Determination of a method for extraction of coenzyme Q_{10} in human plasma: optimization of the use of surfactants and other variables

Determinação de um método de extração de coenzima Q_{10} em plasma humano: otimização do uso de surfactantes e outras variáveis

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ABSTRACT

Objective: To establish a routine for the extraction of the total levels of CoQ_{10} in human plasma through the Ultra High Performance Liquid Chromatography (UHPLC). **Methods:** Two extraction protocols were tested: a) methanol: hexane and b) 1-propanol. The following parameters were analyzed: extraction temperature (19ºC and 4ºC), extraction tubes (glass and polypropylene), and surfactants (SDS, Triton X-100, Tween-20) at different concentrations, i.e., 1%, 3%, 5% and 10%. **Results:** The results showed that the method of extraction of CoQ_{10} in a sample of human plasma at 4ºC, using solvents methanol: hexane (85:15, v/v) in the presence of surfactant Tween-20 at 3% and polypropylene tubes showed better efficiency and reproducibility when compared to the method with 1-propanol. **Conclusion:** By the analyses performed, it was possible to observe that the addition of the surfactant Tween-20 promoted an increase in the recovery of CoQ_{10} by the methanol:hexane extraction method. This method showed good reproducibility, with a low coefficient of variation and high sensitivity, since CoQ_{10} was detected in samples of plasma of a control individual using a UV-type detector. The use of UHPLC equipment allowed a total analysis with total run time of 3.5 minutes, enabling the rapid achievement of results, considered mandatory for laboratory routines.

Keywords: Coenzymes; Surfactants; Chromatography, liquid

RESUMO

Objetivo: Estabelecer uma rotina de extração dos níveis totais de CoQ_{10} em plasma humano por meio da análise por Cromatografia Líquida de Ultra Alta Eficiência (UHPLC). **Métodos:** foram testados dois protocolos de extração: a) metanol:hexano e b) 1-propanol. Os seguintes parâmetros foram analisados: temperatura de extração (19ºC e 4ºC), tubos de extração (vidro e polipropileno), surfactantes (SDS, Triton X-100, Tween-20) em diferentes concentrações 1%, 3%, 5% e 10%. **Resultados:** Os resultados mostraram que o método de extração de CoQ_{10} em amostra de plasma humano, a 4ºC, utilizando-se os solventes metanol:hexano (85:15, v/v) na presença do surfactante Tween-20 a 3% e tubos de polipropileno apresentou melhor eficiência e reprodutibilidade quando comparado ao método com 1-propanol. **Conclusão:** A adição do surfactante Tween-20 no processo de preparação de amostra promoveu um aumento na recuperação da CoQ_{10} pelo método de extração metanol:hexano observada pela boa reprodutibilidade das prelicatas, pelo baixo coeficiente de variação e alta sensibilidade uma vez que a CoQ_{10} foi detectada em amostras de plasma de um indivíduo controle utilizando-se um detector do tipo UV. Além disso, a utilização de um equipamento de UHPLC proporcionou a obtenção de uma análise com tempo total de corrida de 3,5 minutos, o que viabiliza a obtenção rápida de resultados, considerado mandatório para rotinas laboratoriais.

Descritores: Coenzimas; Surfactantes; Cromatografia líquida

INTRODUCTION

Coenzyme Q_{10} (CoQ_{10}), also known as ubiquinone, is a lipid molecule essential for aerobic organisms. It participates in the production of ATP through the oxidative phosphorylation process by transferring electrons from respiratory complexes I and II to complex III in the respiratory chain, located in the inner mitochondrial membrane. Additionally, CoQ_{10} participates in many other vital functions within the cell: it acts as an antioxidant of lipoproteins and cell

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membranes; it is required for the biosynthesis of pyrimidine affecting replication and repair of cellular DNA; it modulates the process of apoptosis through the regulation of the membrane transition pores, and aids in maintenance of body temperature resulting from its function in uncoupling proteins[1].

CoQ_{10} is composed of a ring of benzoquinone associated with a polyprenyl chain derived from the mevalonate pathway, the same synthesis route of cholesterol. The size of this polyprenyl chain varies among organisms; the human species has 10 repetitions (CoQ_{10}), while mice have 9 (CoQ_9), and *Saccharomyces cerevisiae* have 6 (CoQ_6).

The endogenous synthesis of CoQ_{10} occurs in the mitochondria[1] and it is expressed in all tissues, but in greater concentration in the heart, kidneys, liver and muscles, since they are organs that require a greater quantity of energy (ATP) for their function, and decrease their expression during the process of cellular aging[2].

