Vol. 95, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1980 Pages 971-977

SIMULTANEOUS DETERMINATION OF UBIQUINONE-10 AND UBIQUINOL-10 IN TISSUES AND MITOCHONDRIA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Kouichi Katayama, Masahiro Takada, Teruaki Yuzuriha, Kouichi Abe* and Satoru Ikenoya*

Department of Drug Metabolism and *Department of Analytical Chemistry, Research and Development Division, Eisai Co., Ltd, Koishikawa, Bunkyo-ku, Tokyo, Japan

Received May 28,1980

SUMMARY: A method of high performance liquid chromatography with both of a UV detector and an electrochemical detector for the simultaneous determination of ubiquinone and ubiquinol was established. This method could sensitively and specifically measure the redox state of ubiquinone in mitochondria and tissues.

Ubiquinone has been well known as a redox carrier in mitochondrial respiratory chain and its significance for proton-gradient generation has come to be one of the central themes of energy-coupling theory (1,2). Some reports indicated that local deficiencies of ubiquinone were observed in patients with periodontal diseases or heart diseases (3,4). It was also suggested that reduced ubiquinone, ubiquinol, might act as an antioxidant of lipid peroxidation in the bovine heart mitochondria (5).

It is, therefore, important to determine not only ubiquinone but also ubiquinol in biological samples. In recent years, quantitative analysis of ubiquinone homologues using high performance liquid chromatography or Mass spectrometry were reported (6-8). An electrochemical detector for liquid chromatography was indi-

0006-291X/80/150971-07$01.00/0

cated to be quite sensitive for the determination of ubiquinone (9). However, only ubiquinone was determined by these methods.

This paper deals with the method of high performance liquid chromatography with both of a UV detector and an electrochemical detector for the simultaneous measurement of ubiquinone and ubiquinol in tissues and mitochondria. This method is more simple, sensitive and specific than the methods reported previously (10-12).

MATERIALS AND METHODS

Ubiquinol-10 was obtained by the reduction of ubiquinone-10 (Nisshin Chemicals, Japan) with sodium borohydride.

Male guinea pigs were sacrificed by decapitation. The liver, heart and kidneys were quickly removed, rinsed with ice-cold 0.15M NaCl and homogenized at 4°C with 4 vol. (v/w) of aqueous water using a Polytron homogenizer (Hijiriseiko, Japan) at a setting 6 for 20 seconds. One ml of the homogenate was poured into a test tube containing 7 ml of the mixture of ethanol and n-hexane (2:5 by vol), and then the tube was rapidly shaken to extract the quinone and quinol (7,11). Hexane extraction was repeated twice and the extract was dried down under a stream of nitrogen.

The hearts of guinea pigs were homogenized with 20mM Tris-HCl containing 0.25M sucrose (pH 7.4) and the mitochondrial fraction prepared by the method (13) was suspended in the buffer described above at a protein concentration of 0.25-0.5 mg/ml. The quinone and quinol in the suspension were extracted by the afore-mentioned method.

The residue of n-hexane phase was dissolved in isopropanol or ethanol, and 2.5-10 μl of the solution was injected into a column. Liquid chromatography was performed using a Yanako L-2000 pump (Yanagimoto Manufactory Co., Japan), a Rheodyne loop injector and Nucleosil C-18 reversed phase column (25cm x 4.6mm, 10 μm, Macherey-Nagel Co., West Germany). The UV and electrochemical detectors consisted of a Jasco UVIDEC 100 UV detector (Japan Spectroscopic Co., Japan) and Yanako VMD-101 electrochemical detector (9), respectively. The UV detector was

connected with the downward electrochemical detector. The mobile phase was prepared by dissolving 7.0 g of $NaClO_4 \cdot H_2O$ in 1000 ml of ethanol-methanol (9:1 by vol) and mixing with 1.0 ml of 70% $HClO_4$ solution. The flow rate was 1.2 ml/min and the measurement was performed at 25 ± 0.1 °C. The concentrations of ubiquinone-10 and ubiquinol-10 were estimated from calibration curves at the peak height against the concentration of each compound.

