Urinary Excretion Study of Coenzyme Q_{10} in Rats by Ultra-Performance Liquid Chromatography–Mass Spectrometry

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Abstract

A method based on ultra-performance liquid chromatography mass spectrometry (UPLC–MS) applying atmospheric pressure chemical ionization in the positive ion mode is developed for the determination of coenzyme Q_{10} (CoQ_{10}) in rat urine. The assay involves the extraction of crude urine, fast liquid chromatography on a Waters Acquity UPLC BEH C_{18} column (1.7 µm, 1.0 × 50 mm), and selected ion monitoring detection using mass transition. The calibration range is found to be 0.05–25 µg/mL, with the lower limit of quantitation of 0.05 µg/mL. Intra- and inter-day precision (relative standard deviation) for CoQ_{10} in rat urine range from 0.7% to 15%, and accuracy expressed in recovery rates in urine is between 83% and 118%. The recovery of this method is found to be between 80% and 95% at three concentrations. The total cumulative recovery of CoQ_{10} is 1.16 ± 1.05% (percentage of dose intake, *n* = 4) from rat urine collected over 30 h after oral administration of the drug. The UPLC–MS method described allows the quick determination of CoQ_{10} in rat urine with good precision and accuracy. It is suitable for further excretion studies of CoQ_{10} in animals.

Introduction

Coenzyme Q_{10} (CoQ_{10}), also known as ubiquinone, is a lipid-soluble compound mainly located in the mitochondria. In addition to playing a key role in the mitochondrial electron transport chain, it is a critical coenzyme in the synthesis of adenosine triphosphate (1–3). It also serves as an important lipid-soluble antioxidant as well as a membrane stabilizer. Due to the increasing importance of CoQ_{10} in medical and nutritional usage, there is a growing demand for research on its absorption, distribution, metabolism, and excretion. Over the past few years, a number of studies have been performed on its pharmacokinetics, especially on its absorption (4–12). However, limited data is found in the literature pertaining to its excretion in animals or humans. Nakamura et al. examined the biliary and urinary metabolites of CoQ_{10} after intravenous administration of ^{14}C-coenzyme Q_{10} to guinea pigs (13). The cumulative recovery of total radioactivity excreted in bile and urine was reported by Yuzuriha et al. (14) after intravenous injection of radio-labeled CoQ_{10} to guinea pigs. While these radioimmunoassay methods provided accurate concentrations in urine, they were time-consuming and involved tedious pretreatment of the urine samples.

Liquid chromatography coupled to tandem mass spectrometry (LC–MS–MS) is known to be a powerful separation and detection technique in a large number of analytical fields. There have been publications with regard to the quantitation of CoQ_{10} in biological fluids or dairy products by LC–MS or LC–MS–MS (15). The retention time of CoQ_{10} was 5 min with this high-performance LC (HPLC)–MS–MS method, which is not short enough for sample high throughput. More recently, a new technology termed ultra-performance liquid chromatography (UPLC) coupled with MS–MS was proven able to reduce analysis time with increased sample throughput, sensitivity, and resolution. The objective of this paper was to develop a robust and rapid method for the quantitation of CoQ_{10} in urine after oral dosing to rats. Special emphasis was given to the rapidity of the method so that larger series of urine samples can be analyzed in a reasonable amount of time. The urinary excretion study of CoQ_{10} was also designed to further characterize its pharmacokinetics in rats after oral administration of CoQ_{10}.

Experimental

Chemicals and reagents

CoQ_{10} and CoQ_9 standards were purchased from Sigma (St. Louis, MO, purity > 98%); CoQ_{10} soft gels were purchased from Nature's Bounty, Inc. (Bohemia, NY); HPLC-grade acetonitrile, methanol, 2-propanol, hexane, and water were from Fisher Scientific (Fair Lawn, NJ); reagent grade formic acid was pur-

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chased from Sigma-Aldrich (95%, St. Louis, MO).

