Chapter 18

Coenzyme Q10 Assessment and the establishment of a neuronal Cell Model of CoQ_{10} Deficiency

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Abstract

Coenzyme Q10 (CoQ10) plays a key role as an electron carrier in the mitochondrial respiratory chain and as a cellular antioxidant molecule. A deficit in CoQ10 status may contribute to disease pathophysiology by causing a failure mitochondrial energy metabolism as well as compromising cellular antioxidant capacity. This chapter outlines the analytical methods used for determine cellular CoQ10 status using HPLC-UV detection. In addition, we present a pharmacological procedure for establishing a human neuronal cell model of CoQ10 deficiency, for use in research studies.

Key words Coenzyme Q10, CoQ10, mitochondrial respiratory chain, antioxidant, disease, neuronal cell model

1 Introduction

Ubiquinones are a group of homologous quinones that are found in animals, plants and micro-organisms [**1**]. They consist of the benzoquinone nucleus and an isoprenoid side chain which

can consist of between six to twelve isoprenoid units [2]. In man, the predominant ubiquinone species has a side chain composed of ten isoprenoid units and it is known as coenzyme Q10 (CoQ10) [1]. CoQ10 is a lipophilic molecule made up of a benzoquinone ring and a side chain composed of ten isoprenoid units. CoQ10 exists in two major forms, the oxidised, ubiquinone form (CoQ10), and fully reduced, ubiquinol form (CoQ10H2) [2]. CoQ10 is found in almost all cells of the human body where it plays a fundamental role in the mitochondrial respiratory chain (MRC), where it acts as a pivotal electron carrier accepting electrons derived from complex I (NADH ubiquinone reductase; EC 1.6.5.3) and complex II (succinate ubiquinone reductase; EC 1.3.5.1) and then transferring them to complex III (ubiquinol cytochrome c reductase; EC 1.10.2.2) [1] (**Fig. 1**). This process allows a continuous passage of electrons in the MRC allowing the process of oxidative phosphorylation to occur with the concomitant production of ATP [1]. The CoQ10-H2 form of COQ10 serves as an important lipid soluble anti-oxidant, protecting cellular membranes and circulatory lipoproteins against free radical induced oxidative damage [3]. In addition to these roles, CoQ10 has a plethora of other cellular functions such as the regulation of DNA replication and repair though its involvement in pyrimidine synthesis, the regulation of apoptosis through its control of mitochondrial membrane permeability and it has also been reported to be involved in the acidification of lysosomes [2, 4-6].

In view of the role of CoQ10 as an electron carrier within the MRC as well as its cellular antioxidant function, a deficit in CoQ10 status may contribute to disease pathophysiology by causing a failure mitochondrial energy generation as well as compromising cellular antioxidant capacity [2]. Lowered blood and tissue levels of CoQ10 have been reported in a number of diseases and conditions, although whether the CoQ10 deficiency is the cause or consequence of the disease process has yet to be elucidated in the majority of cases [2, 7]. Clinical assessment of patient CoQ10 status is generally based on plasma determinations [2]. However, since the level of circulatory CoQ10 is influenced by a number of physiological factors, skeletal muscle, fibroblasts and blood mononuclear cells have been suggested as more appropriate surrogates for this determination [1, 8]. The most common methods used for the determination of CoQ10 are based on high pressure liquid chromatography with ultraviolet (HPLC-UV) or electrochemical detection (HPLC-ED) systems [6]. Although it is possible to determine COQ10 and CoQ10-H2 concomitantly using HPLC-ED, the determination of only COQ10 is sufficient to determine evidence of a CoQ10 deficiency [8]. For this reason, HPLC-UV

detection appears to be more widely utilised both clinically and in the research environment to determine cellular CoQ10 status [**8**].

In the following chapter will outline the analytical methods used to determine cellular CoQ10 status employing HPLC-UV detection together with the pharmacological procedure required for establishing a human neuronal cell model of CoQ10 deficiency, which has been utilised for research purposes [**8, 9**].

2 Materials

2.1 Preparation of cell and tissue samples (see Note 1)

1. Muscle biopsy samples.
2. Glass hand-held homogenizer.
3. Muscle isolation media: 320 mM sucrose, 1 mM ethylenediaminetetraacetic acid dipotassium salt (K_2EDTA), and 10 mM Trizma-base (pH 7. 4).
4. 1.5 mL-capacity microcentrifuge tubes.
5. Hanks Balanced Salt (HBS) medium or phosphate buffered saline (PBS).
6. Cell culture medium: Dulbecco's modified eagles medium/Ham's F-12 Nutrient Mixture (DMEM/F-12).
7. 25 cm^2 cell culture flasks.

