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NOTE

A novel non-invasive sampling method using buccal mucosa cells for determination of coenzyme Q10

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Abstract Coenzyme Q10 (CoQ10) is an important cofactor in the mitochondrial respiratory chain and a potent endogenous antioxidant. CoQ10 deficiency is often associated with numerous diseases, and patients can benefit from CoQ10 supplementation, being more effective when diagnosed and treated early. Due to the increased interest in CoQ10 deficiency, several methods for CoQ10 analysis from plasmatic, muscular, fibroblast, and platelet matrices have been developed. These sampling techniques are not only highly invasive but also too traumatic for periodic clinical monitoring. In the present work, we describe the development and validation of a novel non-invasive sampling method for quantification of CoQ10 in buccal mucosa cells (BMCs) by microHPLC. This method is suitable for using in a routine laboratory and useful for sampling patients in pediatry. CoQ10 correlation was demonstrated between BMCs and plasma levels (Spearman r, 0.4540; $p<0.001$). The proposed method is amenable to be applied in the post treatment monitoring, especially in pediatric patients as a non-invasive sample collection. More studies are needed to assess whether this determination could be used for diagnosis and if this matrix could replace the traditional ones.

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Keywords Coenzyme Q10 · MicroHPLC-UV · Buccal mucosa cells · Pediatry

Abbreviations

BMCs	Buccal mucosa cells
CoQ9	Coenzyme Q9
CoQ10	Coenzyme Q10
LOD	Limit of detection
LOQ	Limit of quantification
RSD	Relative standard deviation

Introduction

Coenzyme Q10 (CoQ10), a lipid-soluble endogenous compound of cell membranes, plays an important role as electron carrier in the mitochondrial respiratory chain and as a powerful antioxidant agent [1]. Numerous disease processes associated with CoQ10 deficiency can benefit from CoQ10 supplementation, being more effective when diagnosed and treated early [2].

The hallmark in the diagnosis of CoQ10 deficiency syndrome is a decreased CoQ10 concentration in muscle and/or fibroblast. In spite of being more sensitive, these sampling techniques are not only highly invasive but also too traumatic for periodic clinical monitoring. Although plasmatic and platelet CoQ10 measurements may provide a useful estimation for tissue CoQ10 content, the way in which these samples are obtained is still invasive, especially for infants undergoing continuous monitoring of their treatment [2].

In the present work, we describe the development and method validation for CoQ10 quantification in buccal mucosa cells (BMCs), suitable for using in a routine laboratory and useful for sampling patients in pediatry. The objectives of this

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work are (1) to describe the performance of a simple microHPLC-UV method and a sample pretreatment for the analytical determination of CoQ10 in BMCs, (2) to correlate the CoQ10 content in BMCs and plasma following oral CoQ10 supplementation in healthy volunteers and patients with mitochondrial diseases under treatment, and (3) to compare levels of CoQ10 in BMCs in healthy volunteers with those in patients suffering from mitochondrial diseases under and without treatment.

Materials and methods

Reagents

CoQ10, coenzyme Q9 (CoQ9), and *p*-benzoquinone were provided by Sigma (St. Louis, MO, USA). Methanol and ethanol were supplied by Sintorgan (Buenos Aires, Argentina); 1-propanol was purchased from J.T. Baker (Xalostoc, Mexico). All chemicals employed were HPLC grade. All solutions were filtered through a 0.45-μm nylon membrane (Micron Separations Inc., Westborough, MA, USA) and degassed before use.

Preparation of standard solutions

Stock solution of 1 mM was prepared in ethanol, protected from light, and stored at−20 °C for 2 months. An accurate concentration of CoQ10 in the working standard solution was obtained by spectrophotometry at 275 nm, ε=14, 020 L mol^{-1} cm^{-1}. Stock solution of CoQ9 in ethanol (1 mM) was used as internal standard.

