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Analysis of CoQ_{10} in rat serum by ultra-performance liquid chromatography mass spectrometry after oral administration

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Abstract

A UPLC–MS method for determining Coenzyme Q_{10} (CoQ_{10}) levels in rat serum was developed. CoQ_{10} was quantitatively extracted into 2-propanol using a fast extraction procedure. The separation of CoQ_{10} was performed on a Waters Acquity UPLC™ BEH C_{18} column (1.7 μm, 1.0 mm × 50 mm) with the mobile phase containing acetonitrile, 2-propanol, and formic acid (90:10:0.1) over 5 min. The sensitivity of this method allows for the quantitation of 50 ng/mL CoQ_{10} in serum (S/N = 10). The linearity of this method was found to be from 50 to 20,000 ng/mL. The precision was less than 10% (intra- and inter-day), and the average extraction recovery was between 90 and 105%. This procedure provides a precise, sensitive and direct assay method for the determination of CoQ_{10} in rat serum after oral administration. This method could be applied to further pharmacokinetic studies of CoQ_{10}.

Keywords: Coenzyme Q_{10}; UPLC; MS; Rat serum; Pharmacokinetic

1. Introduction

Coenzyme Q_{10} (CoQ_{10}), also known as ubiquinone, is a lipid-soluble compound that concentrates in the mitochondria and acts as an electron carrier in the electron transport chain [1]. Its reduced form has also been implicated as the only endogenously synthesized and lipid-soluble antioxidant protecting cellular membranes, particularly those of the mitochondria and plasma lipoproteins from free radical damage [2]. Elevated levels of CoQ_{10} in blood may have several important functions. Among these are an enhanced protection of LDL from oxidation and prevention of free-radicals caused by neutrophils in inflammatory diseases and prevention of oxidative injury by endothelial cells resulting from ischemia-reperfusion. It is largely for these reasons, together with its apparently nontoxic nature, that attention has been drawn to the possible clinical and therapeutic uses of CoQ_{10} especially in the field of cardiology. It has been used as a therapy for a number of cardiac dysfunctions and has been available for clinical use in Japan for more than 20 years [3].

Methods of obtaining levels of CoQ_{10} in blood and plasma are of clinical significance in regard to oxidative stress and antioxidant defense. A number of studies have focused on the determination of plasma and tissue levels of CoQ_{10} in patients prior to and after treatment [4–15]. Several methods have been described for assaying either total CoQ_{10} or the reduced and oxidized forms in plasma. HPLC with electrochemical or UV detection techniques were used for identification and quantification of CoQ_{10} in biological samples [4–12]. Spectrophotometric [13,14] and chemiluminescent [15] techniques were also used in the quantitative determination of CoQ_{10} in human plasma. The previous methods of assaying the concentration of CoQ_{10} in plasma or serum have shortcomings. Various methods require that the CoQ_{10} be extracted from the plasma and concentrated. Losses in CoQ_{10} can occur during the drying and concentration steps. HPLC–UV methods employed for assaying CoQ_{10} usually quantify the oxidized coenzyme at 275 nm. It is commonly known that CoQ_{10} is completely oxidized during the extraction and HPLC procedure, but this is not necessarily the case when the sample is fresh, and the reduced form of CoQ_{10} largely predominates. Accordingly, these methods can result in the underestimation of the CoQ_{10} concentration if all of the CoQ_{10} is not oxidized. Liquid chromatography coupled to tan-

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dem mass spectrometry is known to be a powerful separation and detection technique in a large number of analytical fields. The quantification of CoQ_{10} in biological fluids or dairy products by LC/MS or LC–MS/MS has been reported [16–20]. However, in these reported LC–MS methods, only Hansen et al. determined the concentration of CoQ_{10} in human serum. The retention time of CoQ_{10} was 5 min or so and the whole running time for the serum sample was 8 min, which was not short enough for high sample throughput. Recently, the search for a method with a significantly reduced analysis time and increased sample throughput has resulted in the development of a new technology termed ultra-performance liquid chromatography coupled with tandem mass (UPLC–MS/MS). This paper presents the development and validation of an assay method for the determination of CoQ_{10} in rat serum by UPLC–MS with an APCI interface operated in positive mode. The analytical procedure described herein will enable investigators and others to reliably determine concentrations of CoQ_{10} in rat serum. In this method, high sample throughput is achieved by simple sample preparation and short chromatographic run times less than 5 min. This analytical method was successfully applied to pharmacokinetic studies of CoQ_{10} in rats after oral dosage.