2.2 Extraction of cellular CoQ_{10}

1. Extraction solvent: 5:2 (v:v) hexane/ethanol.
2. Rotary evaporator.
3. 100% ethanol.
4. 4 mm syringe filter (0.2 um polyvinylidene fluoride).
5. Internal standard: 2 μmol/L dipropoxy-CoQ10 in ethanol.

2.3 HPLC analysis

1. Techsphere ODS (5µ, 150 x 4.6 mm) C18 reversed phase column (HPLC Technology; Welwyn Garden City, UK) or similar.
2. HPLC system (Jasco).
3. PG-975-50 UV/VIS detector or similar.
4. Mobile phase: 57 mM sodium perchlorate in 60:40:1.2 (v:v) ethanol:methanol: 60(v:v) perchloric acid.
5. Working standard: 400 nM CoQ10 and 200 nM dipropoxy-CoQ10.

2.4 Total protein determination

1. DC-protein assay (Bio-Rad Laboratories Ltd; Hemel Hempstead, UK).
2. 0 to 1 mg/mL bovine serum albumin (BSA) standards (n=6) in distilled water.
3. 96-well microtitre plates
4. FLUOstar omega plate reader (BMG Labtech Ltd; Aylesbury, UK) or similar.

2.5 Citrate synthase (CS) assay

1. CS assay buffer: 0.1 mM acetyl-coenzyme A, 100 mM Tris (pH 8.0) on 0.1% Triton X-100.
2. 5, 5'-dithio-bis(2-nitrobenzoic acid) (DTNB).
3. Polystyrene cuvettes.
4. Oxaloacetate.
5. Uvikon 941 spectrophotometer (Northstar Scientific; Potton, UK) or similar.

2.6 Cell model of CoQ_{10} deficiency

1. HS-SY5Y cells.
2. Culture medium: Dulbecco's modified eagles medium/Ham's F-12 Nutrient.
3. Para-aminobenzoic acid (PABA).
4. T75 culture dishes.

3 Methods

3.1 Preparation of tissue and cell samples

1. Homogenize 50-100 mg muscle biopsies in 1:9 (w/v) muscle isolation media using the glass homogenizer (pre-chilled).
2. Transfer 50 μL of each homogenate to a microcentrifuge tube (**see Note 2**).
3. Re-suspend cell samples in HBS or PBS prior to CoQ_{10} determination.
4. For cultures, seed cells at a density of 10^5/mL in 25 cm^2 flasks and culture 5 days at 37°C in 95% air and 5% CO_2 to provide sufficient material for CoQ_{10} determination.

3.2 Extraction of cellular CoQ_{10}

1. Disrupt cellular membranes by three cycles of freeze thawing followed by vigorous mixing using a vortexer (**see Note 3**).
2. Add 30 μL internal standard to all samples (**see Note 4**).
3. Add 5:2 (v/v) hexane/ethanol to the samples followed by vigorous mixing using a vortexer.
4. Centrifuge samples at 15,000 x g for 3 min at 5˚C.
5. Collect the top hexane layer and store on ice.
6. Re-extract the lower aqueous layer two more times as above.
7. Evaporate the resulting samples to dryness using a rotary evaporator.
8. Re-suspend in 300 μL ethanol and pass through the 4 mm syringe filter prior to injection.

3.3 Coenzyme Q_{10} quantification using HPLC-UV detection (Fig. 2) (see Note 5)

1. Maintain HPLC column at 25˚C.
2. Set UV detector to 275 nm.
3. Run the mobile phase at 0.7 mL/min.
4. Inject 50 μL of working standard and analyze over a 20 min run (**see Note 6**).
5. To ensure linearity between UV absorbance at 275 nm and coenzyme Q_{10} concentration, analyze a range of standards of varying CoQ_{10} concentrations and calculate the areas of the resulting CoQ_{10} peaks (**Fig. 3**).
6. Following the calibration of the HPLC with the CoQ_{10} standards, run a blank sample by injecting only ethanol (**see Note 7**).
7. Inject 50 μL volumes of the samples onto the HPLC for analysis (**Fig. 4**) (**see Note 8**).
8. Calculate CoQ10 concentrations in the samples using the following equation:

Concentration CoQ_{10} (pmol/mL) = (sample peak height/internal standard peak height) x internal standard concentration (μM)

Dilution factor: Concentration CoQ_{10} (pmol/mL) = (Conc CoQ_{10} (pmol/mL) x resuspension volume) / volume of extracted sample

9. For determination of intracellular CoQ_{10} status, CoQ_{10} expressed in pmol/ml is divided by the total protein concentration (mg/mL) of the cell or tissue sample and expressed as pmol/mg of protein (**see Note 9**).

3.4 Total protein determination (see Note 10)

1. Dilute all samples and standards in HPLC grade water.
2. Add 10 μl volume of samples in triplicate to a 96 well plate.
3. Add 25 μL DC assay reagent A and 200 μL reagent B to each sample.
4. Incubate 15 min at 25˚C in the dark.
5. Measure the absorbance of the samples at 750 nm using the plate reader.
6. Determine total protein by linear regression of sample absorbance units against the BSA standards (**Fig. 5**).

3.5 CS assay (see Note 11)

1. Add DNTB to give a final concentration of 0.2 mM in CS assay buffer.
2. Add 20 μL of each sample (tissue homogenate/cell suspension) in duplicate to polystyrene cuvettes containing the CS assay buffer/DNTB solution to give a final volume of 1 mL.
3. Gently mix the samples by double inversion and insert into the spectrophotometer into either the reference or sample compartments as appropriate.
4. Initiate the reactions by the addition of 10 μL of 20 mM oxaloacetate.
5. Measure at 412 nm for 5 min at 30˚C.
6. Convert the resulting absorbance/min values into molar concentrations using the Beer-Lambert law:

   $A = C\varepsilon L$

Absorbance/min (A) = concentration (C); the product of the reaction of coenzyme A with DTNB) X (ε; the extinction coefficient (13.6 x 10^3 M^{-1} cm^{-1}) x path length (L; 1 cm).

7. Express results as nmol/min/mg of protein.

3.6 The establishment of a human neuronal cell model of CoQ_{10} deficiency (see Note 12)

1. Seed HS-SY5Y cells into T75 culture flask at approximately 10^5 cells/mL culture medium and approximately 20,000 cells/well.
2. Treat HS-SY5Y cells with escalating concentrations of PABA (0.25, 0.5, 0.75 and 1 mM, final concentrations) (**see Note 13**).
3. Grow cells for 5 days (**see Note 14**).
4. After 5 days of culture with PABA, extract the cells and measure the CoQ_{10} status as described above (**Fig. 6**) (**see Note 15**).

4 Notes

1. In human studies, obtain and use all samples with informed consent. In addition ensure that the study is approved and performed under the ethical guidelines issued by the local ethical committees and that they comply with the Declaration of Helsinki. In animal model studies, ensure that the procedures are approved by the local ethical committees and the equivalent of the UK Home Office guidelines under the Animals (Scientific Procedures) Act of 1986.
2. Only 50 μL of muscle homogenate is required for CoQ10 determination and so any remaining sample can be stored at -80^{o}C until further use [**10**].
3. Freeze thawing involves snap freezing the samples in microcentrifuge tubes in liquid nitrogen and then thawing in a 30^{0}C water bath.
4. To account for the loss of analyte during sample preparation an internal standard is added before CoQ_{10} is extracted from each sample. Dipropoxy-CoQ_{10} was first proposed for use as an internal standard in 2005 by Duncan, et al [**9**]. Previously,

naturally occurring ubiquinones have been used as internal standards (i.e. Coenzyme Q9), although contamination with both dietary sources and endogenous synthesis is possible. Dipropoxy-CoQ10 is advantageous because it is a chemically synthesized, un-physiological compound with similar characteristics to CoQ10 [**9**]. The internal standard is added to the sample prior to CoQ_{10} extraction.