Instrumentation and chromatographic conditions

The quantification of CoQ10 was performed in an HPLC Spectra System SCM1000 (Thermo Scientific, Waltham, MA, USA) with a quaternary pump, a P4000 degasser, AS3000 autosampler, and a UV2000 Dual λ Absorbance detector. Chromatograms were processed using ChromQuest Chromatography Data System software. Separation was achieved using an analytical microcolumn XTerra C18 (50 mm×2.1 mm, 3.5 μm) with an XTerra C18 guard column (Waters Corp., Milford, MA, USA).

Chromatographic conditions were as follows: an injection volume of 10 μL, 30 °C column temperature, an isocratic mobile phase of methanol (100 %), and a flow rate of 0.4 mL min^{-1}, and UV detection was performed at 275 nm. Determination of CoQ10 in both plasma samples and BMCs was accomplished in less than 10 min.

Patients

The study was conducted on healthy volunteers and patients from Jose de San Martin Hospital, associated to the University of Buenos Aires. This work was carried out on 36 control subjects without CoQ10 treatment, 27 healthy volunteers treated with 250 mg of CoQ10, 15 patients with neurological disease treated with CoQ10 according to their deficiency, 3 confirmed patients having CoQ10 deficiency, and 2 patients with neurological disease not confirmed as a CoQ10 deficiency (these last two without CoQ10 treatment at the moment of the study). It was performed according to the principles of the Declaration of Helsinki, and it was approved by the Institutional Review Board and the Bioethical Committee of our institution. Written consent was obtained in every case.

Sample collection

Plasma sample Heparinized blood samples were obtained after a fasting period of 8 h, and aliquots were centrifuged at 2000*g* for 10 min. Plasma was stored at −80 °C until analysis.

BMCs The BMC collection was performed as previously detailed [3], slightly modified. Briefly, BMCs were collected by brushing the inside of the cheeks during 1 min with swabs on each side and then collected in saline solution with 20 % of ethanol. The swabs were rotated to promote cell detachment. The volume of this suspension was accurately measured and an aliquot was taken for cell count. For CoQ10 quantification assay, BMCs were centrifuged at 9000*g* for 10 min at room temperature, the supernatant was removed, and the pellet was immediately stored at −80 °C until use. Cell count was carried out with a hemocytometer filled with blue methylene dye.

Sample preparation and quantitative analysis of CoQ10

Before analysis, the samples were allowed to thaw at room temperature. Assessment of CoQ10 in plasma was performed by an optimized microHPLC-UV method as it has been previously detailed [4].

The BMC pellet was supplemented with 50 μL of the *p*-benzoquinone solution in 1-propanol (4 mg mL^{-1}). Then, 150 μL cold 1-propanol was added and centrifuged at 9000*g* for 2 min, and the organic layer was evaporated to dryness under a nitrogen stream. The dry residue was redissolved in 50 μL of ethanol and injected into the equipment.

The quantification of CoQ10 in BMCs was performed using a six-point calibration curve ranging from 0.06 to 1.3 μM of CoQ10. Working standard levels were selected based on the concentration range expected for CoQ10 in BMCs with a cell count higher than 1.510^{5} in the final pellet. The above-mentioned cell count was standardized for an appropriate CoQ10 quantification. However, in previous works,

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the chromatographic linearity was confirmed at least up to 15.0 μM [4]. In the case of BMCs, the CoQ10 content was expressed as nanomoles of CoQ10 in 10^9 BMCs.

Validation of the proposed method for BMCs

The validation of the proposed method for BMCs was performed according to international guidelines [5]. The following analytical parameters were evaluated: specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, and stability.

Specificity of the current method was determined from a comparison of chromatographic runs between a standard solution of CoQ10 and the biological sample. The retention time of CoQ10 in a standard solution was used to confirm the presence of CoQ10 in real samples. The linearity of the chromatographic method was determined by linear least-squares regression analysis obtained on three different days with triplicates (n=9) at six different levels. Values of LOD and LOQ in BMCs were calculated as signal-to-noise ratios of 3:1 and 10:1, respectively.

