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Highly sensitive and selective determination of redox states of coenzymes Q_9 and Q_{10} in mice tissues: Application of orbitrap mass spectrometry

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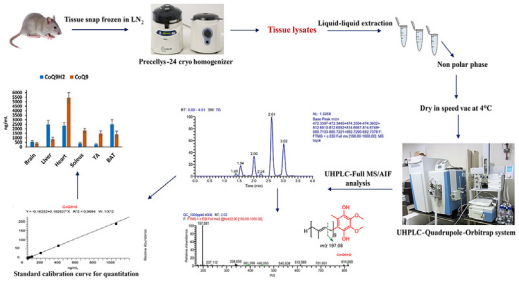
HIGHLIGHTS

- A high-resolution/accurate-mass UHPLC-MS/MS method was developed and validated.
- It was successfully applied to measure redox states of CoQ_9 and CoQ_{10} in mice tissues.
- Different extractive solvents and diluents were evaluated concerning the instability of reduced CoQs.
- Present method offers enhanced sensitivity and selectivity compared to existing methods.

GRAPHICAL ABSTRACT



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ABSTRACT

Coenzyme Q (CoQ) is a redox active molecule that plays a fundamental role in mitochondrial energy generation and functions as a potent endogenous antioxidant. Redox ratio of CoQ has been suggested as a good marker of mitochondrial dysfunction and oxidative stress. Nevertheless, simultaneous measurement of redox states of CoQ is challenging owing to its hydrophobicity and instability of the reduced form. In order to improve the analytical methodology, paying special attention to this instability, we developed a highly sensitive and selective high-resolution/accurate-mass (HR/AM) UHPLC-MS/MS method for the rapid determination of redox states of CoQ_9 and CoQ_{10} by ultra-performance liquid chromatography-hybrid quadrupole-Orbitrap mass spectrometry. CoQs were extracted using hexane with the addition of butylated hydroxytoluene to limit oxidation during sample preparation. Chromatographic separation of the analytes was achieved on a Kinetex C_{18} column with the isocratic elution of 5 mM ammonium formate in 2-propanol/methanol (60:40) within 4 min. A full MS/all ion fragmentation (AIF) acquisition mode with mass accuracy < 5 ppm was used for detection and determination of redox states of CoQ_9 and CoQ_{10} in healthy mice tissues using reduced and oxidized CoQ_4 as internal standards. The validated method showed good linearity ($r^2 \geq 0.9991$), intraday, inter-day precision (CVs $\leq$ 11.9%) and accuracy (RE $\leq \pm$15.2%). In contrast to existing methods, the current method offers enhanced sensitivity (up to 52 fold) with LOD and LOQ ranged from 0.01 to 0.49 ng mL^{-1} and 0.04 −1.48 ng mL^{-1}, respectively. Moreover, we evaluated various diluents to investigate bench top stability (at 4 °C) of targeted analytes in tissue samples during LC-MS assay up to 24 h. Ethanol was determined to be an optimum diluent without any significant oxidation of reduced CoQ up to 24 h. The developed

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method offers a rapid, highly sensitive and selective strategy for the measurement of redox states of CoQs in clinical studies.

1. Introduction

Coenzyme Q (CoQ), also known as ubiquinone, is a lipophilic molecule consisting of a redox active benzoquinone head conjugated to a polyisoprenoid side chain of species-specific length (6–10 subunits; within mammals only CoQ_9 and CoQ_{10} are found) [1,2]. It presents mainly in the inner mitochondrial membrane (IMM) of eukaryotic cells and exists in three redox states, *i.e.* oxidized (CoQ), free-radical intermediate (semiquinone, $CoQH^{\cdot}$) and reduced (CoQH2) [1,3]. CoQ plays a central role in cellular bioenergetics and functions as an electron carrier in the mitochondrial electron transport chain (ETC) for ATP production. Moreover, it acts as a potent endogenous antioxidant in its reduced form that prevents lipid peroxidation, protein carbonylation and oxidative damage to DNA, also capable of regenerating other powerful antioxidant e.g. α-tocopherol and ascorbate [1,4]. Therefore, CoQ has been suggested as a good marker of mitochondrial dysfunction and oxidative stress. In addition, it has been used as a nutritional supplement or therapeutic agent in several diseases, *i.e.* cancer [5], cardiovascular [6] and neurodegenerative diseases [7].

As the vital functions of CoQ depend on its concentration and redox ratio, measurement of redox states has recently gained significant interest in clinical research [8–11]. A variety of analytical methods including high-performance liquid chromatography (HPLC) coupled with ultraviolet (UV) [11–16], chemiluminescence [17] and electrochemical detection (ECD) [15], ultra/high-performance liquid chromatography-tandem mass spectrometry (HPLC/UHPLC-MS/MS) [18–22] have been reported for assessment of CoQ_9 and CoQ_{10} in biological fluids, cells, and tissues. Nevertheless, due to the hydrophobicity and instability of the reduced form of CoQ, highly sensitive and selective analytical methods should be further explored for the rapid measurement of redox states.