CoQ_9 was chosen as the internal standard in this method. Individual standard stock solutions of CoQ_{10} (1 mg/mL) and internal standard (20 µg/mL) were prepared by accurately weighing the required amounts and placing it into separate volumetric flasks and dissolving in 2-propanol. A calibration standard for CoQ_{10} was prepared by diluting 50 µL CoQ_{10} solution in a 2-mL volumetric flask with blank rat urine. It provided a working solution with a concentration of 25 µg/mL. Further dilutions were made from this working solution with blank urine to afford urine standards in the range of 0.05–25 µg/mL. Quality control (QC) samples at three different concentrations of 0.5, 2, and 20 µg/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.

Instrument and conditions

All chromatographic experiments were conducted using a Waters Acquity UPLC system (Milford, MA). Chromatographic separations were performed on a Waters Acquity UPLC BEH C_{18} column (1.7 µm, 1.0 × 50 mm). The mobile phase consisted of acetonitrile–2-propanol–formic acid (90/10/0.1, v/v/v). The flow rate was 0.1 mL/min, the column temperature was at room temperature, the pressure was around 5500 psi, the sample injection volume was 2 µL, the injection type was partial loop with needle overfill, and the duration of the run was 3 min.

The MS equipment consisted of a Waters Micromass Quattro Micro triple-quadrupole system (Manchester, UK). The MS system was controlled by MassLynx software version 4.1. Ionization was performed in positive atmospheric pressure chemical ionization (APCI) mode. MS conditions were the following: corona voltage, 15 µA; cone voltage, 35V; extractor voltage, 5V; RF lens voltage, 0.5 V. The source and APCI probe temperatures were 150°C and 450°C, respectively, and the desolvation and cone gas flows were 550 and 25 L/h, respectively.

The selected *m/z* ratio transitions of the CoQ_{10} and Coenzyme Q_9 ions $[M+H]^+$ used in the selected ion monitoring (SIM) were as follows: CoQ_{10}, *m/z* 863; Coenzyme Q_9, *m/z* 795. The dwell time was set at 200 ms. MS 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.

Sample preparation

Frozen urine samples were thawed at room temperature. After the transfer of 4 mL of rat urine and 50 µL internal standard solutions to a separatory funnel, the urine sample was extracted three times with 4 mL hexane. The organic layers were combined and dried under a gentle stream of nitrogen at 25°C. The residue was constituted in 200 µL of 2-propanol–methanol (7:3, v/v). The sample solution was filtered through a 0.45-µm filter (Waters 13 mm GHP 0.2 µm) before analysis.

Oral formulation

The oral formulation of CoQ_{10} used in this experiment was purchased from the market, as a soft gel. Each soft gel contained 50 mg of CoQ_{10}, vitamin E, rice bran oil, gelatin, glycerin, soy lecithin, yellow beeswax, titanium dioxide color, and annatto color. The recovery of the CoQ_{10} from the soft gel is 90%. The oral formulation was administered to rats at a dose of 100 mg/kg. The concentration of CoQ_{10} in the soft gel was analyzed by the UPLC–MS method.

Calibration and lower limit of quantitation

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/×) linear least-squares regression was used as the mathematical model. The calibration was processed with the MassLynx4.1 QuanLynx software. Seven calibration standards were analyzed triplicate at each concentration. The lower limit of quantitation was defined as the concentration of the CoQ_{10} at which the response of CoQ_{10} is ten times the response compared to the blank noise.

Accuracy and precision and recovery

The method was qualified by analysis of rat urine quality control samples prepared as previously described. 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 were measured over three days. Precision was measured by inter- and intra-assay relative standard deviation (%RSD). The accuracy was evaluated by the deviation or bias (%) of the observed concentration from the actual concentration.

The recovery of the method was obtained in triplicate at three final concentration levels (20, 2, and 0.5 µg/mL) from a detector response of the analyte added to and extracted from the urine, compared to the detector response of the analyte spiked in the organic solvents.

