Quantitation of Ubiquinone (Coenzyme Q₁₀) and Retinyl Palmitate in Serum/Plasma using Liquid Chromatography Electrospray Tandem Mass Spectrometry

Richard E. Mathieu, Catherine P. Riley and Carmen L. Wiley,
Pathology Associates Medical Laboratories, Spokane, WA

Abstract

Introduction: The monitoring of vitamin levels in patients utilizing supplementation therapy is important for the prevention of potential toxicities and measurement of treatment outcomes. Vitamins play an essential role in human health and wellness. While essential, many vitamins cannot be synthesized within the body and must be obtained externally, most commonly from dietary intake. Retinyl Palmitate (RP) and Ubiquinone (Coenzyme Q₁₀) are powerful antioxidants that are responsible for maintaining a variety of essential functions throughout the body. Coenzyme Q₁₀ is considered an essential co-factor in the mitochondrial respiratory chain, responsible for oxidative phosphorylation and plays a unique role in the electron transport chain. Retinyl Palmitate (RP) is a synthetic alternative form of vitamin A (retinol) and is widely used in nutritional supplements and cosmetics.1,2 Vitamin A serves in multiple roles: it is important for growth and development, maintenance of the immune system and good vision.

Mindful of the numerous unique features and the idiosyncratic nature of these molecules we set out to improve their detection utilizing positive electrospray ionization tandem mass spectrometry (ESI-MS/MS). Our aim was to improve the sensitivity and simplify the extraction process and create a robust method suitable for the routine analysis from serum or plasma samples.

Method: Aliquots of 200 µL of standard, control and patient serum/plasma samples were spiked with Coenzyme Q₁₀ – [D₉] serving as an internal standard. The extraneous proteins were precipitated from the samples with ethanol and followed by the addition of hexane. Samples were analyzed on an API 3200 triple quadruple mass spectrometer (Applied Biosystems) equipped with a TurboIonSpray® source and a Shimadzu Prominence 20A HPLC system. Chromatographic separation was achieved using a LUNA 3µm PFP2 50 X 2.0 mm, 100Å column (Phenomenex) at a flow rate of 0.85mL/min, for a total run time of 5 minutes. The MS/MS was operated in positive electrospray and multiple reaction monitoring (MRM). Two transitions were monitored for each analyte: 880.7 > 197.3 and 880.7 > 237.3 for Coenzyme Q₁₀ ([CoQ₁₀] + m/z 269.4 > 93.0 and 269.4 > 107.0 for Retinyl Palmitate and 889.7 > 206.3 and 889.7 > 189.1 for Coenzyme Q₁₀ – [D₉]).

Calibration material was prepared in ethanol and the concentration confirmed by using a UV spectrophotometer. The respective extraction coefficient and corresponding wavelength absorption data was used to verify the integrity and concentration of the calibration material.

Results: Validation was performed on 20B previously run samples, adhering to CLSI-derived protocols. Data analysis was performed using EP Evaluator\(^\text{™}\) for the following parameters: precision (inter-, intra-, LOD/LOQ), linearity, recovery, carryover, interference and stability. CoQ₁₀ and RP demonstrated the assay was linear from 0.22 - 12.0 µg/mL with a slope of 0.954 and an observed error of 6.2%. RP demonstrated linearity from 0.03 – 2.00 µg/mL and a slope of 0.994 with an observed error of 8.8%. Both analytes recovered between 93.0 - 106.0% and the correlation coefficient (r) between CoQ₁₀ and RP was 0.9205 and 0.9958 respectively. Correlation of our assay demonstrated no significant bias and all precision studies had CDVs ≈ <0.0%. No significant interfering substances were observed and there was no evidence of instrument carry-over observed.

Conclusion: We have successfully developed and validated a robust, simple and effective method for extracting and monitoring Coenzyme Q₁₀ and Retinyl Palmitate levels in patient samples.

Introduction and Background

Coenzyme Q₁₀ and Retinyl Palmitate are lipophilic molecules that serve as powerful antioxidants and are responsible for maintaining a variety of essential functions throughout the body. Coenzyme Q₁₀ is responsible for oxidative phosphorylation, functions as an electron carrier and is an essential component of the electron transport chain. Retinyl Palmitate is a common synthetic variant of Vitamin A and is broken down in the small intestine to Retinol. It is important in the prevention of cancer, night blindness, and infections. The measurement of these compounds has been primarily performed utilizing high pressure liquid chromatography (HPLC) and spectrophotometry. The HPLC methodology can lend itself to extended run times, large sample volumes, subjective interpretations and inadequate sensitivities. Mindful of the unique features and the idiosyncratic nature of these molecules we set out to improve the detection utilizing positive electrospray ionization tandem mass spectrometry (ESI-MS/MS). Our goal was to improve the sensitivity and to simplify the extraction process to create a robust method suitable for the routine analysis from serum or plasma samples.

