Simultaneous Determination of Oxidized and Reduced Coenzyme Q and α -Tocopherol in Biological Samples by High Performance Liquid Chromatography with Platinum Catalyst Reduction and Electrochemical Detection

Hiroyuki WAKABAYASHI, Susumu YAMATO, Masaharu NAKAJIMA and Kenji SHIMADA*

Department of Analytical Chemistry, Niigata College of Pharmacy, 5-13-2 Kamishin'ei-cho, Niigata 950-21, Japan.

Received February 14, 1994; accepted April 1, 1994

A convenient and sensitive high performance liquid chromatographic method with post-column platinum catalyst reduction and electrochemical detection was applied to the simultaneous determination of oxidized and reduced coenzyme Q (CoQ and CoQH₂) and α -tocopherol (α -TP) in human and rat samples. After the separation of oxidized and reduced CoQ homologues (CoQ₈–CoQ₁₀ and CoQ₈H₂–CoQ₁₀H₂) and α -TP on a reversed-phase column, oxidized CoQ homologues were reduced on a platinum catalyst reduction column, and then all the reduced forms were quantified with an electrochemical detector operated in the oxidation mode (+0.6 V vs. Ag/AgCl). The order of elution was α -TP, CoQ₈H₂, CoQ₉H₂, CoQ₈, CoQ₁₀H₂, CoQ₉, and CoQ₁₀ and all were well separated within 13 min. The detection limits at a signal-to-noise ratio of 3 were 20 pg for α -TP, 70 pg for CoQ₈ and CoQ₈H₂, and 100 pg for CoQ₉, CoQ₉H₂, CoQ₁₀ and CoQ₁₀H₂. Quantitative recoveries (93–102%) from serum and tissues were obtained for all the analytes studied. This method was applied satisfactorily to the simultaneous determination of CoQ, CoQH₂ and α -TP in biological materials.

Keywords coenzyme Q; α -tocopherol; HPLC-ED; platinum catalyst reduction; serum; tissue

Coenzyme Q (CoQ) is a lipid-soluble compound consisting of a redox-active quinoid nucleus and a hydrophobic side-chain containing a number of monosaturated *trans*-isoprenoid units. The hydroquinone form (CoQH₂) is the two-electron reduction product of CoQ. The main function of CoQ in biological systems is to act as the redox component of transmembrane electron transport systems, such as the respiratory chain of mitochondria.¹⁾ CoQ is also known to play a role in controlling the physical properties of phospholipid bilayer membranes.^{2,3)} Several studies have suggested that CoQH₂ has an anti-oxidative effect like α -tocopherol (α -TP).^{4–8)} Booth *et al.* showed that CoQH₂ is at least as active as α -TP in scavenging free radicals because of its anti-oxidant efficiency.⁹⁾ Therefore, it is of importance to be able to determine not only CoQ and α -TP but also CoQH₂ in biological samples in order to elucidate the biochemical and physiological roles of these compounds.

Several reports have been published relating to the determination of CoQ and/or CoQH₂ using high performance liquid chromatography (HPLC).^{10–15)} Two types of detector (UV and electrochemical detector)^{13,14)} or twin electrochemical detectors¹⁵⁾ are needed for the simultaneous determination of CoQ and CoQH₂. Lang *et al.*^{16,17)} and Edlund¹⁸⁾ have independently developed HPLC methods with the electrochemical detection (ED) and simultaneous determination of CoQ, CoQH₂ and α -TP. However, the former method requires both UV and electrochemical detectors, while the latter needs a coulometric detector with three working electrodes housed in cells, which is not available in many laboratories.

In a previous paper, we demonstrated that a platinum catalyst reduction column is a useful tool for hydrogenating the quinone ring to give the corresponding hydroquinone. By connecting the reduction column to a sep-

aration column, we have been able to determine 6-(10-hydroxydecyl)-2,3-dimethoxy-5-methyl-1,4-benzoquinone (idebenone), a drug for the treatment of senile dementia, and its reduced form, 6-(10-hydroxydecyl)-2,3-dimethoxy-5-methyl-hydroquinone.¹⁹⁾

In this paper, we report a convenient and sensitive method for the simultaneous determination of CoQ, CoQH₂ and α -TP using HPLC-ED, in which a platinum catalyst reduction column and an electrochemical detector with a glassy carbon electrode are incorporated in the detection system. We have also applied this system to the simultaneous determination of CoQ, CoQH₂ and α -TP in human and rat serum and tissue samples.