Freeze-thaw and short-term stabilities

The QC samples at three final concentrations (20, 2, and 0.5 µg/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, kept at this temperature from 12 to 24 h, and analyzed.

Animal study

All the experimental procedures were approved and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Mississippi. The Sprague Dawley rats (200–220 g) were obtained from Harlan Company (Indianapolis, IN). The rats were housed in standard metabolicages and allowed free movement and access to water 12 h before and during the experiment. Urine samples were collected over a 4 h period after administration. Urine samples were put into a polyethylene tube and stored at –20°C.

Application of the method to biological samples

The assay method described was applied to study the pharma-

cokinetics of CoQ_{10} in rat urine after oral administration. The pharmacokinetic parameters were calculated by WinNonlin professional software version 5.0.1 (Pharsight, Mountain View, CA) using the non-compartment model.

Results and Discussion

Even though the HPLC technique combined with UV, MS, or photodiode array detector has been widely used for the analysis, the retention time of CoQ_{10} in biological samples was between 5 and 25 min with those published conventional HPLC methods (5–7,12,15–18). With the new UPLC method, the retention time of CoQ_{10} was shortened to 1.75 min, which may result in higher sample throughput. The mobile phase optimization was accomplished by comparing various solvent systems composed of 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 the presence of 5% water in the 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 the separation and ionization of CoQ_{10} and the internal standard CoQ_9.

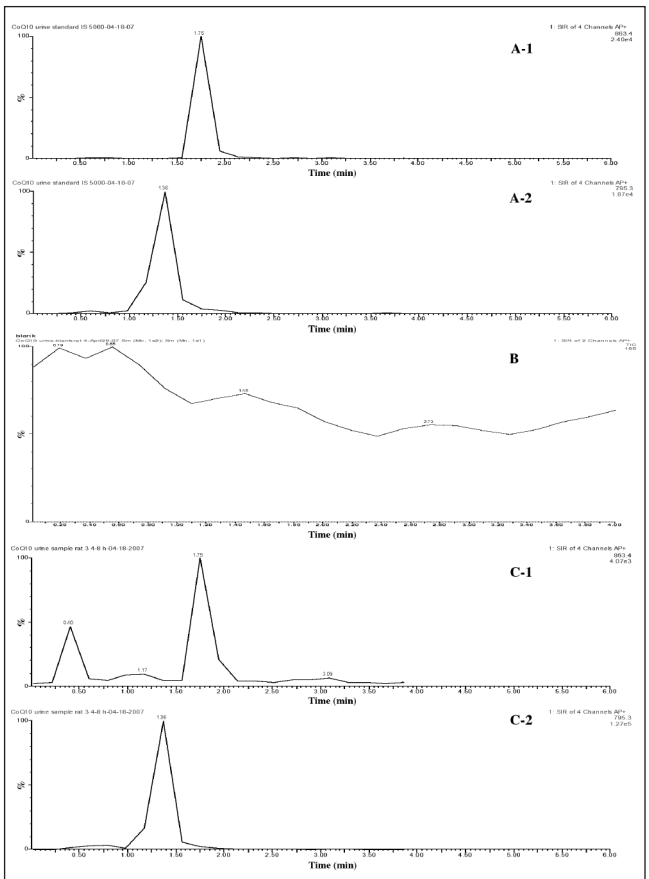

Figure 1. Chromatograms of blank urine spiked with CoQ10 (5 µg/mL) and internal standard (A), blank urine (B), urine samples collected between 4 and 8 h after oral administration of CoQ_{10} to rats (C). CoQ_{10} (*m/z* 863) (1), internal standard (*m/z* 795) (2).