Clinically, patients with a CoQ_{10} deficiency are very heterogeneous, present variable phenotypes with neurological commitment in most cases[3]. Therefore, some subtypes of clinical presentation seem to be more common: (1) encephalomyopathy, with mitochondrial myopathy and recurring myoglobinuria[4]; (2) multisystem involvement in childhood; (3) Leigh syndrome; (4) myopathic form[5]; (5) ataxic form, probably the most common among the five forms[6]; (6) steroid-resistant nephrotic syndrome[7]. Its association with other clinical phenotypes, such as seizures, muscle weakness, mental retardation, ophthalmoplegia, peripheral neuropathy, pyramidal signs and scoliosis is quite frequent. Hypergonadotrophic hypogonadism seems to be more common in patients with late onset of the disease[8].

The primary deficiency of CoQ_{10} is autosomal recessive and is included in the group of mitochondrial diseases. It is caused by a defect in the biosynthetic route of ubiquinone which is not yet completely elucidated[6]. Among the mitochondrial diseases described, to date, CoQ_{10} deficiency is the only one that has chances of effective treatment through the exogenous CoQ_{10} administration[9].

Much of what is known about the biosynthesis of coenzyme Q comes from studies carried out with *Escherichia coli* and *S. cerevisiae*[10]. There are at least a dozen genes named *COQ1*, *COQ2*, *COQ3*... *COQ10* in *Saccharomyces cerevisiae*, necessary for the respiratory function of coenzyme Q[10-13].

In the human species, patients were described with mutations in genes *COQ2*, *COQ4*, *COQ6*, *COQ8*, *COQ9, PDSS1* and *PDSS2*[14-20].

The secondary deficiency in the metabolism of CoQ_{10} was discussed in the pathophysiology of myopathy by statins, toxicity by anthracyclin and Parkinson's disease, but the relative contribution of CoQ_{10} in triggering these diseases is still unknown[21,22].

However, supplementation with CoQ_{10} is amply recommended at high doses (30mg/kg in children and at least 600mg daily in adults) for all patients with mitochondrial diseases, since it is a supplement devoid of side effects and it brings many benefits to cellular metabolism[23,24]. Since it is lipophilic, CoQ_{10} is transported in circulation by particles of lipoproteins, and its concentration in the plasma has been correlated with the total levels of cholesterol, especially LDL, in patients who take statins[25,26].

Considering this effective possibility of treatment, interest in determining the levels of CoQ_{10} in the plasma and other types of cells or tissues were the target of various studies. The diagnosis of CoQ_{10} deficiency is performed through the analysis of the enzymatic activity of mitochondrial complexes I+II and II+III[1], as well as by quantification of the total levels of the enzyme directly in the muscle or fibroblasts. The levels of CoQ_{10} determined in the blood are not used for diagnosis, since CoQ_{10} is present in certain foods and is absorbed through the diet. However, it is used for therapeutic monitoring in patients with confirmed deficiency of the coenzyme.

There are various methods described in scientific literature for the quantification of CoQ_{10} in plasma, cells and tissues. The use of reverse phase high performance liquid chromatography (HPLC) equipment coupled with electrochemical or ultraviolet (UV)-type detectors is very frequent. Currently, most of the methods involve the preparation of the sample using a single stage of dilution with 1-propanol, followed by the direct injection of the sample into an HPLC system[27,28]. However, this method is more commonly used in the electrochemical-type detector that displays greater sensitivity, allowing the detection of minimal concentrations of CoQ_{10}. Due to the lower level of sensitivity of ultraviolet detectors, their use requires a more elaborate sample processing, with extraction stages using organic solvents such as hexane[29,30], hexane/methanol[31,32] or ethanol[33], followed by the concentration of the sample in order to then inject it into the HPLC system.

In this study, two CoQ_{10} extraction methods in human plasma were tested: methanol:hexane, and 1-propanol, both with UV detection. In the attempt to improve the methods of CoQ_{10} extraction by UV detection, the addition of some surfactants such as

SDS (sodium dodecyl sulfate), Triton X-100 and Tween-20 was tested as to the possibility of obtaining a better recovery at the end of the extraction process. Other variables that may directly influence the result of CoQ_{10} quantification were also studied: temperature (4°C and 19°C) and types of tubes (glass and polypropylene).

OBJECTIVE

To establish a routine for extraction of the total levels of CoQ_{10} in human plasma through the ultra high performance liquid chromatography (UHPLC) analysis.

