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DETERMINATION OF REDUCED AND TOTAL UBIQUINONES IN BIOLOGICAL MATERIALS BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

TADASHI OKAMOTO, YUKO FUKUNAGA, YUKAKO IDA and TAKEO KISHI*

Faculty of Pharmaceutical Sciences, Kobe-Gakuin University, Nishi-ku, Kobe 673 (Japan)

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SUMMARY

A convenient and reliable liquid chromatographic (LC) method with electrochemical detection (ED) was developed for the determination of reduced (ubiquinol) and total ubiquinones in biological materials. After extraction of samples with *n*-hexane, ubiquinol was separated on a reversed-phase column and assayed directly by ED. In order to determine the total amount of a ubiquinone in biological samples, the unbiquinone was converted into the corresponding reduced form by treatment with sodium borohydride. No significant interfering peak (plastoquinol-9, ubichromenol-9, etc.) was observed in the elution areas of ubiquinol-7 to -11. This LC–ED method was about 70 times more sensitive than the previous LC–UV method and was able to detect 150 pg of ubiquinol-10. The method was applied satisfactorily to the determination of the contents of ubiquinol homologues in biological materials. The content of ubiquinols is a major component of the total ubiquinones in human plasma and urine and rat plasma and liver, but a minor component in rat heart and kidney.

INTRODUCTION

It is well known that ubiquinone plays an important role as an essential electron carrier in the mitochondrial respiratory chain [1, 2]. Several reports have indicated that exogenous ubiquinone can give protection against myocardial cellular damage caused by ischaemia [3, 4] and adriamycin toxicity [5, 6], and that one of the proposed mechanisms for such cellular damage is an increase in lipid peroxides of the myocardium [7]. On the other hand, the protective effect of reduced ubiquinone (ubiquinol) against lipid peroxidation has been demonstrated in isolated liver mitochondria [8], in bovine heart submitochondrial particles [9] and in egg yolk phosphatidylcholine liposomes [10]. Mellors and Tappel [8] reported that ubiquinol-6 was more effective than ubiquinone-6 and almost as effective as α-tocopherol in the removal of free radicals. Takeshige et al. [11] also suggested that lipid peroxidation in bovine heart mitochondria may be con-

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trolled according to ubiquinol-10 level. However, it is still obscure how ubiquinol contributes to the physiological function of ubiquinone, except for mitochondrial respiration, in the animal body. In order to investigate such problems, a reliable and sensitive method for the determination not only of ubiquinone but also of ubiquinol in biological materials is required.

For the determination of ubiquinol in biological samples, the dual-wavelength spectrometric method which was developed by Chance [12], Hatefi [13] and Crane and Barr [14] has often been employed in the past. However, these methods cannot easily distinguish each homologue of ubiquinol and need a large amount of sample because of the low sensitivity. Liquid chromatography (LC) with UV detection is a useful method for the separation and determination of ubiquinone homologues in serum and animal tissues because of its excellent selectivity and sensitivity [15–20]. We have also reported an LC method for ubiquinone-related compounds in biological materials [21]. However, in view of the low molar absorptivities of ubiquinols at conventionally used detection wavelengths (275–290 nm), these compounds could not be determined by the LC–UV method. For this purpose we introduced LC with electrochemical detection (ED). In recent years, LC–ED methods capable of determining ubiquinols have been developed by Ikenoya et al. [22], Tsai et al. [23] and Lang and co-workers [24, 25]. The method of Tsai et al. [23], however, requires additional equipment for the flow–quench system which is not available in many laboratories. Moreover, in the methods of Ikenoya et al. [22] and Lang and co-workers [24,25], two types of detector (ED and UV) are also needed because of the simultaneous determination of ubiquinol and ubiquinone.

This paper reports a convenient and precise method for the separation and determination of reduced and total ubiquinone homologues using LC–ED, which does not require any additional detector or equipment. In addition, the application of this method to the determination of ubiquinols and total ubiquinones in various biological specimens is described.

EXPERIMENTAL

Chemicals

Ubiquinone homologues from ubiquinone-7 to -10, plastoquinone-9 and ubichromenol-9 were kindly donated by Mr. S. Osono (Eisai, Tokyo, Japan). Ubiquinone-11 was supplied by Dr. H. Fukawa (Nisshin Chemical, Saitama, Japan). α-Tocopherol and retinyl palmitate were purchased from Nakarai Chemicals (Kyoto, Japan). All LC solvents were purchased from Wako (Osaka, Japan) and were of analytical-reagent grade for LC. Other reagents were of the best grade commercially available.