2. Experimental

2.1. Chemicals

CoQ_{10} and CoQ_9 standards were purchased from Sigma (St. Louis, MO, USA, purity >98%); CoQ_{10} soft gels were purchased from Nature's Bounty, Inc. (Bohemia, NY, USA); HPLC grade acetonitrile, methanol, 2-propanol and water were from Fisher (Fairlawn, NJ, USA); Reagent grade formic acid was purchased from Sigma–Aldrich (95%, St. Louis, MO, USA); Drug-free rat serum was bought from Innovative research Inc. (Southfield, MI, USA).

2.2. Equipment

All chromatographic experiments were conducted using a Waters Acquity UPLC system (Milford, MA, USA). Chromatographic separations were performed on a Waters Acquity UPLC™ BEH C_{18} column (1.7 μm, 1.0 mm × 50 mm). The mobile phase consisted of acetonitrile:2-propanol:formic acid (90:10:0.1, v/v). The flow rate was 0.25 mL/min, the sample injection volume was 10 μL, and the duration of the run was 6 min.

2.3. Mass spectrometry

The MS equipment consisted of a Waters Micromass Quattro Micro™ triple-quadrupole system. The MS system was controlled by version 4.0 of MassLynx software. Ionization was performed in positive APCI mode. MS conditions were the following: Corona voltage 15 μA, cone voltage 35 V, extractor voltage 5 V, and RF lens voltage 0.5 V. The source and APCI probe temperatures were 150 and 450 °C, respectively, and the desolvation and cone gas flow were 550 and 25 L/h, respectively.

The selected mass-to-charge (m/z) ratio transitions of the CoQ_{10} and CoQ_9 ions $[M+1]^+$ used in the selected ion reaction (SIR) were as follows: CoQ_{10}, m/z 863 and CoQ_9, m/z 795. The dwell time was set at 200 ms. The conditions were optimized by direct infusion of standard solutions prepared in 2-propanol and delivered by a syringe pump at a flow rate of 10 μL/min.

2.4. Standard and working solutions

Individual standard stock solutions of CoQ_{10} (1 mg/mL) and internal standard CoQ_9 (20 μg/mL) were prepared by accurately weighing the required amounts into separate volumetric flasks and dissolving in 2-propanol.

Calibration standard for CoQ_{10} was prepared by diluting 100 μL CoQ_{10} solution in a 5 mL volumetric flask with blank rat serum. It provided a working solution with a concentration of 20,000 ng/mL. Further dilutions were made from this stock solution with blank serum to afford serum standards in the range of 50–20,000 ng/mL. Quality control (QC) samples at three different concentrations (50, 500, and 5000 ng/mL) were prepared separately. The QC samples were used to assess the accuracy and precision of the assay method. All the calibration and QC samples were then extracted by the method described in the subsequent section and analyzed. The QC samples were stored along with the test samples at −20 °C until needed.

2.5. Sample preparation

A mixture consisting of 50 μL of serum and a 10 μL aliquot of internal standard solution was placed in an Eppendorf microtube for processing. 2-Propanol (100 μL) was added to precipitate the proteins, and the microtube was vortexed for 1 min and centrifuged for 10 min at 12,000 rpm (Eppendorf 5415C centrifuge). The supernatant was filtered through a 0.45 μm filter (Waters 13 mm GHP 0.2 μm) before analysis.

2.6. Oral formulation

The oral formulation of CoQ_{10} used in this experiment was purchased over the counter as a soft gel. Each soft gel capsule contained 50 mg of CoQ_{10}, vitamin E, rice bran oil, gelatin, glycerin, soy lecithin, yellow beeswax, titanium dioxide color and annatto color. The oral formulation was administered to rats at a dose of 200 mg/kg by a gavage tube, and the dose was administered into the stomach of the rat. The concentration of CoQ_{10} in the soft gel was analyzed by UPLC–MS method.