ANALYTICAL METHODS

Reagents and Solvents

Chromatographic grade solvents methanol, hexane and 1-propanol were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich (Missouri, USA), respectively. Reagents such as SDS (sodium dodecyl sulfate), Triton X-100 and CoQ_{10} standard (> 98% purity) were obtained from Sigma-Aldrich (Missouri, USA) and Tween 20 was obtained from Amresco (Solon, USA). Human albumin solution at 20% was provided by Grifols Brasil Ltda.

Standard solution

The standard solution of CoQ_{10} was prepared at the concentration of 1mg/mL using 1-propanol as dilution solvent, and storing it at -20°C protected from light.

Sample preparation

For initial optimization and standardization studies of the CoQ_{10} extraction process, we used a 4% human albumin solution with the objective of simulating plasma samples, which then received the addition of the standard solution of CoQ_{10} resulting in the final concentration of 1000mg/mL. After definition of the best method for extraction, the blood collection protocol was followed as described below.

Samples of blood were collected from the same control individual by peripheral venous puncture into glass tubes containing anticoagulant citric acid, sodium citrate and dextrose, and maintained at 4°C. The plasma was obtained after centrifugation at 894.2g for 10 minutes at 4°C, transferred to Eppendorf-type tubes and stocked at -80°C for further analysis. Sample collection was performed after obtaining the signature on the informed consent form approved by the Research Ethics Committee of the *Hospital Israelita Albert Einstein* (No.10/1465).

Methods for extraction of CoQ_{10}

CoQ_{10} extraction with methanol:hexane

The initial protocol was used[34], with the following modifications: 700mL of plasma, 100mL of detergents (SDS, Triton X-100 and Tween-20) at concentrations of 1%, 3%, 5% and 10% separately, 1400mL of methanol and 1500mL of hexane were added to a polypropylene tube and a glass tube. The samples were submitted to mechanical shaking for one minute and centrifuged at 1752.8g for 10 minutes at 10°C. The supernatants were transferred to a new tube and evaporated without heating under a flow of nitrogen for 20 minutes. Next, the residues were resuspended in 60mL of mobile phase of methanol:hexane (85:15, v/v), homogenized under vortex mechanical shaking for 15 seconds and orbital shaking for 15 minutes. For the chromatographic analysis, 20mL were used. Extractions were performed in triplicate at 19°C (controlled temperature) and at 4°C (ice bath).

CoQ_{10} extraction with 1-propanol

The initial protocol was used[27] with the following modifications: 700mL of plasma, 100mL of detergents (SDS, Triton X-100 and Tween 20) at the concentrations of 1%, 3%, 5% and 10% separately, 1400mL of 1-propanol were added to a polypropylene tube and a glass tube. The samples were submitted to shaking for one minute and were centrifuged at 894.2g for 10 minutes at 10°C, then transferred to an "amber vial" with the help of a filtrating unit with 0.22mm, 13mm. The chromatographic analysis was performed by means of a 20mL injection of the sample. The extractions were performed in triplicate at 19°C (controlled temperature) and at 4°C (ice bath).

Chromatographic analysis and detection of CoQ_{10}

The equipment used for Ultra High Performance Liquid Chromatography (UHPLC) was HP1290 (Hewlett-Packard) constituted by an automatic sampler, binary pump, and UV detector with variable wavelength. The detection was made at 275nm. For the chromatographic separation, an analytical column was used, Zorbax Eclipsy C18® (50 x 2.1mm, 1.8μm), with an equivalent guard column, both obtained from Hewlett-Packard and maintained at 45°C during the analysis. The CoQ_{10} displayed a retention time of 1.5 minutes and the total time of chromatographic analysis was 3.5 minutes. The

Table 1. Analysis of the variants that interfere in the process of CoQ_{10} extraction: addition of detergents, tubes and temperature conditions

Variables	C V%	Mean mUA	Response UA
Samples with no addition of detergent, in polypropylene tubes, 4ºC	14.2	8.4	-
Samples with addition of 10% Triton X-100 detergent at 10%, in polypropylene tubes, 4ºC	5.8	11.5	3.1
Samples with addition of Tween-20 detergent at 3%, in polypropylene tubes, 4ºC	4.3	12.7	4.3
Samples with no addition of detergent in polypropylene tubes, 19ºC	3.1	7.3	-
Samples with the addition of Triton X-100 detergent at 10%, in polypropylene tubes, 19ºC	3.3	8.1	0.8
Samples with addition of Tween-20 detergent at 3%, in polypropylene tubes, 19ºC	8.7	7.6	0.3
Samples with no addition of detergent in glass tubes, 4ºC	4.8	7.5	-
Samples with addition of Triton X-100 detergent at 10%, in glass tubes, 4ºC	9.9	11.2	3.7
Samples with the addition of Tween-20 detergent at 3%, in glass tubes, 4ºC	8.9	9.0	1.5
Samples with no addition of detergent, in glass tubes, 19ºC	3.6	5.6	-
Samples with the addition of Triton X-100 detergent at 10%, in glass tubes, 19ºC	3.3	8.1	2.5
Samples with the addition of Tween-20 detergent at 3%, in glass tubes, 19ºC	5.6	7.3	1.7

