. Linearity

Stock solution 1 mM CoQ_{10} was prepared in 2-propanol, sonicated 45 min, and serially diluted with methanol to provide different standard solutions: 0.001, 0.01, 0.05, 0.1, 0.2, 0.4, 0.8, 1, 1.6, 2.5, 5, and 10 μM. Stock solution 0.75 mM CoQ_9 was prepared in 2-propanol, sonicated 15 min, and diluted with methanol to the working IS solution (1 μM). These solutions were stored at $-20\,^\circ$C. Calibration curves were constructed by linear regression analysis of the ratio of CoQ_{10}/CoQ_9 areas to CoQ_{10} concentration. To calculate

the precision of the linearity, the calibration curve was evaluated six times.

2.4.4. Limit of detection (LOD) and limit of quantification (LLOQ)

LOD and LLOQ were estimated both with standard solutions and by progressive dilutions of 100 μM of CoQ_{10} spiked ILL. The LOD was considered when signal-to-noise ratio (S/N) was at least 3 and the LLOQ when S/N > 10.

2.4.5. Precision

The intra-assay precision (coefficient of variation, CV) was evaluated by performing 10 analyses of the same spiked matrix, on the same day, at low (0.05 μM), medium (0.3 μM) and high concentrations (1 μM). To establish the inter-assay variability, the same spiked sample was processed in 10 independent preparations on 10 different days, at the same low, medium and high concentrations.

2.4.6. Recovery

Recovery was evaluated by the addition of known concentrations of CoQ_{10} (0.01, 0.05, 0.1, 0.2, 0.4, 0.8, 1 μM) to a pool of ILL, followed by 10 min shaking. These preparations were processed as a sample. All the analyses were performed in triplicate.

2.5. Subjects

The control group consisted of 33 apparently healthy subjects: 20 males and 13 females; median age, 20 years; and range, 1–62 years. CoQ_{10} values were expressed as mean and 95% confidence interval.

In addition, to study the influence of CoQ_{10} ingestion on plasma and ILL 10 healthy volunteers from our laboratory (median age, 28 years; range, 25–62 years; and ratio of males to females, 3:7) were studied. The entire group was recruited to take 100 mg CoQ_{10} daily for 6 days. None of them was taking any other vitamin supplement before or during the study. Venous blood samples were collected before and after 6 days of CoQ_{10} supplementation.

Informed consent was obtained from all the subjects or the patients' parents. The study was approved by the Ethics Committee of the Hospital Clinic – Barcelona, Spain. Samples were obtained in accordance with the Helsinki Declaration of 1964.

2.6. Statistical analysis

Statistical analyses were performed using the SPSS version 18.0 software. Kolmogorov–Smirnov test was used to check variables, which were under a normal distribution. Statistically significant differences between groups before and after supplementation with CoQ_{10} were analyzed using a nonparametric Wilcoxon test. A p-value < 0.05 was considered significant.

3. Results and discussion

The optimized conditions for the detection of both CoQ_9 and CoQ_{10} by HPLC–ESI–MS/MS in the MRM mode were CV: 15 V and CE: 20 eV. The selected transitions for CoQ_{10} and CoQ_9 were (m/z) 826 > 197 and 894 > 197, respectively. The precursor ions for both CoQ_{10} and CoQ_9 were the corresponding methyl ammonium adduct molecules $[(M+CH_3\ NH_3)^+]$. The MRM results using these conditions showed good resolution (Fig. 1a and b) and high sensitivity, as it has previously been reported [9].