2.7. Animal study

All the experimental animal procedures were approved and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Mississippi. The Sprague–Dawley rats (200–220 g) were obtained from the Harlan Company (Indianapolis, IN) and already had a polyethylene cannula inserted into the right jugular vein. The rats were housed in standard cages and allowed

free movement and access to water 12 h before the experiment and during the experiment.

Blood samples were taken from the indwelling cannula. An initial blood volume of 0.1 mL was withdrawn to clear the line of heparinised saline. A fresh syringe was then used to withdraw a 0.15 mL blood sample which was placed in a microtube. The blood samples were allowed to clot at 0 °C for 30 min. Serum was separated by centrifugation at 3000 rpm for 10 min at 4 °C and was processed immediately.

3. Method validation

The validation of the LC–MS method included linearity, selectivity, inter- and intra-assay precision and accuracy, stability and recovery.

3.1. Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. The potential interfering substances include endogenous matrix components, metabolites and decomposition products.

3.2. Linearity and lower limit of quantification (LLOQ)

Calibration curves were calculated based on the relationship between the ratio of the peak area of CoQ_{10} to that of the internal standard and the theoretical concentration of analyte. Weighted ($1/x$) linear least-squares regression was used as the mathematical model. The calibration was processed with MassLynx4.0 QuanLynx software. The lower limit of quantification was defined as the concentration of the CoQ_{10} at which the response of CoQ_{10} is 10 times the response compared to the blank noise.

3.3. Accuracy and precision

Intra-day accuracy and precision were evaluated by analysis of the three QC samples with five determinations per concentration in the same day. The inter-day accuracy and precision was measured over 3 days. Precision was measured by inter- and intra-assay %R.S.D. The accuracy was evaluated by the deviation or bias (%) of the observed concentration from the actual concentration.

3.4. Recovery

The recovery of the method was obtained in triplicate at three final concentration levels (10,000, 1000, and 100 ng/mL) from a detector response of the analyte added to and extracted from the serum, compared to the detector response of the analyte spiked after extraction into serum extracts.

3.5. Freeze–thaw and short-term stabilities

The QC samples at three final concentrations (20,000, 5000, and 500 ng/mL) were stored at −20 °C for 24 h and thawed at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. After three cycles, the percent loss of the analyte was determined by comparing the concentrations with those obtained before freezing.

For the short-term stability test, the QC samples at the same three concentrations were thawed at room temperature and kept at this temperature from 12 to 24 h and analyzed [21].

3.6. Application of the method to biological samples

The assay method described here was applied to study the pharmacokinetics of CoQ_{10} in rat serum after oral administration of the drug. The pharmacokinetic parameters were calculated with the aid of WinNonlin professional software version 5.0.1 (Pharsight, Mountain View, CA, USA) using the noncompartment model. Peak plasma concentration (C_{max}) was obtained from observed data. Plasma $AUC_{0\rightarrow t}$ values (t being the time of the last plasma concentration measured) were calculated by the linear trapezoidal rule. The first order rate constant, λ_z, was estimated by linear regression of time versus log of the concentration. Plasma clearance (CL_p), terminal half-life ($t_{1/2}$), and apparent volume of distribution (V_d) were also calculated.

4. Results and discussion

4.1. Ultra-performance liquid chromatography and sample preparation

Recent technological advances have made available reversed phase chromatographic media with a 1.7 μm particle size along with a liquid handling system that can operate such columns at much higher pressures (max: 16,000 psi). This technology, termed ultra-performance liquid chromatography (UPLC), offers significant theoretical advantages in resolution, speed and sensitivity for analytical determinations due to the use of smaller column diameter, particularly when coupled with mass spectrometers capable of high-speed acquisitions.