MATERIALS AND METHODS

Chemicals and Solutions CoQ₁₀ was purchased from Merck (Darmstadt, Germany). α -TP was obtained from Sigma (St. Louis, MO, U.S.A.). CoQ₈, CoQ₉, and menaquinone-8 (vitamin K₂, *n*=8) were kindly donated by Eisai Co., Ltd. (Tokyo, Japan). Sodium borohydride and anhydrous sodium perchlorate were obtained from Kanto Chemical (Tokyo, Japan). All other chemicals and solvents employed were of analytical reagent or HPLC grade, and were used without further purification. Stock solutions of CoQ homologues (0.5 mg/10 ml), α -TP (0.5 mg/10 ml), and menaquinone-8 (internal standard, 0.1 mg/10 ml) were prepared by dissolving each compound in HPLC-grade ethanol and diluting to the appropriate concentrations prior to use. The stock solutions were shielded from light during storage. CoQ₈H₂, CoQ₉H₂, and CoQ₁₀H₂ were freshly prepared by the reduction of the corresponding CoQ with 0.25% sodium borohydride in water according to the method of Okamoto *et al.*¹¹⁾

Apparatus and Chromatographic Conditions The equipment consisted of an LC-6A HPLC system

(Shimadzu, Kyoto, Japan) and a Rheodyne 7125 injection valve (Cotati, CA, U.S.A.) with a 100 μ l sample loop. A separation column of Capcell Pak C₈ (SG 120, 5 μ m particle size, 150 $\times$ 4.6 mm i.d., Shiseido, Japan) was operated at ambient temperature. The electrochemical detector was an EICOM ECD 100 (Eicom, Kyoto, Japan) with a glassy carbon working electrode (G-25 gasket) and an Ag/AgCl reference electrode; the applied oxidation potential was set at +0.6 V. A mixture of ethanol-methanol-water (8.2:0.8:1.0, v/v) containing 0.05 M sodium perchlorate was used as the mobile phase at a flow-rate of 0.8 ml/min. For the post-column reduction, a stainless steel column (10 $\times$ 4.6 mm i.d.) was packed with platinum catalyst (5% on alumina, 10 μ m, Toa Electronics, Tokyo, Japan) in accordance with the previous report.¹⁹⁾ Briefly, a stainless-column was uniformly packed with platinum catalyst by tapping, and then purged with water at a flow-rate of 10 ml/min for 5 min. This reduction column was connected to the outlet of the separation column and the effluent from the reduction column was passed into the detector. Data were recorded and evaluated using a Shimadzu C-R 3A integrator. The amounts of CoQ, CoQH₂ and α -TP were calculated from the ratios of the peak areas of these compounds relative to the internal standard (I.S., menaquinone-8).

Extraction of CoQ, CoQH₂ and α -TP from Serum

Human and rat blood samples were collected by venipuncture, transferred to centrifuge tubes individually and centrifuged at 1500 *g* for 10 min. The serum thus obtained was used for the assay. An aliquot of 0.1 ml of human or rat serum was pipetted into a 10 ml glass-stoppered centrifuge tube, and then 0.1 ml distilled water was added followed by 0.5 ml ethanol, 50 μ l menaquinone-8 solution as an I.S. (1 μ g/ml in ethanol) and 3 ml hexane. The solution was vortex-mixed for 5 min and centrifuged at 1500 *g* for 10 min. The hexane layer was removed and evaporated to dryness under nitrogen gas. The residue was dissolved in 100 μ l ethanol which was then filtered through the Column Guard (Millipore, 0.45 μ m) and a 10 μ l aliquot of the clear filtrate was applied to the HPLC system.