Only a handful of methods have been reported for simultaneous assessment of redox states of CoQ_9 or CoQ_{10} however, these methods are limited by poor sensitivity and long analysis time [19,23–25]. Moreover, the reported LC-MS/MS methods are mainly based on the triple quadrupole mass spectrometer (TQ-MS) that is prone to false compliant and non-compliant results due to low resolution [26–28]. Recent advancements in high-resolution mass spectrometry (HRMS) instrumentation resulted in the quadrupole-orbitrap (Q-Exactive) hybrid tandem mass spectrometer that combines the mass selection/isolation capability of the quadrupole with the high resolution/accurate mass (HR/AM) measurement capacity of Orbitrap, thereby potentially avoiding false positive and false negative findings [28–30]. Furthermore, several studies have demonstrated that in contrast to TQ-MS, HR/AM methods with resolution >50,000 full width at half-maximum (FWHM) and accuracy < 5 ppm offers higher selectivity for trace level detection and quantification of compounds in complex biological matrices [28–31].

In this context, we developed and validated a highly sensitive and selective HR/AM UHPLC-MS method in full MS/all ion fragmentation (AIF) acquisition mode for rapid assessment of redox states of CoQ using UHPLC-Q-Orbitrap. The developed method was successfully applied to determine redox states of CoQ_9 and CoQ_{10} in various tissues, *i.e.* brain, heart, liver, brown adipose tissue (BAT), soleus and tibialis anterior muscle (TA) of healthy mice using reduced and oxidized CoQ_4 as internal standards (IS). Moreover, we have evaluated full MS/AIF and targeted SIM/data-dependent MS/MS (tSIM/ddMS/MS) acquisition modes of Q-Orbitrap with respect to sensitivity and different extraction solvents as well as diluents concerning the instability of reduced CoQ in biological matrices.

2. Materials and methods

2.1. Chemicals and reagents

LC-MS grade methanol, 2-propanol, 1-propanol, ammonium formate, chloroform and ACS reagent grade hexane, ethanol, butylated hydroxytoluene (BHT), sodium borohydride ($NaBH_4$) and 6 N hydrochloric acid (HCl) solution were obtained from Fisher Scientific (Pittsburgh, PA, USA). Mass spectrometry calibration solutions (Thermo Scientific Pierce LTQ Velos ESI positive and negative ion calibration solutions) were procured from Thermo Fisher Scientific (San Jose, CA, USA). Coenzyme Q_4 (purity ≥ 90%), used as an internal standard, coenzyme Q_9 (purity ≥ 95%) and coenzyme Q_{10} (purity ≥ 98%) were procured from Sigma–Aldrich (St. Louis, MO, USA), Cayman Chemical Company (Ann Arbor, MI, USA), Fisher Scientific, respectively. Ultrapure water was obtained from Milli-Q system (Millipore Co., Billerica, MA, USA).

2.2. Instrumentation and analytical conditions

The UHPLC-MS/MS analysis was performed on a Hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive, Thermo Scientific, Waltham, MA, USA) hyphenated with a Thermo Scientific Accela 1250 UHPLC system via electrospray ionization source. The Accela UHPLC system equipped with a quaternary pump, vacuum degasser and an open autosampler with a temperature controller. Chromatographic separation of targeted analytes and internal standard was achieved on a Kinetex C_{18} 150 × 2.1 mm (2.6 μm, 100 Å) column (Phenomenex, Torrance, CA) with isocratic elution of 5 mM ammonium formate in 2-propanol/methanol (60:40) at a flow rate of 260 μL min^{-1} within 4 min. The sample injection volume was 5 μL.

Detection and determination was performed in full MS/AIF mode with positive electrospray ionization mode. Optimized MS parameters were as follow: spray voltage, 4.0 kV; capillary temperature, 300 °C; sheath gas, 50 (arbitrary units); auxiliary gas, 10 (arbitrary units); microscans, 1; maximum injection time, 200 ms; AGC target, 1e6/5e5; mass resolution, 140,000/70,000 FWHM; *m/z* range, 150–1000, higher energy collisional dissociation (HCD) energy; 22 eV. Nitrogen was employed as a collision gas. The mass spectrometer was calibrated before analysis using commercial calibration solutions to maintain mass accuracy below 5 ppm. The Xcalibur 2.2 software (Thermo Scientific, Waltham, MA, USA) was used to control the instrument and for data acquisition and processing. Q Exactive 2.2 SP 1 (Thermo Scientific) was used to control the tuning window of mass spectrometer.