The mobile phase optimization was accomplished by comparing various solvent systems composed by mixtures of methanol, acetonitrile, 2-propanol, formic acid, acetic acid, ammonium acetate and ammonium hydroxide. The retention time was 8 min using 100% acetonitrile as the mobile phase. Even 5% water in mobile phase (acetonitrile:2-propanol:water = 85:10:5) can increase the retention time to 10 min and increase the column pressure to over 10,000 psi. The mobile phase consisting of a mixture of acetonitrile:2-propanol:formic acid (90:10:0.1) was found to be suitable for separation and ionization of CoQ_{10} and the internal standard. The retention time for CoQ_{10} was 4.10 min and for the internal standard was 2.55 min. The selection of SIR mode and associated acquisition parameters were evaluated for best response under positive mode by infusing a standard solution via a syringe pump into the mobile phase. MRM mode was also tested during

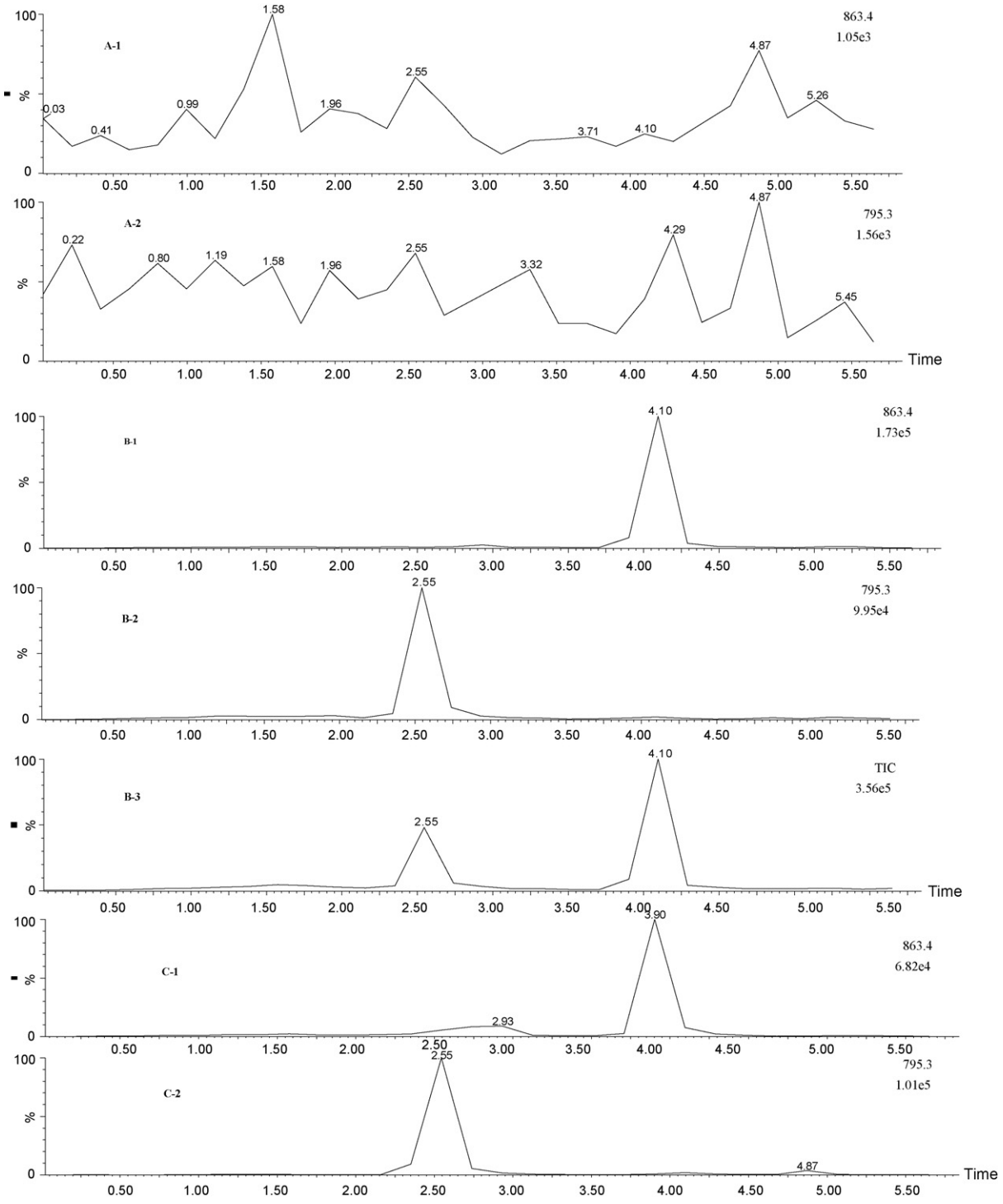

Fig. 1. Chromatograms of (A) blank serum, (B) blank serum spiked with CoQ_{10} (10,000 ng/mL) and internal standard, (C) serum samples 2 h after oral administration of CoQ_{10} to rats. (1) CoQ_{10} (*m*/*z*: 863), (2) internal standard (*m*/*z*: 795) and (3) CoQ_{10} and the internal standard.