Extraction of CoQ, CoQH₂ and α -TP from Rat Tissues

A male Wistar rat, weighing 250–300 g, was sacrificed. The brain, lungs, heart, liver and kidneys were quickly removed, rinsed with ice-cold saline, and the wet weight recorded. Tissues (*ca.* 0.1 g wet weight) were minced and homogenized in 1 ml distilled water. An aliquot of 0.5 ml homogenate was pipetted into a glass-stoppered centrifuge tube, followed by 0.5 ml ethanol, 50 μ l I.S. solution (1 μ g/ml in ethanol), and 3 ml hexane. The solution was vortex-mixed for 5 min and centrifuged at 1500 *g* for 10 min. The hexane layer was removed and evaporated to dryness under nitrogen. The residue was dissolved in 100 μ l ethanol which was then filtered through the Column Guard and a 10 μ l aliquot of the clear filtrate was injected into the HPLC.

RESULTS AND DISCUSSION

Optimization of HPLC-ED Conditions In order to determine the optimal applied-voltage for ED, hydrodynamic voltammograms were obtained by analyzing each solution of CoQ homologues (5 ng) and α -TP (5 ng) at different voltage settings (Fig. 1). Each compound exhibited the same electrochemical characteristics and reached a maximum response at an applied voltage of +0.6 V. When the applied voltage was set at +0.6 V, the background current was always less than 5 nA. Hence, the detection potential was maintained at +0.6 V vs. the Ag/AgCl reference electrode. As a result of a compromise involving the mobile phase composition, flow-rate and concentration of the supporting electrolyte, an excellent separation of an authentic mixture of CoQ, CoQH₂ and α -TP was obtained on a Capcell Pak C₈ column using a mobile phase of ethanol-methanol-water (8.2:0.8:1.0, v/v) containing 0.05 M sodium perchlorate at a flow-rate of 0.8 ml/min. As shown in Fig. 2, CoQ₈–CoQ₁₀, CoQ₈H₂–CoQ₁₀H₂ and α -TP were clearly separated from each other. The retention times were 3.8 min for α -TP, 4.9 min for CoQ₈H₂, 6.0 min for CoQ₉H₂, 6.7 min for CoQ₈, 7.5 min for CoQ₁₀H₂, 8.6 min for CoQ₉, and 12.1 min for CoQ₁₀. Under these conditions, the detection limits at a signal-to-noise ratio of 3 were 20 pg for α -TP, 70 pg for CoQ₈ and CoQ₈H₂, and 100 pg for CoQ₉,

Fig. 1. Hydrodynamic Voltammograms of CoQ and α -TP

Injection amount, 5 ng; $\blacktriangle$ — $\blacktriangle$, CoQ₈; $\blacksquare$ — $\blacksquare$, CoQ₉; $\bullet$ — $\bullet$, CoQ₁₀; $\circ$ — $\circ$, α -TP.

Fig. 2. Chromatogram of an Authentic Mixture of CoQ, CoQH₂, α-TP and Menaquinone-8 (I.S.)

Peaks: 1=α-TP (2.5 ng); 2=CoQ₈H₂ (10 ng); 3=CoQ₉H₂ (10 ng); 4=CoQ₈ (10 ng); 5=CoQ₁₀H₂ (10 ng); 6=CoQ₉ (10 ng); 7=I.S. (7 ng); 8=CoQ₁₀ (10 ng).

CoQ₉H₂, CoQ₁₀ and CoQ₁₀H₂. These detection limits with the present method are superior to those in HPLC methods reported earlier.^{12,13,16,17,20,21} In order to reduce errors during the extraction procedure and to increase the analytical precision, several compounds having electrochemical characteristics similar to CoQ were examined as candidates for a suitable I.S. Okamoto and co-workers reported that CoQ₉ and CoQ₁₁ were the most convenient I.S. for measuring CoQ₁₀ in human samples and CoQ₉ in rat samples, respectively.¹⁰ As shown in Figs. 3 and 4, the CoQ₉ peak was clearly visible on the chromatograms of rat serum and tissues with the detection system employed here. Menaquinone-8, a vitamin K₂ homologue, was not detected to any great extent in rat tissues,²² and was eluted at 10.5 min and was separated from the other analytes which were of interest to us. Menaquinone-8 was chosen as the best I.S. for further investigation.