Accuracy was evaluated by means of a recovery assay. For extraction recovery assay, the BMC samples were harvested from a pooled BMC suspension. The recovery assay was carried out by spiking the pellets of BMC samples with three different levels of CoQ10, each one by triplicate, on three different days (n=9) and comparing between the peaks obtained by spiking pellets with increasing concentration of CoQ10 and the corresponding peak of unextracted standards that represent 100 % recovery. The three spiked concentrations used in the assays for precision and accuracy were studied at high (1.20 μM), low (0.06 μM), and middle (0.50 μM) levels of CoQ10.

Precision was evaluated on the basis of intraday (n=3) and interday (n=9) analysis repeatability on spiked pellets of BMC samples at the three different levels above mentioned. Results were expressed as relative standard deviation (RSD).

Processed sample stability was evaluated as follows: immediately after collection and after 8 and 24 h at 4 and 25 °C.

BMC pellet stability was carried out at 7, 15, and 30 days storage at−80 °C. Stability of the evaporated sample was also evaluated at 1 and 7 days storage at −80 °C. The percent stability was calculated as follows: %stability=$(S/S_0)*100$, with S being the concentration of the CoQ10 at a given condition and S_0 the CoQ10 concentration from a fresh sample.

Statistical analysis

The results were expressed as median±SEM. Shapiro–Wilk's W test of normality was performed. Differences between groups were analyzed by Student's t test, and the Spearman r coefficient was calculated for correlations. Levels of significance were established at $p<0.05$.

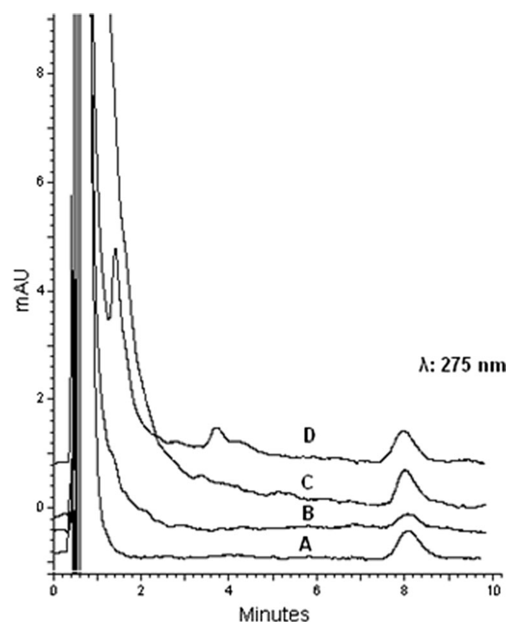

Fig. 1 HPLC chromatogram of *A* CoQ10 standard (0.9 μM), *B* patient with CoQ10 deficiency untreated (37.5 nmol CoQ10/10^9 BMCs), *C* patient with CoQ10 deficiency treated (105 nmol CoQ10/10^9 BMCs), and *D* control subject (53.1 nmol CoQ10/10^9 BMCs)

Results and discussion

Sample preparation

The time and number of centrifugations to obtain the BMC pellet was evaluated by counting the remaining cells in the

Table 1 CoQ10 in BMCs in the selected population. Results are expressed as median±SEM

	CoQ10 (nmol/10^9 BMCs)
Healthy control	
Non-treated[a]	
Adult (n=21)	69.3±4.2
Pediatric (n=15)	56.3±5.0
Treated	
Adult[b] (27)	95.3±10.6*
Neurological disease	
Non-treated[a, c]	
CoQ10 deficiency (n=3)	33.8±2.1**
Not CoQ10 deficiency (n=2)	68.4±9.0***
Treated	
CoQ10 deficiency treated (n=12)	147±28.2****

*$p<0.05$ with respect to healthy non-treated patients; **$p<0.05$ with respect to healthy controls; ***$p<0.05$ with respect to CoQ10 deficiency; ****$p<0.05$ with respect CoQ10 deficiency non-treated

[a] Non-treated with CoQ10

[b] Treated with CoQ10 250 mg, single dose

[c] Adult

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supernatant. The centrifugation time between 2 and 10 min and the number of centrifugations between 1 and 3 were also studied, and the absence of cell count in the supernatant with 1 centrifugation during 10 min was observed.