RESULTS AND DISCUSSIONS

Ubiquinone homologues were detected by high performance liquid chromatography with both UV and electrochemical detectors, and the latter detector was reported to be ten to twenty times more sensitive than the former detector (9). The electrochemical detector was, thus, used to measure a small quantity of ubiquinol since the molecular extinction coefficent ($E_{mM} \approx 4.1$) at 290 nm for ubiquinol is lower than that ($E_{mM} = 14.6$) at 275 nm for ubiquinone. Fig. 1 shows the chromatograms of authentic ubiquinone-10 by UV detector at 275 nm and ubiquinol-10 by electrochemical detector at a applied poteatial of 0.7 V.

As shown in Fig. 1, ubiquinone and ubiquinol were clearly separated each other and eluted at the retention time of 7.4 min and 5.7 min, respectively. The detection limit for ubiquinone-10 was 1 ng and that for ubiquinol-10 was 0.1 ng. A conversion from ubiquinol to ubiquinone during the liquid chromatography was less than 2% of the injected ubiquinol.

Fig. 2 shows representative chromatograms of the extracts from heart and heart mitochondrial fraction of guinea pigs. The chromatographic peaks observed for ubiquinone-10 and ubiquinol-10 were identified from the retention time, co-chromatography with the appropriate standard, hydrodynamic UV spectra and hydrodynamic voltammograms. Furthermore, the peak corresponding

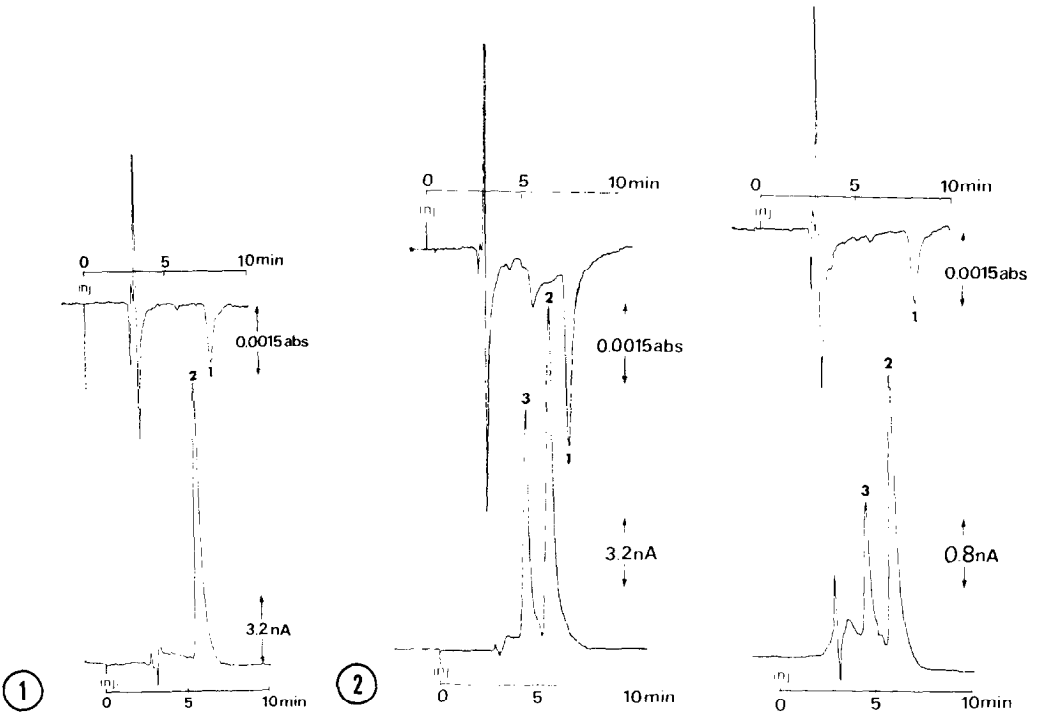

Fig. 1. Chromatograms of the mixture of authentic ubiquinone-10 and ubiquinol-10. A mixture of 25.4 ng ubiquinol-10 and 48.0 ng ubiquinone-10 in 5 μl of isopropanol was injected into the column and detected by electrochemical detector (lower chart) and UV detector (upper). The peak-1 and 2 were ubiquinone-10 and ubiquinol-10, respectively.