Reproducibility and Recovery The reproducibility of the present method was assessed by analyzing a standard mixture containing CoQ₈, CoQ₉, CoQ₁₀ and α-TP, ten times a day for five consecutive days. The average coefficients of variation of within-day and between-day assays were respectively 3.0 and 3.6% for CoQ₈, 3.2 and 3.9% for CoQ₉, 3.1 and 4.0% for CoQ₁₀, and 2.7 and 3.3% for α-TP. To determine the amounts of CoQ, CoQH₂ and α-TP in biological samples, calibration curves were constructed by plotting the peak-area ratios of these

Fig. 3. Typical Chromatograms of Extracts from Healthy Human Serum (A) and Normal Rat Serum (B)

Peaks: 1=α-TP; 2=CoQ₈H₂; 3=CoQ₈; 4=CoQ₁₀H₂; 5=CoQ₉; 6=I.S.; 7=CoQ₁₀. The injection volume was 10 μl.

compounds relative to the I.S. against the amount of these compounds injected. The calibration curves for all analytes were linear over the range of 0.5–100 ng. The regression equations were $y=0.1318x+0.034$ ($r=0.998$) for CoQ₈, $y=0.0997x+0.016$ ($r=0.996$) for CoQ₉, $y=0.0561x+0.076$ ($r=0.996$) for CoQ₁₀, $y=0.7495x-0.214$ ($r=0.998$) for CoQ₈H₂, $y=0.6038x-0.289$ ($r=0.997$) for CoQ₉H₂, $y=0.5021x-0.186$ ($r=0.997$) for CoQ₁₀H₂, and $y=0.3907x+0.693$ ($r=0.996$) for α-TP. The recoveries of CoQ and α-TP added to human and rat samples are shown in Table I. The extraction of CoQ and α-TP from biological materials has generally been performed with hexane.^{10-17,20,21} We have examined the best conditions for extraction, i.e., volume of hexane, mixing time and the number of extractions. A single extraction with 3 ml hexane for 5 min gave a satisfactory recovery from all human and rat samples. As shown in Table I, CoQ and α-TP were recovered satisfactorily in all cases with an efficiency of 93–102% (relative standard deviation (R.S.D.) 2.4–6.5%).

Concentration of CoQ, CoQH₂ and α-TP in Serum and Tissues Figures 3 A and B show representative chromatograms of serum extracts from human and rat, respectively. Typical chromatograms of extracts from rat cerebellum (A), heart (B), lungs (C), liver (D) and kidneys (E) are shown in Fig. 4. Other rat brain tissues (cerebral cortex and hippocampus) showed similar

TABLE I. Recoveries of Co Q and α -TP from Tissues and Serum

		CoQ ₈		CoQ ₉		CoQ ₁₀		α -TP	
		Added ^{a)}	Recovery (%)	Added ^{a)}	Recovery (%)	Added ^{a)}	Recovery (%)	Added ^{a)}	Recovery (%)
Rat	Cerebral cortex	1	95.9 $\pm$ 2.9	10	97.2 $\pm$ 3.8	10	98.5 $\pm$ 2.4	5	97.7 $\pm$ 3.2
	Cerebellum	1	95.3 $\pm$ 3.1	10	96.5 $\pm$ 3.9	10	98.1 $\pm$ 2.9	5	97.2 $\pm$ 3.0
	Hippocampus	1	96.0 $\pm$ 2.6	10	97.3 $\pm$ 3.5	10	98.0 $\pm$ 3.0	5	96.9 $\pm$ 3.1
	Heart	5	97.1 $\pm$ 3.0	50	98.4 $\pm$ 3.6	10	97.7 $\pm$ 3.2	5	101.5 $\pm$ 3.6
	Lung	1	94.4 $\pm$ 5.1	10	96.8 $\pm$ 3.5	10	95.8 $\pm$ 6.2	5	98.7 $\pm$ 3.0
	Liver	5	93.5 $\pm$ 4.7	50	98.5 $\pm$ 3.9	10	96.2 $\pm$ 5.1	5	99.8 $\pm$ 3.5
	Kidney	1	94.2 $\pm$ 5.2	50	98.8 $\pm$ 4.0	10	97.8 $\pm$ 4.7	5	102.1 $\pm$ 3.1
	Serum	0.1	97.3 $\pm$ 4.8	0.3	98.2 $\pm$ 3.9	0.3	97.4 $\pm$ 4.2	5	97.5 $\pm$ 3.0
Human Serum		0.1	95.1 $\pm$ 5.6	0.3	98.0 $\pm$ 4.5	0.3	97.8 $\pm$ 4.4	5	97.4 $\pm$ 3.1

