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Journal of Chromatography B, 805 (2004) 297–301
JOURNAL OF CHROMATOGRAPHY B
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Analysis of coenzyme Q_{10} in human plasma by column-switching liquid chromatography

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Received 11 November 2003; received in revised form 18 February 2004; accepted 8 March 2004

Available online 15 April 2004

Abstract

A new method of determining coenzyme Q_{10} in human plasma was developed based on column-switching high performance liquid chromatography (HPLC). CoQ_{10} was quantitatively extracted into 1-propanol with a fast one-step extraction procedure, after centrifugation, the supernatant was cleaned on an octadecyl-bonded silica column and then transferred to reversed-phase column by a column-switching valve. Determination of CoQ_{10} was performed on a reversed-phase analytical column with ultraviolet detection at 275 nm and the mobile phase containing 10% (v/v) isopropanol in methanol at a flow-rate of 1.5 ml/min. The sensitivity of this method allows the detection of 0.1 μg/ml CoQ_{10} in plasma (S/N = 3). The linearity between the concentration and peak height is from 0.05 to 20 mg/l. The reproducibility (R.S.D.%) of the method is less than 2% (within day) and less than 3% (between day), the average recovery is 100.9 + 2.1%, it takes only 30 min to complete an analysis procedure, suitable for the determination of CoQ_{10} in human plasma especially for batch analysis in clinical laboratories. Finally, the method was applied to determine the plasma CoQ_{10} levels in healthy subjects, hyperthyroid and hypothyroid patients.

Keyword: Coenzyme Q_{10}

1. Introduction

Coenzyme Q_{10} (CoQ_{10}), also known as ubiquinone, is a lipid soluble compound that mainly locates in the mitochondria and acts as an electron carrier in the electron transport chain [1], so it is essential for the production of cellular energy in the form of phosphate adenosine triphosphate (ATP). Moreover, CoQ_{10} has been studied as an antioxidant agent, together with other lipophilic antioxidants CoQ_{10} plays an intrinsic role in protecting circulating lipoproteins against oxidative damage [2]. Therefore, its concentration in lipoproteins and plasma may be a useful marker of oxidative stress and antioxidant defense. Since CoQ_{10} can be used as a food supplement or as an adjunctive therapy in several diseases [3,4], it is necessary to assay plasma levels of CoQ_{10} to monitor the bioavailability of orally administered CoQ_{10}.

Many analytical methods have been reported for quantitative determination of CoQ_{10} in human plasma [5–15], such as spectrophotometric [5,6], voltammetric [7] and chemiluminescent [8]. Among these publications, the most popular tools used for assaying are HPLC [9–15], because of the complexity of biological fluid specimens, they usually required samples purified before injection. Thin layer chromatography (TLC) and solid phase extraction (SPE) pretreatment method have been described in several literatures [11–14], those manual purification methods are time and money consuming, unsuitable for routine determination, especially for clinical chemistry laboratories. In this paper, a procedure of determining CoQ_{10} with on-line sample purification is investigated based on column-switching HPLC, resulting in an automatic analysis method, in the meantime, the CoQ_{10} levels in plasma of healthy subjects and the thyroid patients were determined.

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doi:10.1016/j.jchromb.2004.03.008

2. Experimental

2.1. Chemicals

CoQ_{10} was purchased from Sigma (St. Louis, MO, USA), methanol, 1-propanol, isopropanol were purchased from Tedia (Fairfield, OH, USA). All chemicals were HPLC-grade and were used without further purification. Ethanol was analytical purification obtained from China.

2.2. Equipment

All chromatographic experiments were conducted using a Shimadzu LC system (Shimadzu scientific instruments, Japan) incorporating a LC-10Atvp pumping system, SIL-10Advp autoinjector, SPD-10Avp UV detector set at 275 nm, SCL-10Avp system controller and Shimadzu Class-VP version 6.10 software. Column-switching was achieved using electric six-port valve (Rheodyne, USA). Valve switching was controlled using Shimadzu SCL-10Avp system controller and Shimadzu Class-VP version 6.10 software. The precolumn (20 mm × 4.6 mm i.d.) was packed with 5 μm Hypersil C_{18} (Elite, Dalian, China). The analytical column (150 mm × 4.6 mm i.d.) was packed with 5 μm Hypersil ODS_2 (Elite, Dalian, China). The automatic analytical system was assembled in Fig. 1.