Fig. 2. Chromatograms of the extracts from the heart and heart mitochondrial fraction of guinea pigs. An extract of the heart (200 mg) was dissolved in 500 μl of isopropanol and 2.5 μl of the solution was injected to the column (left figure). A mitochondrial extract (0.4 mg of mitochondrial protein) after adding succinate was dissolved in 200 μl of ethanol and 10 μl of the solution was injected (right figure). The peak-1, 2 and 3 were ubiquinone-10, ubiquinol-10 and α-tocopherol, respectively.

to ubiquinol-10 was characterized by the following experiments. The oxidation of the extract with PbO_2 or the acetylation with acetic anhydride induced the disappearance of the peak, and the oxidation of the extract generated the increase of peak height of ubiquinone-10 corresponding to the amount of the decreased ubiquinol-10.

TABLE I

CONCENTRATIONS OF UBIQUINONE-10 AND UBIQUINOL-10 IN TISSUES OF GUINEA PIGS

Tissues	Concentration (μg/g)*		% of ubiquinol-10 to the total**
	ubiquinone-10	ubiquinol-10	
Liver	21.2 ± 3.9	31.1 ± 4.7	59.3 ± 5.4
Heart	124.1 ± 22.4	95.7 ± 14.5	44.4 ± 8.1
Kidney	51.1 ± 4.2	121.4 ± 10.5	70.3 ± 1.5

* Mean ± standard error of 4 guinea pigs.

** The total means the sum of ubiquinone-10 and ubiquinol-10.

A substance at the peak of retention time of 4.4 min was identified as α-tocopherol. An oxidisable compound or retinyl palmitate was eluted at the same retention time as that of ubiquinol-10, but the applied potential (0.7 V) was low enough to eliminate the contamination of retinyl palmitate in the determination of ubiquinol-10. Ubichromenol, which was eluted at 6.7 min, was not detected as shown in Fig. 2.

Table I shows the concentrations of ubiquinone-10 and ubiquinol-10 in some tissues of guinea pigs. The total concentrations of ubiquinone or the sum of ubiquinone-10 and ubiquinol-10 were 52.3 ± 6.6 μg/g of liver, 219.8 ± 9.9 μg/g of heart and 172.5 ± 13.6 μg/g of kidney (mean ± S.E. of 4 experiments), which were in good agreement with the values reported previously (8). The percentages of the reduced form to the total were 59.3% in the liver, 44.5% in the heart and 70.3% in the kidney. A physiological significance of these values needs to be clarified in comparison with values of guinea pigs with experimental model disease.

Table II shows the concentrations of total ubiquinone-10 in heart mitochondrial fraction and the percentages of the reduced

TABLE II

OXIDATION-REDUCTION LEVELS OF UBIQUINONE-10 IN THE HEART MITOCHONDRIAL FRACTION OF A GUINEA PIG

	Exp-1*	Exp-2**
total ubiquinone-10 (μg/mg of protein)	3.75	3.75
% of ubiquinol-10 to the total		
only buffer	3.9	3.2
add succinate	45.2	43.1
add malate	44.6	19.1

* fresh mitochondrial fraction of a guinea pig.

** mitochondrial fraction aged at -20°C for 1 day.

form after adding succinate or malate. The ubiquinol-10 in the mitochondrial fraction was 3.2-3.9% before adding the substrates. It is indicated that ubiquinol might change to the oxidized form during the preparation of mitochondrial fraction. The reduced form was increased by the addition of succinate or malate as substrates for succinate-ubiquinone reductase or NADH-ubiquinone reductase, respectively. The percentage of ubiquinol-10 after adding malate was lower in the aged mitochondrial fraction than in the fresh one. This was probably due to partial damage of the mitochondrial fraction during the storage.

The concentrations of ubiquinone-10 and ubiquinol-10 can be simultaneously determined by the method reported in this paper, and the method is so sensitive that the concentrations can be determined in extracts from 2-5 mg of heart and 0.05 mg protein of mitochondria. In addition to the measurement of substrates in tissues (13), this method for the determination of ubiquinone-10 and ubiquinol-10 may be useful to measure the redox state

which should be related to the cell energy state in mitochondria and tissues in animals with experimental model diseases such as anoxia and ischemia.

ACKNOWLEDGEMENTS The authors thank Drs. S. Ohtake, K. Kawabe, J. Tsutsumi, T. Nakamura and M. Ohmae of Eisai Co., Ltd. for their valuable assistance and discussion.

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