Chromatography

Apparatus. The LC device consisted of a Shimazu LC-3A pump (Shimazu Seisakusho, Kyoto, Japan) with a Rheodyne loop injector and an Irica E-502 amperometric detector (Irica, Kyoto, Japan).

Chromatographic conditions. The analytical conditions for the determination

of ubiquinol were as follows: stationary phase, Finepak SIL C_{18-5} (5-μm particles); column, stainless-steel, 250 mm×4.6 mm I.D., Japan Spectroscopic); mobile phase, a mixture containing 0.05 *M* sodium perchlorate in ethanol–methanol–acetonitrile–70% perchloric acid (400:300:300:1, v/v); flow-rate, 1.0 ml/min; applied potential, +0.6 V vs. Ag/AgCl; sensitivity, 8 nA; and chart speed, 0.5 cm/min. The LC measurements were performed at room temperature.

Calculation. The contents of ubiquinol homologues in the samples were calculated from calibration graphs of peak height versus the concentration of each homologue.

Preparation of authentic ubiquinone and ubiquinol. Authentic ubiquinone homologues from ubiquinone-7 to -11 were prepared by dissolution in ethanol to yield concentrations of 10 μg/ml. Authentic ubiquinol homologues were freshly prepared from the corresponding ubiquinone by adding 25 μg of sodium borohydride (10 μl of a 0.25% solution of sodium borohydride in water) to give a concentration of 1 μg/ml prior to LC analysis.

Samples

Human blood samples. After informed consent had been obtained, blood was obtained from normal human volunteers by venipuncture using heparin-coated tubes to prevent coagulation.

Rats. Four-week-old male Wistar rats (specific pathogen-free, body mass 60–80 g) were purchased from Shizuoka Laboratory Animal Centre (Shizuoka, Japan). They were fed a ubiquinone-free diet [26] for two weeks in advance and grown to about 150 g body mass. Rats were anaesthetized with diethyl ether, injected with 0.2 ml of heparin (1000 U/ml) into the femoral vein and then laparotomized. First, blood was drawn from each abdominal aorta and each liver was perfused with 20 ml of 0.9% ice-cold saline from the hepatic portal vein to the inferior vena cava. Each tissue was cut out as quickly as possible.

Preparation of samples for LC

All processes for the preparation of the samples were carried out in the dark.

Plasma. An aliquot of 0.2 ml of plasma from heparinized venous blood was pipetted into a brown glass-stoppered centrifuged tube and 0.3 ml of distilled water, 2 ml of ethanol and 5 ml of *n*-hexane were added in that order. The mixture was shaken vigorously reciprocally at a rate of 80 min^{-1} for 10 min and centrifuged at 500 *g* for 10 min. This extraction procedure was repeated three times. Subsequently, the combined *n*-hexane layer was concentrated in vacuo under a stream of nitrogen. The resulting residue was dissolved in 0.5 ml of ethanol and an aliquot of 10 μl of the ethanol solution was injected into the column to determine ubiquinol level. Separately, an aliquot of 10 μl of 0.25% sodium borohydride solution was added to 0.4 ml of the ethanol solution and the mixture allowed to stand for 10 min at room temperature. An aliquot of 10 μl of the solution was injected into the column to determine total ubiquinones.

Urine. An aliquot of 2 ml of urine collected at random was placed in a brown glass-stoppered centrifuged tube and 2 ml of ethanol and 5 ml of *n*-hexane were

added. The solution was extracted as described above for plasma samples and assayed by LC.

Rat tissues. Rat tissues were homogenized with 0.9% saline at 4°C using a Polytron homogenizer (Type PT 10/35; Kinematica, Lucerne, Switzerland) at a setting of 7 for 30 s. The final volume of the homogenate was adjusted so as to contain about 1–2 μg of ubiquinol. An aliquot of 0.5 ml of the tissue homogenate was pipetted into a brown glass-stoppered centrifuged tube and then 2 ml of ethanol and 5 ml of *n*-hexane were added. The solution was extracted as described above for plasma samples and determined by LC.

Measurement of urinary creatinine content

The creatinine content in urine was determined spectrophotometrically by the Folin–Wu method [27].

RESULTS AND DISCUSSION

Separation of ubiquinol homologues

Finepak SIL C_{18-5} reversed-phase material was chosen as the stationary phase because it offered the best separation from ubiquinol-7 to -11. Fig. 1A shows the chromatogram of authentic ubiquinol-7 to -11. The retention times of ubiquinol homologues decreased with decreasing proportion of acetonitrile in the mobile phase.