2.3. Preparation of standards

All sample preparation work was carried out under a dim light to avoid photochemical decomposition of CoQ_{10}. Ten milligrams CoQ_{10} was completely dissolved in 25 ml ethanol, the exact concentration was calculated by reading the absorbance on a spectrophotometer (275 nm wavelength; 1 cm quartz cuvette) using its molar absorption ($\varepsilon = 14{,}600$). The stock solution (100 mg/l) of CoQ_{10} was prepared by diluting above solution in appropriate volume of ethanol. A series of standard solutions were obtained by diluting stock solution to final CoQ_{10} concentration of 0.05, 0.2, 0.4, 0.8, 1.2, 2, 4, 10, 20 mg/l. Working solutions of standards were prepared in ethanol: water (1:0.3), All solutions were stored in icebox kept at −20 °C.

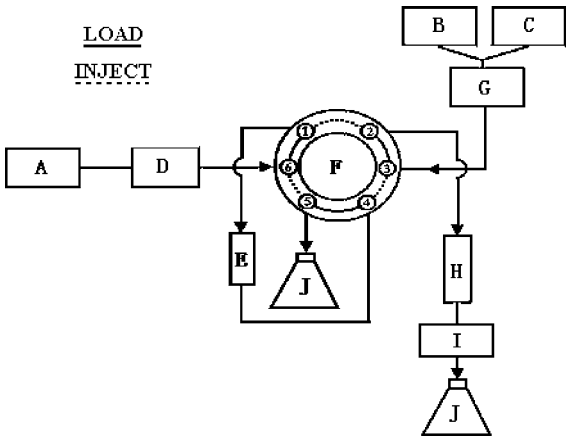

Fig. 1. The diagram of automatic analysis system. A: pump 1; B: pump 2; C: pump 3; D: autoinjector; E: precolumn; F: electric six-port valve; G: mixing container; H: analytical column; I: detector; J: waste drain.

2.4. Preparation of samples

All heparined plasma samples were kindly supplied by Dalian Center Hospital (Dalian, China). Samples collection was performed in a dim condition. Plasma samples were stored in icebox and kept at −20 °C until analysis.

In the day assigned for analysis the samples were thawed for 2 h at room temperature. Three hundred microliters of plasma was supplemented with 1 ml of 1-propanol and vortexed for 2 min, after centrifuged at 8000 rpm for 10 min (to spin down the protein precipitate), 400 μl of the supernatant was injected into HPLC.

2.5. Column-switching HPLC conditions

A short precolumn was used to eliminate both polar compounds including reagents and strongly retained solutes. The mobile phase for the precolumn was pure methanol employed at a flow-rate of 0.5 ml/min by pump 1 (Fig. 1). The fraction containing the analyte of interest was transferred from the procolumn to the analytical column by a mobile phase containing 10% (v/v) isopropanol in methanol at a flow-rate of 1.5 ml/min delivered by pumps 2 and 3. The separation was performed on the analytical column with the same mobile phase keeping the same flow-rate and the detection was performed with UV set at 275 nm, while the precolumn was washed and activated with pure methanol, preparing for the next analysis. These switching events are listed in Table 1.

2.6. Quantification and validation methods

The quantification of CoQ10 was performed with an external standard method. Recovery of CoQ_{10} was determined by spiking three known concentrations (1–3 mg/l) of CoQ_{10} to a plasma sample, and the result was calculated by the comparison of spiked amounts and the measured amounts. The accuracy was evaluated by determining a same sample repeatedly for three times in 1 day at the same conditions. To obtain the day-to-day reproducibility, a same plasma sample was assayed five times (one time per week) at the same conditions.

3. Results and discussion

CoQ_{10} is a lipophilic molecule, which always lies in plasma associated with kinds of lipoproteins and other lipophilic compounds. Usual methods for determination of CoQ_{10} in plasma or biological tissues were based on

Table 1
Scheme of column-switching events

Time after injection (min)	Valve position	Flow-rate of mobile phase in column E	Flow-rate of mobile phase in column H	Event
0–4	LOAD	0.5	1.5	Sample is injected onto precolumn and then be purified
4–6.5	INJECT	0	1.5	The analytes are eluted from column E to column H
>6.5	LOAD	0.5	1.5	Separation on column H, while column E is being activated

alcohol–hexane extraction [9,10,12,13], the extract was brought to dryness and redissolved before injection. Those methods need more handling time, resulting in artificial error and poor reproducibility. Based on Mosca et al. [16], a simple and fast method with one-step extraction procedure was possible.