The selection of the SIM mode and associated acquisition parameters were evaluated for the best response under the positive mode by infusing a standard solution via a syringe pump into the mobile phase. The optimized responses for CoQ_{10} were obtained. SIM mode 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 SIM mode. Full scans of the two compounds were performed at *m/z* 200–1000. The CoQ_{10} was at *m/z* 863, and internal standard was *m/z* 795.4. The dwell time was set at 200 ms. APCI mode and electrospray ionization mode were compared in this experiment. The APCI positive ion mode was chosen because it displays fewer matrix effects and has higher sensitivity in detecting CoQ_{10} in urine. The applied sample clean-up procedure proved to be very suitable for urine samples. The water-soluble endogenous impurities could not be extracted with hexane. Clear colorless solutions were obtained after liquid–liquid extraction. The efficiency of CoQ_{10} extraction by this method appears to be satisfactory as it leads to a good recovery.

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. In this method, no significant endogenous interfering peaks with CoQ_{10} or internal standard were observed in urine samples. Representative chromatograms of CoQ_{10} in rat urine samples are shown in Figure 1.

The peak area ratios of CoQ_{10} to internal standard versus concentrations were calculated. The calibration model was selected

Table I. Intra- and Inter-Assay Precision and Accuracy

Nominal conc. (µg/mL)	Intra-assay precision and accuracy (*n* = 4)		Inter-assay precision and accuracy (*n* = 3)	
	Mean recovery* (%) ± SD	RSD† (%)	Mean recovery (%) ± SD	RSD (%)
10	107.09 ± 2.23	2.1	107.37 ± 15.65	14.6
1	82.87 ± 0.31	0.4	93.57 ± 11.83	12.6
0.1	118.41 ± 0.87	0.7	117.25 ± 1.13	1.0

* Recovery: 1 – (nominal concentration – measured concentration) / nominal concentration.
† RSD: relative standard deviation.

Table II. Extraction Recovery of CoQ_{10} from the Rat Serum (*n* = 3)

Concentration (µg/mL)	Recovery (%)* Mean value ± SD	RSD %
20	94.83 ± 10.43	11.0
2	85.78 ± 0.46	0.5
0.5	86.64 ± 3.05	3.5

* Recovery: response of standard spiked before extraction/response of standard spiked after extraction.

based on the analysis of the data by linear regression with intercepts and 1/× weighting factor. The linear equation was $Y = 0.23X - 104.82$. The coefficient (r) for CoQ_{10} was 0.998. The calibration range was selected according to the concentrations anticipated in the samples to be determined. The final calibration range was 0.05–25 µg/mL. The lower limit of quantitation was 0.05 µg/mL, which is equivalent to the lowest point of the standard curves.

The results of intra- and inter-assay precision and accuracy are presented in Table I. Inter-assay recoveries were in the range of 93.57% to 117.25% with precision (RSD) 1.0–14.6%. The deviation of the retention time for QC samples was less than 5%. Intra-assay recoveries were in the range of 82.87–118.41% with precision (RSD) 0.4–2.1%.

The mean recovery of CoQ_{10} was 86.64% for low level samples (0.5 µg/mL), 86.64% for medium level samples (2 µg/mL), and 94.83% for high level samples (20 µg/mL). The results are shown in Table II.

The aim of the stability study was to obtain information on the stability of the analyte in real study samples and to establish storage conditions and lengths of storage as well as sample processing conditions. The results of freeze-thaw and short-term storage stability are shown in Table III. The CoQ_{10} was found to be stable after three freeze-thaw circles at low and medium concentrations. However, CoQ_{10} was found to be decreased by 30% at high concentration. Ideal storage for CoQ_{10} urine samples was found to be at –70°C. CoQ_{10} in rat urine at three concentrations stored in room temperature was found to be stable at least for 6 h. The mean recoveries from the nominal concentrations were between 70% and 90% at three different concentrations. Therefore, sample preparation steps before hexane extraction can be performed at room temperature. The QC samples were found to be stable on the auto-sampler at room temperature for at least 24 h.