CV: coefficient of variation; mUA: unit of area of chromatographic peak.

mobile phase used was methanol-hexane (85:15; v/v) with a flow rate of 0.45mL/min.

RESULTS

Establishment of the CoQ_{10} extraction protocol

For the establishment of the CoQ_{10} extraction protocol, initially performed were tests using 4% human albumin solution with added CoQ_{10} solution of 1000mg/mL. The extraction methods methanol:hexane and 1-propanol were tested in the presence and absence of surfactants such as SDS (anionic), Triton X-100 (non-ionic) and Tween-20 (non-ionic) at concentrations of 1%, 3%, 5% and 10%, at 4°C and 19°C. The first results indicated that the extraction method using methanol:hexane in the presence of the surfactants Triton X-100 and Tween-20 showed better rates of CoQ_{10} recovery, while with the use of SDS, the reproducibility and efficiency were much lower (data not shown).

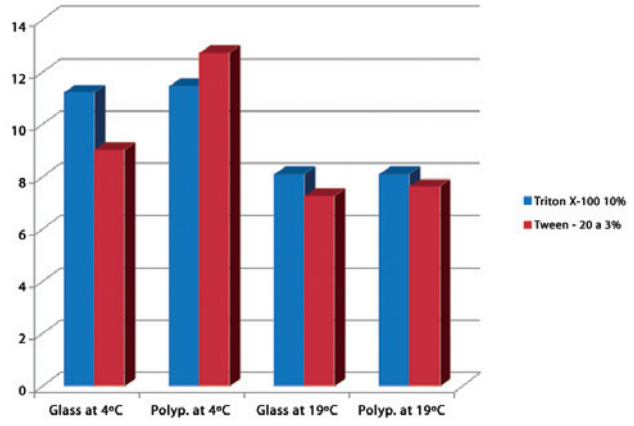

Figure 1. Result of the extraction of CoQ_{10} in plasma samples: comparison of the effect of Triton X-100 at 10% and Tween-20 at 3%

The same extraction protocol used in the tests with CoQ_{10} added to albumin was applied to samples of plasma from control individuals without the addition of CoQ_{10} to verify the reproducibility of the method in real samples. The samples were analyzed as to temperature of extraction (controlled at 19°C and at 4°C, ice bath)

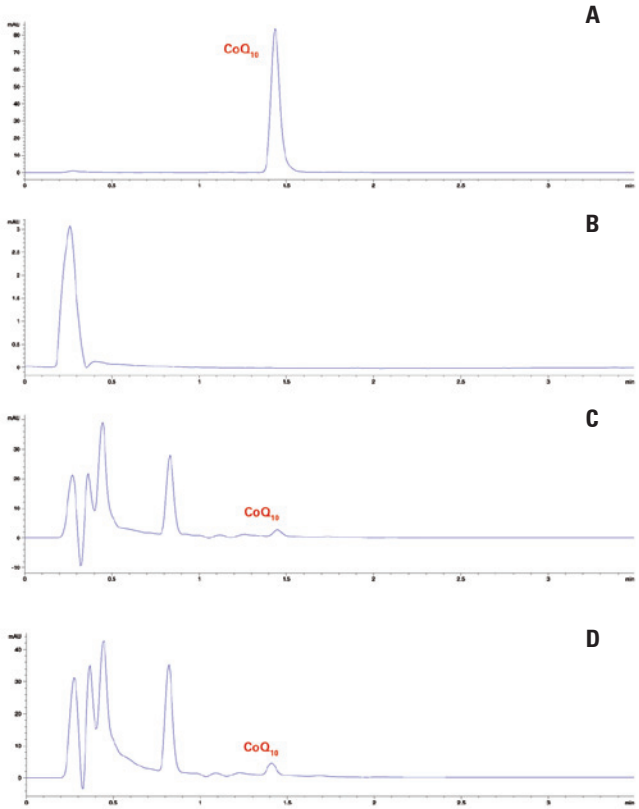

Figure 2. Chromatograms. (A) standard solution of CoQ_{10} at the concentration of 1mg/mL; (B) control sample of reactive substances; (C) control sample of plasma with no addition of detergent; (D) sample of plasma control using the detergent Tween-20 at 3%

and as to the composition of the tubes used for the extraction (polypropylene and glass), as per table 1.