The most satisfactory result was obtained with an eluent composition of 0.05 *M* sodium perchlorate in ethanol–methanol–acetonitrile–70% perchloric acid

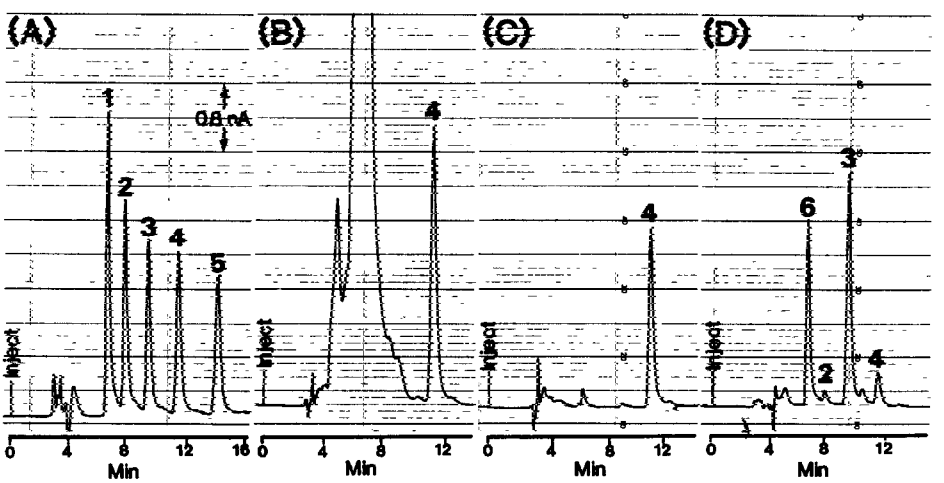

Fig. 1. Chromatograms of human plasma, urine and rat liver samples. (A) Standard ubiquinol homologues (ethanolic mixture containing 5 ng of each ubiquinol); (B) human plasma sample; (C) human urine sample; (D) rat liver sample. Peaks: 1 = ubiquinol-7; 2 = ubiquinol-8; 3 = ubiquinol-9; 4 = ubiquinol-10; 5 = ubiquinol-11; 6 = α-tocopherol. LC conditions: stationary phase, Finepak SIL C_{18-5}; mobile phase, a mixture containing 0.05 *M* sodium perchlorate in ethanol–methanol–acetonitrile–70% perchloric acid (400:300:300:1, v/v); flow-rate, 1.0 ml/min; applied potential, +0.6 V vs. Ag/AgCl; attenuation, 8 nA.

TABLE I

RETENTION TIMES AND DETECTION LIMITS OF UBIQUINOL-RELATED COMPOUNDS

LC conditions as described in the text.

Compound	Retention time (min)	Detection limit* (pg)
Uniquinol-7	6.92	100
Uniquinol-8	8.17	150
Uniquinol-9	9.77	150
Uniquinol-10	11.25	150
Uniquinol-11	14.00	200
Retinyl palmitate	9.50	2000
α-Tocopherol	6.87	50
Ubichromenol-9	13.37	150
Plastoquinol-9	8.87	150

*The signal-to-noise ratio was 5.

(400:300:300:1, v/v), giving a flow-rate of 1.0 ml/min. As shown in Table I, the retention times were 6.92 min for ubiquinol-7, 8.17 min for ubiquinol-8, 9.77 min for ubiquinol-9, 11.25 min for ubiquinol-10 and 14.00 min for ubiquinol-11. Under these conditions, the calibration graphs were linear up to at least 20 ng of ubiquinol homologues. The detection limits from ubiquinol-7 to -11 were in the range 100–200 pg (signal-to-noise ratio = 5). In terms of the detection limit for ubiquinone-10, the LC–ED method was 67 times more sensitive than the LC–UV method set at 275 nm as described previously [21].

When biological materials were extracted with *n*-hexane, ubiquinol-related compounds such as plastoquinol-9 and ubichromenol-9 can be also extracted. However, these compounds could be separated from ubiquinol homologues under the LC conditions described above (Table I). Recently, Lang and co-workers [24, 25] reported the simultaneous determination of tocopherols and ubiquinols in blood and tissue homogenate by LC–ED. Under our LC conditions, α-tocopherol was eluted at the same retention time as ubiquinol-7. However, this is not a problem in practice with animal samples, because ubiquinone-7 (including ubiquinol-7) is not synthesized in the rat [26]. According to the method of Katayama et al. [28], the peak of ubiquinone-10 was overlapped by that of retinyl palmitate. However, the applied potential of 0.6 V in our method was low enough to eliminate the contamination of retinyl palmitate in the ubiquinol homologues.