To extract CoQ_{10} from plasma, different organic solvents that are miscible with water in all proportions were tested. The recoveries of CoQ_{10} in such solvents were given in Table 2. This result was consistent with that of Edlund [17]. Methanol, acetonitrile and 10% (w/v) trichloroacetic acid were not suitable because of strong polarity, the recoveries of CoQ_{10} in these solvents were zero. Propanol was chosen.

Quantitative determination of CoQ_{10} in the solvent-extracted plasma without any purification was found unsuitable for routine determinations according to some authors [11,18] owing to the large solvent front, the dirty injected sample and the short column lifetime. TLC [14] had been used as an effective tool to purify the extraction of plasma, but a poor recovery and accuracy were obtained. Grossi et al. [12] and Kaplan et al. [13] took SPE as an alternative method of prepurification, but two steps of SPE are required, this manual pretreatment method was also tired and more expensive. Kommuru [11] had simplified the procedure of purification with only a step of SPE, but the solvent front was so large that it cannot be thought as an ideal method of prepurification.

In this paper, a simple and rapid method of on-line prepurification using column-switching is developed. To avoid the clogging of the frit, the extract was centrifuged at 8000 rpm for 5 min to spin down the protein precipitate as completely as possible. After the supernatant was injected by autoinjector, the short column before analysis column packed with octadecyl-bonded silica was used to eliminate both polar compounds including reagents and strongly retained solutes, producing the clean profile (Fig. 2). Since the events of injection and the scheme of column-switching were controlled by a system controller, resulting in an automatic analytical system of CoQ_{10} determination, which made the prepurification be completed on-line and the internal standard unnecessary. This automatic analysis system has significantly minimized sample handling. It only took less than 30 min to complete one sample assay.

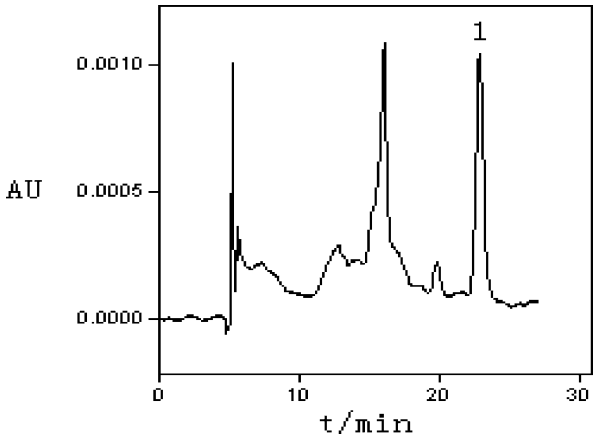

Fig. 2. Analytical profile obtained from automatic analytical system. Column-switching HPLC conditions as Table 1. Identification—1: CoQ_{10} (0.64 mg/l).

Recovery of CoQ_{10} in this method was determined by spiking three known concentrations (1–3 mg/l) of CoQ_{10} to a certain plasma sample, and the result was calculated by the comparison of spiked amounts and the measured amounts. The satisfactory recoveries (Table 3) showed the efficiency of CoQ_{10} extraction and the precision of determination. All processes were automatically and conformably controlled, the method exhibits good reproducibility and good accuracy. R.S.D.% was less than 2% (within day) (Table 3) and less than 3% (between day). These data also mean the CoQ_{10} is stable enough under the dim light during our total research period.

To investigate the linear range of the method, a series of working solutions that contain different concentration of

Table 2
Extraction recovery using different solvents

Solvent	Volume of plasma (ml)	Volume of solvent (ml)	Recovery (mean ± S.D.) (%)	*n*
Ethanol	0.3	1	31	1
Acetone	0.3	1	88	1
2-Propanol	0.3	1	108.3 ± 14.4	3
1-Propanol	0.2	1	96 ± 9.2	3
1-Propanol	0.3	1	98 ± 3.5	3
1-Propanol	0.4	1	95.3 ± 4.0	3
1-Propanol	0.5	1	90.7 ± 10.7	3

Table 3
Recovery of CoQ_{10} added to a plasma sample ($N = 3$)

	Measured (mg/l)	R.S.D. (%)	Recovery (%)
Basal concentration	0.80 ± 0.01	1.2	
Spiking with 1 mg/l	1.79 ± 0.01	0.6	99 ± 2
Spiking with 2 mg/l	2.83 ± 0.04	1.4	101.5 ± 2.3
Spiking with 3 mg/l	3.87 ± 0.06	1.6	102.3 ± 2