It was also observed that the sample must be processed on the day of collection. CoQ10 in the collected sample falls significantly after 24 h at 25 or 4 °C (%stability, 54.6 and 62.7, respectively). However, it could be maintained for 8 h at 4 °C (%stability>90). The pellet can be stored at −80 °C for at least 30 days, in accordance with a previous stability study [3]. For practical reasons, the evaporated sample can be stored at −80 °C for at least 7 days (%stability>90).

The CoQ10 extraction of the BMC pellet was optimized by evaluating various organic solvents (methanol, acetonitrile, and 1-propanol) for cell disruption, protein precipitation, and extraction of CoQ10. Cold 1-propanol showed the best CoQ10 recovery with short-time sample pretreatment.

Quantification and validation of the procedure

In this work, the chromatographic method previously reported [4] was applied for the quantification of CoQ10 in BMCs. Considering that CoQ10 was evaluated in a different biological matrix, a complete validation was performed.

Three quantification methods were compared: external standard, standard addition, and internal standard. Comparing concentration levels of CoQ10 in the analyzed samples, there are no differences among the three quantitative methods. Taking into account the results obtained, we decided to use non-matrix calibration curves for quantification in further analysis.

Specificity of the method for analysis of CoQ10 in BMCs was verified without interference of any endogenous compound (Fig. 1). The calibration curve was linear in the range 0.06–1.3 μM ($y=17{,}853x+2436$, $Sy{\cdot}x$ 1159, $S_{intercept}=340.9$, $S_{slope}=582.3$, r^2 0.9711). The LOQ was 0.06 μM and the LOD 0.018 μM. Accuracy results were between 96.0 and 98.1 % (RSD 2.39–8.58). The intraday (RSD 0.84–9.69, $n=3$) and interday (RSD 1.86–11.7, $n=9$) precision of results for CoQ10 analysis in BMCs was tested by analyzing three concentration levels of the calibration curve at low, middle, and high levels of CoQ10.

Intra- and interday accuracy and precision data for BMCs were in compliance with international guidelines for validation of analytical assays.

Application

The developed and validated microHPLC method proposed in this study was applied in the assessment of CoQ10 in BMCs in the different populations as described in "Materials and methods" (Fig. 1). CoQ10 levels in BMCs provide a significant correlation ($p<0.001$) with plasmatic CoQ10 levels (Spearman r 0.4540, 95 % confidence interval 0.1929–0.6551). The results observed in Table 1 show that there is a significant increase ($p<0.05$) of CoQ10 BMC levels in healthy volunteers treated with CoQ10 compared to non-treated subjects. Moreover, and although more studies will be needed to establish references values, CoQ10 levels in BMCs are decreased in patients with neurological disease diagnosed as CoQ10 deficiency compared to those of control patients ($p<0.05$) and of patients with other neurological disease diagnosed as non-CoQ10 deficiency ($p<0.05$). In the same way, patients with neurological disease with CoQ10 deficiency treated with this coenzyme show increments in their BMC CoQ10 levels ($p<0.05$) with respect to treated patients. Although the sample size with neurological disease is small, these observations indicate that CoQ10 quantification in BMCs could discriminate CoQ10 deficiency and could also be applied to its monitoring in patients undergoing treatment, making it a viable alternative as a non-invasive sampling method.

Conclusion

We demonstrate that the CoQ10 determination in BMCs is an alternative, non-invasive, and simple method, with a significant correlation with CoQ10 plasma levels. The analytical method was extensively validated in compliance with international standards. The proposed method is amenable to be applied in the post treatment monitoring, especially in pediatric patients as a non-invasive sample collection. The sampling can be performed by the parents or by a non-trained person, which represents a significant advantage. Moreover, it contributes to ameliorate the discomfort that pediatric patients suffer after several blood extractions. More studies are needed to assess whether this determination could be used for diagnosis and if this matrix could replace the traditional ones.

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Conflict of interest The authors do not have any disclosures to report.

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