the study. There were several possible daughter ions, but no one daughter ion dominated. Therefore, SIR was used as the acquisition mode in order to increase the detector sensitivity of the measurement. No interferences with other compounds that originated from the sample matrix were observed in this method. In the present work, initial analysis was performed with the mass spectrometer in scan mode in order to identify the CoQ_{10} compounds and select the most abundant ions for monitoring with SIR mode. Full scans of the two compounds were performed at *m*/*z* 200–1000. The CoQ_{10} was at *m*/*z*: 863, and the internal standard was *m*/*z*: 795. The dwell time was set at 200 ms. The optimization of MS detection was carried out in the atmospheric pressure chemical ionization (APCI) positive ion mode. The 2-propanol was used to extract the CoQ_{10} from the rat serum. The supernatant of the sample after centrifugation was injected directly to the UPLC. Direct injection of the 2-propanol extract

Table 1
Summary of the accuracy and precision of CoQ10 in rat serum

Spiked concentration (ng/mL)	Intra-day precision and accuracy ($n=5$)		Inter-day precision and accuracy ($n=3$)	
	Mean recovery (%) ± S.D.	R.S.D. (%)	Mean recovery (%) ± S.D.	R.S.D. (%)
50	81.9 ± 2.9	3.6	77.5 ± 3.9	5.0
500	83.0 ± 6.3	7.5	86.0 ± 3.1	3.6
5000	118.6 ± 0.8	0.7	111.5 ± 10.8	9.7

R.S.D.: relative standard deviation. Recovery = measured concentration × 100/nominal concentration.

also makes the procedure particularly simple and fast. The efficiency of the CoQ_{10} extraction by this method appears to be satisfactory as it leads to an acceptable recovery.

4.2. Specificity

UPLC–MS analysis of the serum samples showed no endogenous peak interference with the quantification of CoQ_{10} and the internal standard. Representative chromatograms of blank rat serum spiked with CoQ_{10} and internal standard, blank serum and extracted serum samples are shown in Fig. 1.

4.3. Linearity and lower limit of quantification

The peak area ratios of CoQ_{10} to internal standard versus concentrations were calculated. Weighted linear least-squares ($1/x$) regression was used as the mathematical model. The linear equation was $Y = 1.51E-5X + 0.00197$. The coefficient (r) for CoQ_{10} was 0.9991. The calibration range was selected according to the concentrations anticipated in the samples to be determined. The final calibration range was 50–20,000 ng/mL. The lower limit of quantification was 50 ng/mL.

4.4. Accuracy and precision

The results of accuracy and precision determinations at the three concentrations are presented in Table 1. The repeatability and reproducibility bias (%) is within the acceptance limits of ±20% at low concentration and ±15% at other concentration levels. The results also show that the analytical method is accurate, expressed with recoveries in the range of 77.5–118.6%.

4.5. Recovery

The mean recoveries of this method were in the range of 90.5–104.2% at three different concentrations. The results are shown in Table 2. These results suggested that there was no relevant difference in extraction recovery at different concentration levels.

Table 2
Mean recovery of CoQ_{10} from rat serum ($n=3$)

Concentration (ng/mL)	Recovery (%)	
	Mean value ± S.D.	R.S.D.
10000	98.8 ± 0.5	0.5
1000	90.5 ± 0.9	1.0
100	104.2 ± 20.4	19.6

Recovery = response of standard spiked before extraction × 100/response of standard spiked after extraction.

4.6. Stability

The results of freeze–thaw and short-term storage stability are shown in Table 3. The CoQ_{10} was found to be stable after three freeze–thaw cycles at three different concentrations. CoQ_{10} in rat serum at three concentrations stored in room temperature was found to be stable for at least 6 h. The mean recoveries from the nominal concentrations were between 73.7 and 104.3% at three different concentrations. The QC samples were found to be stable on the auto-sampler at room temperature for at least 24 h.