5. The HPLC-UV detection method used to determine CoQ_{10}status is based on the method developed by Boitier et al. [**11**],
6. This is used for calibration.
7. This step ensures that there has been no carry over or contamination of CoQ_{10} from the standards onto the HPLC chromatograms of the samples.
8. **Fig. 4** shows a typical HPLC-UV chromatogram for a SH-SYS5 cell sample.
9. The HPLC-UV detection method has been used both clinically [**10**] and in research studies **[8]** to determine the CoQ10 status of cells, tissues and plasma. The observed ranges for skeletal muscle, blood mononuclear cells, plasma and fibroblasts are shown in **Table 1** [**6, 7, 9**]. Approximately 50% of cellular CoQ_{10} status is present within the mitochondrial [**8**] and therefore, it may be important to take into account the abundance of this organelle in cell and tissue samples when determining their CoQ_{10} status. This is especially important in the skeletal muscle tissue of patients with disorders of the MRC which have been associated with a proliferation of mitochondria [**12**]. In these patients expressing skeletal muscle CoQ_{10} status to the total protein content of the tissue may result in a `false negative` result [**6**]. Therefore, it has been suggested that expressing cellular and tissue CoQ_{10} status to both the total protein content as well as to the activity of the mitochondrial marker enzyme, citrate synthase (CS; EC 1.1.1.27) may improve the diagnostic yield and reveal evidence of a possible CoQ_{10} deficiency [**6, 12**].
10. Total protein concentration can be determined by the Bio-Rad DC-protein assay or through use of a similar kit. This assay is a modified method based on that of the Lowry assay [**12**] and is performed as per manufacturer's instructions.
11. CS is an enzyme in the citric acid cycle which catalyses the condensation of oxaloacetate and acetyl-coenzyme A to form citric acid and coenzyme A. It is found within the mitochondrial matrix and is commonly used as an indicator of mitochondrial enrichment [**13, 14**]. The assay spectrophotometric assay to measure CS activity is based on the method described by Shepherd & Garland in 1969 [**15**]. The assay measures the production of coenzyme A via a reaction with 5, 5'-dithio-bis(2-nitrobenzoic acid) (DTNB).

12. In order to investigate the pathogenesis of a CoQ_{10} deficiency, cellular models of this disorder have to be established which will allow the effect of this deficit on mitochondrial metabolism, antioxidant capacity to be investigated as well being important tools to evaluate potential therapeutic strategies to treat this disorder.
13. In order to establish a human neuronal cell model of CoQ_{10} deficiency para-aminobenzoic acid (PABA) was utilized to induce a deficit in CoQ_{10} status in human neuroblastoma SH-SY5Y cells. The ability of PABA to pharmacologically induce a cellular CoQ_{10} deficiency was previously reported in a study by Alam et al (1975) [**16**] where this compound was found to competitively inhibit the activity of CoQ_{10} biosynthetic enzyme, 4-hydroxybenzoate: polyprenyl transferase(COQ2). PABA is soluble in culture medium [**6**].
14. The time of culture was selected because the half-life of CoQ_{10} in the brain has been reported to be 3.75 days [**6**].
15. Using 1 mM PABA treatment, the CoQ_{10} status of the SH-SY5Y cells can be decreased maximally to 46% of control levels or a 54% decrease in cellular CoQ_{10} status. Higher concentrations of PABA or longer term incubations will not induce a further deficit in CoQ_{10} status [**6**]. Importantly, the effect of PABA treatment on cellular CoQ_{10} status is both progressive (**Fig. 6**) and reproducible [**6**].

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Fig. 1 Diagram of the mitochondrial respiratory chain (MRC) highlighting the role of CoQ_{10} as an electron carrier

Fig. 2 Schematic diagram of a high performance liquid chromatography (HPLC) system

Fig. 3 Concentration of CoQ10vs peak area on HPLC chromatogram at a UV absorbance of 275 nm

Fig. 4 Typical HPLC-UV chromatogram for SH-SYS5 cell sample

Fig. 5 BSA standard curve used to calculate protein concentrations in the Bio-Rad DC-protein assay

Fig. 6 Effect of para-aminobenzoic acid (PABA) treatment on SH-SY5Y cellular CoQ_{10} status

Table 1 The observed range of CoQ10 levels in human plasma, skeletal muscle, blood mononuclear cells, fibroblasts and neuroblastoma SH-SY5Y cells

Tissue	Observed range	Units
Plasma	227-1432	nmol/L
Skeletal muscle	140-580	pmol/mg of protein
Blood mononuclear cells	37-133	pmol/mg of protein
Fibroblasts	39-75	pmol/mg of protein
SH-SY5Y cells	75-250	pmol/mg of protein