The calibration equation was linear for concentrations ranging from 0.01 to 1 μM, with good precision and linearity ($n = 6$): $y = 23.2$ (2.85) + 17.9 (2.15), standard deviations of the slopes and intercepts are in parenthesis, linear regression was $r^2 = 0.997$ (SD: 0.002). LOD and LLOQ for CoQ_{10} in diluted standard were 0.001 and 0.01 nmol/L, respectively. LOD and LLOQ for CoQ_{10} in diluted matrix were 0.008
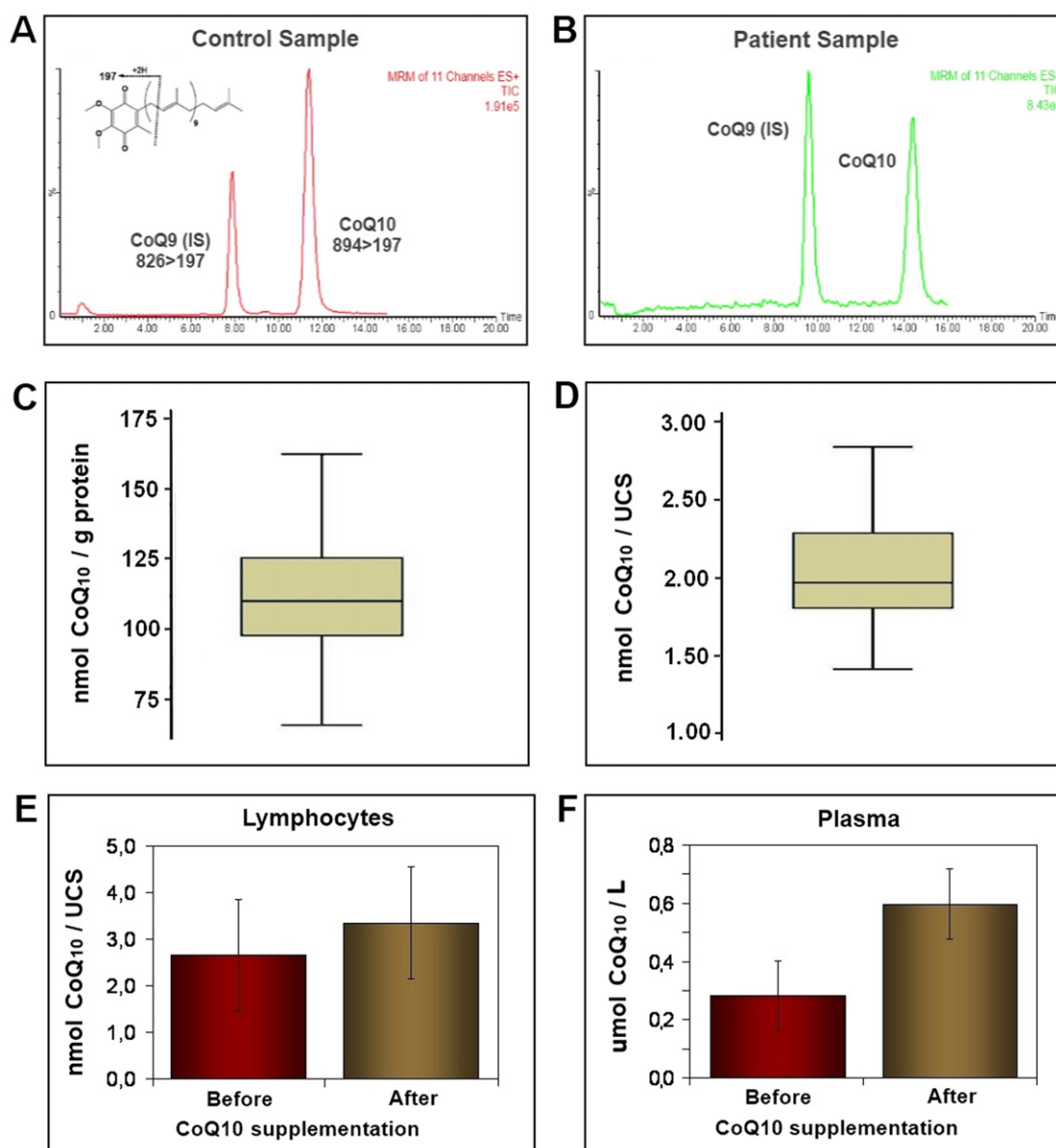
Fig. 1. (a and b) MRM chromatogram of transitions $m/z\ 894 > 197$ (CoQ_{10}) and $m/z\ 826 > 197$ (CoQ_9, IS) corresponding to a control (87 nmol CoQ_{10}/g protein, 2.11 nmol/UCS) and to a deficient patient (69 nmol CoQ_{10}/g protein, 0.7 nmol/UCS), respectively; (c and d) Reference values of CoQ_{10} in ILL normalized to total protein or to CS, respectively; and (e and f) CoQ_{10} concentration in lymphocytes ($p = 0.082$) and plasma ($p = 0.01$) from apparently healthy volunteers ($n = 10$) before and after 6 days of 100 mg CoQ_{10} daily supplementation.

and 0.03 nmol/L, respectively. Intra-assay % CVs were 7.3, 7.4 and 7.7 for low, medium and high concentrations, respectively. Inter-assay % CVs were 8.3, 9.6 and 10.5 for low, medium and high concentrations, respectively. The extraction recovery was greater than 95%. The interference of the endogenous CoQ_9 was negligible.

Reference values showed a normal Gaussian distribution ($p = 0.61$ when normalized to protein and $p = 0.095$ when normalized to CS). The mean of the reference interval of CoQ_{10} in ILL was 107 nmol/g protein (95% confidence interval: 105–120), and 2.0 nmol/UCS (95% confidence interval: 2.06–2.46) (Fig. 1c and d, respectively). As previously reported in fibroblasts [3], the reference interval of CoQ_{10} in ILL is narrower when CoQ_{10} is normalized to CS activity (Fig. 1d compared to 1c), which might be to help for the diagnosis of some patients. In fact, an untreated patient with low levels of CoQ_{10} in fibroblasts, also showed deficient CoQ_{10} in ILL if the value was related to CS (0.96 nmol/UCS), while CoQ_{10} was

within the reference range (82 nmol/g) when related to protein. Nevertheless, this single observation should be confirmed in further studies.

Lymphocyte CoQ_{10} concentration after 6 days supplementation in 10 healthy volunteers was not significantly ($p = 0.086$) increased (Fig. 1e), only 26% were outside the control range, while a significant increase in plasma (three to six-fold increase, $p = 0.018$) was observed (Fig. 1f). Our results differ from previous authors [14], who found increased levels of CoQ_{10} in both plasma and lymphocytes after supplementation during the same period (1 week) and the same doses (100 mg/day). Therefore, in our hands, lymphocytes seem to be a reliable material for the diagnosis of primary CoQ_{10} deficiency, even if treatment had already been started one week before sample collection. However, this might not be true for long term treatment as we did found increased CoQ_{10} concentrations in lymphocytes of two patients treated with CoQ_{10} for over 1 year: 317

and 436 nmol/g protein and 4.8 and 6.7 nmol/UCS, respectively. On the other hand, monitoring of CoQ$_{10}$ in lymphocytes could be a good option for the follow-up of patients, as they reflect the intracellular CoQ$_{10}$ concentration [3,10,11].

Concerning diagnosis, it is important to bear in mind that one patient with genetically confirmed CoQ$_{10}$ defect had normal CoQ$_{10}$ levels in fibroblasts [15] and that the same may happen with lymphocytes. Therefore, as recommended by Rahman et al. [16], it is advisable to test more than one tissue to confirm the diagnosis.

In conclusion, the present procedure to quantify CoQ$_{10}$ in lymphocytes is a minimally invasive procedure, fast and reliable analytical tool and has been implemented in our laboratory for the diagnosis and follow-up of patients with CoQ$_{10}$ deficiency.

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