Values are means $\pm$ S.D. ($n=5$). ^{a)} Added amounts are expressed as $\mu\text{g/g}$ wet weight of tissues and $\mu\text{g/ml}$ of serum.

Fig. 4. Typical Chromatograms of Extracts from Rat Cerebellum (A), Heart (B), Lungs (C), Liver (D) and Kidneys (E)

Peaks: 1 = α -TP; 2 = CoQ₈H₂; 3 = CoQ₉H₂; 4 = CoQ₈; 5 = CoQ₁₀H₂; 6 = CoQ₉; 7 = I.S.; 8 = CoQ₁₀. The injection volume was 10 μl .

chromatographic patterns. The within-day reproducibility of the proposed method was also examined by reanalyzing the same biological samples. Satisfactory reproducibilities were obtained for all analytes with coefficients of variation $< 3.6\%$. When successive injections of various biological samples were made during a one month period (about 500 sample injections), the electrochemical response gradually decreased. This electrode desensitization phenomena resulted from the adsorption of the final oxidized products on the glassy carbon electrode surface. However the sensitivity could be recovered simply by cleaning the

surface with acetone or polishing the electrode. On the other hand, the reduction ability of the platinum catalyst was unchanged by successive injections of biological samples. The proposed method was applied to the simultaneous determination of CoQ, CoQH₂ and α -TP in human and rat samples. Table II shows the concentrations of CoQ, CoQH₂ and α -TP in rat tissues and serum. The extensive data on various tissues were obtained for biochemical and physiological investigations of CoQ, CoQH₂ and α -TP. As shown in Table II, six homologues of CoQ and CoQH₂ in rat brain (cerebral cortex,

TABLE II. Oxidized and Reduced CoQ and α -TP in Rat Tissues and Serum

	CoQ ₈	CoQ ₈ H ₂	CoQ ₉	CoQ ₉ H ₂	CoQ ₁₀	CoQ ₁₀ H ₂	α -TP
	$\mu\text{g/g tissue or ng/ml}$						$\mu\text{g/g tissue or } \mu\text{g/ml}$
Cerebral cortex	n.d.	n.d.	19.5 $\pm$ 3.7	19.1 $\pm$ 3.5	8.2 $\pm$ 2.0	8.3 $\pm$ 1.4	13.6 $\pm$ 1.9
Cerebellum	0.5 $\pm$ 0.1	n.d.	46.9 $\pm$ 8.7	19.5 $\pm$ 4.1	9.7 $\pm$ 2.8	7.1 $\pm$ 1.8	12.3 $\pm$ 2.2
Hippocampus	2.1 $\pm$ 0.3	2.2 $\pm$ 0.7	19.2 $\pm$ 2.4	16.8 $\pm$ 4.6	10.6 $\pm$ 2.9	14.4 $\pm$ 2.3	16.7 $\pm$ 1.6
Heart	8.4 $\pm$ 1.9	n.d.	190.9 $\pm$ 35.4	20.8 $\pm$ 3.8	27.2 $\pm$ 4.9	2.5 $\pm$ 0.4	27.0 $\pm$ 2.8
Lung	0.5 $\pm$ 0.2	n.d.	42.1 $\pm$ 8.0	n.d.	8.4 $\pm$ 2.6	n.d.	28.6 $\pm$ 2.5
Liver	10.6 $\pm$ 2.2	9.6 $\pm$ 2.1	120.6 $\pm$ 35.1	67.8 $\pm$ 18.9	21.7 $\pm$ 0.4	7.1 $\pm$ 2.1	25.5 $\pm$ 3.4
Kidney	1.7 $\pm$ 0.2	1.1 $\pm$ 0.2	100.4 $\pm$ 29.1	63.9 $\pm$ 14.6	15.4 $\pm$ 2.4	9.2 $\pm$ 1.5	17.5 $\pm$ 2.5
Serum	74.1 $\pm$ 15.9	n.d.	471.9 $\pm$ 75.3	35.3 $\pm$ 7.2	124.9 $\pm$ 34.4	n.d.	9.1 $\pm$ 1.6