Recovery, precision and reproducibility

Direct extracts from biological materials contained both ubiquinols and ubiquinones and it is necessary to reduce the ubiquinones to ubiquinols in advance in order to determine the total ubiquinone content. A recovery test was performed by addition of known amounts of ubiquinol-10 or ubiquinone-10 to plasma. The recoveries of ubiquinol added to plasma are shown in Table II. The mean recovery was $100.0 \pm 1.96\%$ ($n = 8$). The ubiquinone-10, therefore, was reduced completely to the corresponding quinol form, ubiquinol-10, by the addition of 10 μl of 0.25% sodium borohydride solution.

TABLE II

RECOVERIES OF UBIQUINOL AND UBIQUINONE ADDED TO HUMAN PLASMA

Compound	Added UQ-10 (A) (μg/ml)	$NaBH_4$ treatment	Observed ubiquinol-10 (μg/ml)		Recovery: $(C)/(A+B)\times 100$ (%)
			Before addition (B)	After addition (C)	
Ubiquinone-10	0.50	–	0.90	0.88	–
	1.00	–	0.90	0.91	–
	0.25	+	1.21	1.43	97.9
	0.50	+	1.21	1.72	100.6
	1.00	+	1.21	2.17	98.2
	2.00	+	1.21	3.23	100.6
Ubiquinol-10	0.50	–	1.16	1.63	98.2
	1.00	–	1.16	2.15	99.5
	0.50	+	1.16	1.72	103.6
	1.00	+	1.16	2.19	101.4

We have reported earlier [21] the determination of the plasma level of ubiquinone-10 by LC with UV detection. In the method, ubiquinol-10 in human plasma was recovered as ubiquinone-10 due to following a thin-layer chromatographic step. Therefore, determinations of total ubiquinone-10 in fifteen human plasma samples were carried out by the LC–ED and LC–UV methods. No significant difference was observed between the two methods (Student's paired t-test, $t=0.937, p>0.20$).

The reproducibility of the proposed procedure was also tested by analysing the same plasma samples in quadruplicate. Samples were taken by repeated sampling from a single batch of human plasma. Excellent reproducibility was obtained for ubiquinol-10 and total ubiquinone-10, with coefficients of variation of 3.07 and 3.03%, respectively.

Stability of ubiquinol-10 in human plasma

It is well known that ubiquinols are not stable and are oxidized easily in air. Ikenoya et al. [22] reported that the levels of ubiquinol homologues in frozen tissues from the guinea pig decreased gradually with time. Therefore, in order to evaluate the stability of ubiquinol-10 in plasma under various storage conditions, a pooled plasma was stored at 24°C (ambient temperature), 2°C and −10°C for up to four days, analysed and compared with a 0-h sample. The results are shown in Table III. The percentage of ubiquinol-10 with respect to the total, i.e., the sum of ubiquinol-10 and ubiquinone-10, was constant for one day when kept at 2 and −10°C. At 24°C, 69% of the ubiquinol-10 disappeared in one day, but the total contents remained unchanged. When extracted from plasma and prepared in ethanolic solution, ubiquinol-10 in the solution rapidly oxidized. Similar results were obtained for urine and animal tissues. Therefore, these results indicate that

TABLE III

STABILITY OF UBIQUINOL-10 IN HUMAN PLASMA

Storage conditions	Period (h)	Ubiquinol-10 (μg/ml)	Total (μg/ml)	Ratio* (%)
2°C	0	0.86	0.99	87
	24	0.84	0.98	86
	48	0.71	1.01	70
	168	0.33	0.97	34
−10°C	0	0.99	1.12	88
	24	1.01	1.10	92
	48	0.88	1.13	78
	168	0.44	1.11	40
24°C	0	0.81	0.92	88
	24	0.56	0.91	62
	48	0.54	0.91	59
Ethanol solution of extracts	0	1.02	1.29	79
	6	0.52	1.29	40
	24	0.24	1.28	19

*Ubiquinol-10/total×100 (%).

blood samples drawn from humans must be analysed on the same day or within 24 h, even if stored below 2°C.