Urine voided between 0 and 30 h after oral administration of CoQ_{10} was collected. Figure 2 summarizes the cumulative amounts of CoQ_{10} recovered in rat urine. During the collecting period, only 1.16 ± 1.05% CoQ_{10} was recovered from urine. The corresponding results of the pharmacokinetic parameters in urine are shown in Table IV. The results showed that the renal excretion of CoQ_{10} is low. This may be due to the fact that most of the CoQ_{10} was excreted into feces by biliary excretion. As CoQ_{10} is a compound with both polar and lipophilic groups, it is more likely to be excreted in the bile rather than in urine. The results suggest that only low levels need to be dosed to maintain their levels in the blood.

Table III. Results of Short-Term and Freeze-Thaw Stability Studies of CoQ_{10} in Rat Urine

		Concentration (µg/mL)					
		0.5		2		20	
Stability	Time (h)/ circle (times)	Recovery (%) ± SD	RSD* (%)	Recovery (%) ± SD	RSD (%)	Recovery (%) ± SD	RSD (%)
Short-time	6	70.85 ± 0.47	0.7	72.82 ± 3.47	4.8	90.57 ± 13.89	15.3
Stability	24	126.27 ± 7.91	6.3	100.78 ± 4.22	4.2	93.95 ± 11.06	11.8
	1	103.03 ± 1.33	1.3	87.45 ± 5.61	6.4	97.20 ± 0.41	0.4
Freeze-thaw	2	109.09 ± 13.69	12.6	118.16 ± 3.23	2.7	73.88 ± 7.26	9.8
stability	3	86.47 ± 4.51	5.2	94.30 ± 16.26	17.2	68.16 ± 5.57	8.2

* RSD: relative standard deviation.

Conclusion

The LC–MS method that we have developed for urinary CoQ_{10} is rapid and leads to reproducible results. An extraction process using hexane as solvent allows rapid and simple sample extraction. This optimized method provides excellent sensitivity, precision, and accuracy for assessment of CoQ_{10} in rat urine. The results are suitable for research and can be easily adapted for further pharmacokinetic studies.

Table IV. Estimated Urinary Pharmacokinetic Parameters after Oral Administration of CoQ_{10} to Rats

Parameter	Unit	Mean	SE
Amount recovered*	mL × µg/mL	236.50	122.61
AURC all†	mL × µg/mL	175.12	83.97
HL λ_z‡	h	13.97	1.50
T_{max} rate§	h	6.67	4.67
Percent recovered**	% × kg	0.24	0.12
λ_z††	1/h	0.05	0.01

* Amount recovered: cumulative amount eliminated. Σ(concentration × volume);
† AURC all: area under the urinary excretion rate curve from time 0 to the last rate;
‡ HL_λ_z: terminal half-life;
§ T_{max} rate: midpoint of collection interval associated with the maximum observed excretion rate;
** Percent recovered: 100 × amount recovered/dose;
†† λ_z: first order rate constant associated with the terminal (log-linear) portion of the curve. This is estimated via linear regression of midpoints vs. log excretion rates.

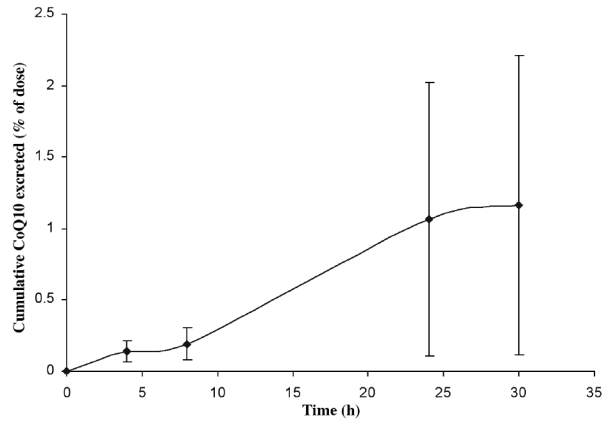

Figure 2. Cumulative recovery of CoQ_{10} in urine from Sprague-Dawley rats following oral administration of 100 mg/kg (n = 4). Data are expressed as the mean percentage of dose recovered.

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