Materials and Methods

Sample: Two hundred and eight previously tested samples (138 CoQ₁₀, and 70 RP) spanning the analytical range of the assay for both analytes were used to correlate our assay against the method performed at a reference laboratory. No distinction was made as to the patient’s condition, age, gender or race.

Calibrators: To monitor for signs of degradation, the concentration of the calibrators prepared were extended run times, large sample volumes, subjective interpretations and inadequate sensitivities. Mindful of the unique features and the idiosyncratic nature of these molecules we set out to improve the detection utilizing positive electrospray ionization tandem mass spectrometry (ESI-MS/MS). Our goal was to improve the sensitivity and to simplify the extraction process to create a robust method suitable for the routine analysis from serum or plasma samples.

Discussion

The analysis of Coenzyme Q₁₀ and Retinyl Palmitate by LC-MS/MS utilizing the highly versatile and robust PFP analytical column coupled with a simplified liquid-liquid extraction is an appealing alternative to the previous methods that were attempted during the initial phases of our validation process. The accurate and reproducible measurement of these compounds was complicated by their idiosyncratic nature and instability in the presence of light and ambient temperatures. Imperative to our study was the ability to quantify these molecules consistently and accurately over time as well as the ability to establish and maintain stable, consistent and robust curves.

Intermediate calibrators were prepared in triplicate and analyzed on a spectrophotometer to determine the concentration of the material. We learned early in the development of this assay that the spectrophotometer could only serve as one of the many tools that we would employ to ensure the integrity of our calibration material. The β-ionone and quinone functional groups on the Retinyl Palmitate and Coenzyme Q₁₀ respectively account for approximately 70% of the reported absorbance; therefore, if this component were present in the solution the measured absorbance and calculated concentration would be higher than the actual amount of available analyte present.

The result shifting in the molecular weight of greater than ±2.5 Daltons from the degradation of the analyte could result in the inability to detect the target compound entirely. This factor becomes more important when considering ion ratios and the measured amount of the parent compound are off or the calibration material is in a state of degradation, and the resulting concentration is higher or lower than the assigned values. Careful handling of the stock reagents was another critical step in quality assurance when preparing the calibration material. We used ethanol stabilized with butyLATED hydrogeniosile (BHA) to prevent the breakdown of the material during storage and use. The conjugated aromatic ring of BHA is able to stabilize free radicals by sequestering them and serves as a free radical scavenger in solution. We learned early in the hopes of preventing further degradation reactions within the calibration material.

The extraction process begins with the addition of a stable isotopic deuterated internal standard, in the form of Coenzyme Q₁₀ – [D₉] to 200 µL of serum. The stock reagents are precipitated using ethanol and mixed, followed by the addition of n-hexane to facilitate the migration of the analytes into the upper organic layer. These samples are centrifuged and placed in the -70°C freezer to isolate the aqueous layer by solidifying it.

To ensure that the liquid organic layer is poured off to a clean tube and placed in a TurboVap®-Dry down unit and removed with dry nitrogen. Extracted concurrently with the samples are six levels of calibration, prepared by serial dilution in ethanol. The curve is weighted 1/s and processed using MultiQuant® software. Each batch of prepared calibration material is run against the previous set several times prior to being put into use; curves are routinely overlaid against reference curves that are stored in the MultiQuant® Vitamin Assay program to monitor for signs of degradation. In addition, QC is closely monitored to watch for trends or spikes, the technical staff is aware that degradation of the QC material will occur at roughly the same rate as that of the calibration material, making the need for vigilant monitoring of all of the components of this assay to ensure integrity and reporting standards are maintained. Numerous other validation studies were performed to ensure the performance of this assay including, but not limited to, linearity, stability, Carryover, LOD/LOQ/UROL, Dilution Studies, Ion Suppression, Precision, and verification with commercially available quality control material.

Conclusion

Our goal was to improve the sensitivity and simplify the extraction process and create a robust method suitable for the routine analysis of Coenzyme Q₁₀ and Retinyl Palmitate from serum or plasma samples. Due to the instability and ease of degradation of these molecules, numerous safeguards were established and are routinely monitored to ensure the integrity of the reported results. We have developed a simple extraction procedure partnered with a highly sensitive and robust ESI-MS/MS method suitable for the routine analysis of Coenzyme Q₁₀ and Retinyl Palmitate.

References