Table 4
Thyroid parameters of the subjects investigated and the CoQ_{10} data obtained

Analyst	Hypothyroid	Healthy	Hyperthyroid
Subject no.[a]	10 (f = 9, m = 1)	58 (f = 37, m = 21)	38 (f = 31, m = 7)
Age (year)	37.3 ± 17.2 (20–79)	45.2 ± 14.8 (6–72)	42.7 ± 15.7 (12–70)
fT_3 (pmol/l)[c]	3.56 ± 0.5 (2.75–4.4)	4.92 ± 1.3 (2.82–11.53)	21.59 ± 13.96[b] (3.63–50)
fT_4 (pmol/l)[c]	9.23 ± 4.61 (3.18–16.05)	16.47 ± 2.56 (12.61–24.16)	54.01 ± 31.28[b] (15.13–100)
TSH (mIu/l)[c]	41.99 ± 41.05[b] (2.35–100)	1.63 ± 0.6 (0.005–3.37)	0.21 ± 0.65[b] (0.002–3.04)
CoQ_{10} (mg/l)	0.88 ± 0.20[b] (0.58–1.16)	0.65 ± 0.25 (0.19–1.25)	0.46 ± 0.20[b] (0.13–0.86)

[a] f: female, m: male.
[b] Significantly different from values of healthy subjects ($P < 0.05$).
[c] fT_3, fT_4 and TSH concentrations were determined by electrochemistry luminescence (ECL), data are reported as mean ± S.D. (distribution range).

Table 5
The correlation between the level of thyroid hormones and that of CoQ_{10}

	fT_3	fT_4
CoQ_{10}	−0.396*	−0.446*

* A significant correlation is found ($P < 0.05$).

CoQ_{10} had been analyzed. It was found that the automatic analysis method described in this paper has a good linearity ($R^2 = 0.9999$) between the concentration and peak height over the range from 0.05 to 20 mg/l, and the calibration curve is $y = 1441.2x - 16.664$ (y—peak height, x—concentration). The lower limit of detection at signal-to-noise ratio of 3:1 was approximately 0.1 μg/ml in human plasma. This minimum detectable level is close to 0.05 mg/l obtained by the method using SPE to purify the plasma reported by Grossi et al. [12] and Kaplan et al. [13].

To show the usefulness, the method developed was applied to determine the CoQ_{10} level in groups of healthy subjects and thyroid patients before any pharmacological treatment. The information of healthy subjects and the thyroid patients and the data of CoQ_{10} obtained were given in Table 4. Because the quantification was carried out using the external standard, the new calibration curve was used in every day of experiment. One working solution was run after every three plasma samples. It was observed from Table 4 that the level of CoQ_{10} determined using automatic analysis method was well correlated with those obtained by other authors [12,19,20] both for healthy subjects and thyroid patients. Our data showed that the levels of CoQ_{10} have a significant difference not only between healthy subjects and hyperthyroidism, but also between healthy subjects and hypothyroidism.

In hospitals, thyroid hormones (fT3 and fT4) have been widely used as bio-markers for thyroid diseases. Our data also showed that there lies a significant inverse correlation between the level of the thyroid hormones (fT_3 and fT_4) and that of the CoQ_{10} (Table 5), which is similar to Pandolfi et al.' results [19].

4. Conclusions

An automatic analysis HPLC system was developed by using a pre-column that completes the purification on-line producing cleaner and more replicable chromatographic profiles. The rapid and simple extraction procedure as proposed here avoided the tired alcohol–hexane extraction, improving the precision and the reproducibility. The analysis method proposed makes all analysis procedure be controlled by the system controller, which results in automatic assaying procedure and short analysis time. Additionally, the method makes internal standard unnecessary. This analytical method was suitable for routine CoQ_{10} determination, especially for clinical chemistry laboratories because of its simplification, rapidness and automation. Finally, the method developed was used to determine the CoQ_{10} level in human plasma, the results showed that the concentration of CoQ_{10} was significantly different from among healthy, hypothyroid and hyperthyroid subjects, and a significant inverse correlation was found between the level of CoQ_{10} and that of the thyroid hormones.

Acknowledgements

This study has been supported by the high-technology development plan "863 project" (2003AA223061) of State Ministry of Science and Technology of China and the Knowledge Innovation Program of the Chinese Academy of Sciences (K2003A16).

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