The results demonstrate that the extractions performed in real samples of plasma presented a similar response as that shown with a solution of albumin in which the use of the detergent Tween-20 at 3%, at 4°C, using methanol:hexane resulted in the best response obtained for the detection of CoQ_{10} (Figure 1). In addition to presenting the best recovery, there was also good reproducibility between the replicates with a CV (coefficient of variation) of 4.3%. Thus, the option of using a surfactant such as Tween-20 resulted in an improvement (51.2%) in efficiency.

The chromatograms demonstrate the chromatographic quality obtained with the mobile phase composed of methanol:hexane (85:15, v/v) with a flow of 0.45mL/min. (Figure 2). The retention time of $CoQ_{10}$was 1.45 minutes and the peak purity (99%) was obtained through the analysis of its spectrum (ChemStation, Agilent).

DISCUSSION

The application of chromatographic techniques has been widely used in studies of enzymatic determination and pharmacokinetics for therapeutic monitoring. Recently, the interest in determining the levels of CoQ_{10} in plasma and other types of cells or tissues has been the target of various studies, given the possibility of effective treatment in neurological patients or those with metabolic diseases through the oral supplementation[35].

CoQ_{10} is present in circulating plasma associated with lipoproteins, and its concentration is directly related to the concentration of cholesterol present in the plasma[36]. Due to this characteristic binding of CoQ_{10} to lipophilic molecules, we chose to use surfactants as aids in the extraction process with the intent of breaking this type of bond and thus increasing the levels of detection of the coenzyme. Some authors demonstrated that the action of SDS in plasma significantly increases the recovery of CoQ_{10} after the extraction process[37]. Conversely, the effect of Triton X-100 on the solubilization of membranes was observed both in proteins and in lipids[38]. Indeed, when initiating the first experiments, low rates of CoQ_{10} were attained without the addition of surfactants during the process of extraction, observed by the small chromatographic peak area (mUA).

In this study, the best results were achieved with the methanol:hexane extraction method when compared to 1-propanol. Some authors have used 1-propanol as a solvent for precipitation of proteins due to its greater lipophilic character, but the success of this technique only occurs when used in electrochemical-type detectors, since they are more sensitive than what was used in this study (UV). This occurs because this technique does not allow the concentration of the sample in the process of extraction, and the addition of 1-propanol merely acts by precipitating the proteins and diluting the sample, making it more difficult to quantify CoQ_{10} with the use of UV detectors. It was also observed that the use of surfactants Triton X-100 and Tween-20 resulted in better rates of recovery of CoQ_{10}, while the use of SDS showed lower reproducibility and efficiency. Additionally, even when used at low concentrations, SDS promoted the formation of a "cloud point" that hindered the separation of the organic and aqueous phases.

When the established protocol using the human albumin solution was applied for the extraction of CoQ_{10} in real samples of plasma, the response was similar to that with the use of surfactant Tween 20 at 3%, at 4°C in methanol:hexane, resulting in a better recovery, good reproducibility among the replicates, a CV of 4.3%, and an increase of 51.2% in efficiency of the extraction process.

Temperature is also a relevant parameter to be evaluated when the substance studied is an enzyme, which since it is thermolabile, may easily suffer denaturation at higher temperatures.

The best results of this study showed that the control of the temperature (4°C) is of vital importance during the extraction process. It was also noted that there was better separation of the organic and aqueous phases when polypropylene tubes were used, contributing, in fact, to an improvement in the efficiency of the extraction.

CONCLUSION

By the analyses performed, it was possible to observe that the addition of the surfactant Tween-20 promoted an increase in the recovery of CoQ_{10} by the methanol:hexane extraction method. This method showed good reproducibility, with a low CV and high sensitivity, since CoQ_{10} was detected in samples of plasma of a control individual using a UV-type detector.

The use of UHPLC equipment allowed a total analysis with total run time of 3.5 minutes, enabling the rapid achievement of results, considered mandatory for laboratory routines.

Since this stage of method optimization was concluded, this extraction protocol is in the validation phase which will allow its applicability for future clinical tests with a guarantee of reliable results.

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