Determination of ubiquinol and total ubiquinone in biological materials

The extraction of ubiquinone from biological materials has generally been performed with organic solvents such as *n*-hexane [21, 22, 24, 25] or after alkaline saponification in the presence of pyrogallol [15, 16]. However, extraction by saponification has been suggested to oxidize ubiquinol to ubiquinone in animal tissues [22]. Therefore, a direct extraction method with ethanol–*n*-hexane was chosen, as described under Experimental. Under this procedure, the efficiency of extraction of ubiquinol homologues was 98.5±0.75% (mean±S.D., $n=5$) when extracted three times with *n*-hexane. No internal standard, therefore, was used in this method. Indeed, the linear correlation coefficients of the calibration graphs of peak height versus concentration were greater than 0.996 for all the ubiquinol homologues.

Fig. 1B and C show representative chromatograms of the extracts from human plasma and urine, respectively. These samples contained only ubiquinol-10. Ubiquinol-9 and other shorter homologues were not detected. The chromatographic peak observed for ubiquinol-10 was identified from the retention time, co-chromatography with authentic ubiquinol-10 and UV spectra. Further, the peak disappeared on oxidation of sample extracts with potassium hexacyanoferrate(III) and it appeared again on re-reduction with sodium borohydride. Similar identifications were carried out for human urine and rat tissues.

The levels of ubiquinol-10 in human plasma and urine are given in Table IV.

TABLE IV

LEVELS OF UBIQUINOL-10 IN HUMAN PLASMA AND URINE

Sample	Subject	Age (years)	Ubiquinol-10	Total	Ubiquinol-10/total (%)
Plasma (μg/ml)	Male A	31	0.99	1.12	88
	Female B	23	0.49	0.57	86
Urine* (μg/mg of creatinine)	Males	21–31 ($n=11$)	0.044 ± 0.015	0.074 ± 0.024	60.9 ± 10.5
	Females	21–23 ($n=11$)	0.050 ± 0.021	0.086 ± 0.037	58.9 ± 12.2
	Total	21–31 ($n=22$)	0.047 ± 0.018	0.080 ± 0.031	59.9 ± 11.2

*Data are expressed as mean ± S.D.

TABLE V

UBIQUINOL CONTENTS IN RAT TISSUES

Percentage of total content of the corresponding homologues in parentheses.

Tissue	n	Ubiquinol content (mean ± S.D.) (μg/ml for plasma, μg/g wet mass for tissues)		
		Ubiquinol-8	Ubiquinol-9	Ubiquinol-10
Plasma	8	0.06 ± 0.01 (69)	0.37 ± 0.06 (78)	0.10 ± 0.01 (70)
Liver	7	2.2 ± 0.6 (44)	67.1 ± 3.9 (55)	11.5 ± 1.5 (42)
Heart	4	0.73 ± 0.09 (11)	16.6 ± 0.5 (9)	1.9 ± 0.08 (8)
Kidney	4	0.44 ± 0.03 (13)	18.3 ± 3.6 (14)	4.4 ± 0.8 (15)

The percentages of the ubiquinol-10 to the total were 86 and 88% in human plasma. The mean urinary levels of ubiquinol-10 in eleven males and eleven females were 0.044 ± 0.015 and 0.050 ± 0.021 μg/mg of creatinine, respectively, which were not significantly different from each other. Daily urinary levels of ubiquinol-10, therefore, were estimated to be 44–75 μg based on the daily urinary excretion levels of creatinine in normal subjects.

A typical LC trace for ubiquinol homologues in a rat liver sample is shown in Fig. 1D. The ubiquinol homologues found in rat liver were ubiquinol-8, -9 and -10. As ubiquinol-7 was eluted at the same retention time as α-tocopherol (Table I), we suspected that peak 6 in Fig. 1D might be ubiquinol-7. However, the peak did not disappear on oxidation of the sample with potassium hexacyanoferrate(III). Moreover, when the oxidized sample was applied to the UV detector, the peak of ubiquinone-7 was not detected in the chromatogram. We reported previously [26] that rats fed on a ubiquinol-free ration did not have ubiquinol-7 in the liver.

Table V shows the contents of ubiquinol homologues in rat tissues. Among the ubiquinol homologues, ubiquinol-9 was predominant, its mean contents (μg/g wet mass, μg/ml) being 0.37 ± 0.06 for plasma, 67.1 ± 3.9 for liver, 16.6 ± 0.5 for heart and 18.3 ± 3.6 for kidney.

The mechanism by which ubiquinone undergoes reduction to yield ubiquinol is a major subject that is of interest to us. We have developed a simple and precise

LC method for the simultaneous determination of ubiquinol and total ubiquinone in biological materials which is sufficiently sensitive for use in clinical and enzymatic studies.

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