4.7. Application to the pharmacokinetic study

Fig. 2 shows the plasma concentration versus time profiles for CoQ_{10} in rats after intravenous administration. The respective estimates of the pharmacokinetic parameters of CoQ_{10} are summarized in Table 4. The PK data analysis showed that CoQ_{10} has moderate clearance and a large volume of distribution. The maximum serum concentration was detected at 4 h, with a mean value of 524.0 ng/mL. After 8 h, CoQ_{10} can still be detected in rat serum. The large volume of distribution (V_d) indicated that the CoQ_{10} quickly leaves the blood stream and is distributed into tissues, mainly the liver and heart [22].

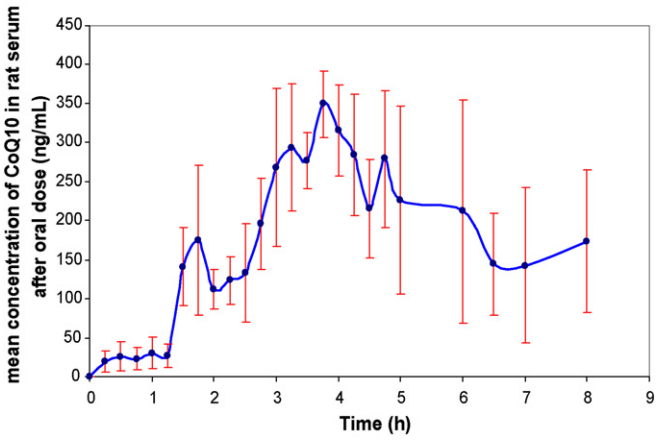

Fig. 2. Mean concentrations of CoQ_{10} in rat serum after oral administration vs. time profile ($n=4$).

Table 3
Results of short-term and freeze–thaw stability studies of CoQ_{10} in rat serum

Stability	Time (h)/cycle (times)	Concentration (ng/mL)					
		500		5000		20,000	
		Recovery (%) ± S.D.	R.S.D. (%)	Recovery (%) ± S.D.	R.S.D. (%)	Recovery (%) ± S.D.	R.S.D. (%)
Short-time stability	12 h	100.1 ± 4.7	4.7	91.3 ± 8.02	8.7	104.8 ± 7.4	7.1
	24 h	85.9 ± 7.2	8.4	102.2 ± 1.9	1.8	110.4 ± 2.7	2.4
Freeze-thaw stability	1	89.7 ± 7.9	8.8	95.5 ± 9.2	9.7	88.6 ± 1.2	1.4
	2	81.3 ± 10.0	12.3	88.9 ± 11.1	12.5	73.7 ± 6.0	8.2
	3	104.3 ± 15.3	14.5	87.6 ± 5.3	6.1	107.2 ± 15.0	14.0

R.S.D.: relative standard deviation.

Table 4
Estimated pharmacokinetic parameters after oral administration of CoQ_{10} (200 mg/kg) to rats (four rats per sampling time points)

Parameters	Units	Mean	S.E.
C_{max}	ng/mL	524.0	152.9
$t_{1/2}$	h	2.4	0.7
T_{max}	h	4.1	0.7
V_d	mL/kg	363108.5	124910.5
CL_p	mL/(h kg)	110990.4	32074.8
$AUC_{0\rightarrow t}$	h ng/mL	1471.6	522.8
λ_z	1/h	0.3	0.1

C_{max}, peak plasma concentration; $t_{1/2}$, terminal elimination half life; T_{max}, time of maximum observed concentration; AUC, area under the plasma concentration–time curve; V_d, apparent volume of distribution; CL_p, plasma clearance; λ_z, first order rate constant; S.E., weighted standard error.

5. Conclusion

A fast, sensitive and specific UPLC–MS method for the determination of CoQ_{10} in rat serum has been developed and qualified. The UPLC method resulted in improvements in terms of run time, resolution and sensitivity. The complete chromatographic process only takes 5 min, which results in high throughput capability. Sample clean-up involves a simple solvent extraction step and shows good reproducibility. The UPLC–MS method has been applied to the pharmacokinetic studies of CoQ_{10} in rats and could also be applied with minor changes to other biological matrices or pharmacokinetic studies.

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