Values are means $\pm$ S.E. ($n=10$). n.d., not detected.

TABLE III. Levels of Oxidized and Reduced CoQ and α -TP in Normal Human Serum

Age	n	CoQ ₉ H ₂	CoQ ₁₀	CoQ ₁₀ H ₂	α -TP
		ng/ml			$\mu\text{g/ml}$
20—30	8	45.5 $\pm$ 11.6 ^{a)}	648.3 $\pm$ 106.2 ^{a)}	1141.8 $\pm$ 215.3 ^{a)}	8.9 $\pm$ 2.3 ^{a)}
30—40	6	37.3 $\pm$ 12.3 ^{a)}	707.6 $\pm$ 89.5 ^{a)}	1366.2 $\pm$ 260.6 ^{a)}	12.3 $\pm$ 3.1 ^{a)}
40—50	3	33.4 $\pm$ 7.1 ^{a)}	728.4 $\pm$ 75.1 ^{a)}	1427.3 $\pm$ 152.7 ^{a)}	12.9 $\pm$ 1.6 ^{a)}
50—60	1	31.5	741.9	1561.2	14.91
Total	18	36.9 $\pm$ 6.2 ^{a)}	706.6 $\pm$ 41.3 ^{a)}	1374.1 $\pm$ 175.0 ^{a)}	12.3 $\pm$ 2.5 ^{a)}

a) Values are means $\pm$ S.E.

cerebellum and hippocampus), heart, lungs, liver, kidneys and serum could be simultaneously determined. The concentration of CoQ₉ was greatest in all samples measured and a tissue difference was observed in the CoQ/CoQH₂ ratios which may reflect the redox state of the mitochondrial transport chain.^{16,17)} The values of CoQ₈, CoQ₉, CoQ₉H₂, CoQ₁₀ and CoQ₁₀H₂ in rat heart and liver are in good agreement with those reported by Okamoto *et al.*^{10,11)} The values of CoQ homologues in rat serum are also in agreement with those in plasma reported by Okamoto *et al.*¹⁰⁾ but the values of CoQH₂ homologues in serum were less than those in plasma reported by Okamoto *et al.*¹¹⁾ The range of α -TP content in tissues was 12—29 $\mu\text{g/g}$ tissue. The concentrations of α -TP in rat samples were 9.1 $\pm$ 1.6 $\mu\text{g/ml}$ serum and 25.5 $\pm$ 3.4 $\mu\text{g/g}$ liver tissue, respectively. These values are in agreement with those reported earlier.^{16,17)} Table III summarizes the average concentrations of CoQ, CoQH₂ and α -TP in human serum obtained from healthy volunteers. Blood was collected by venipuncture and the serum was immediately analysed. One group of investigators¹⁰⁾ reported that normal human plasma and serum contained only CoQ₁₀, however, we found not only CoQ₁₀ but also CoQ₉H₂ and CoQ₁₀H₂. The compound CoQ₁₀H₂ was the predominant component, its mean concentration being 1374.1 $\pm$ 175.0 ng/ml, while the mean level of CoQ₁₀ was 706.6 $\pm$ 41.3 ng/ml. The levels of CoQ₁₀ and CoQ₁₀H₂ in human serum were considerably higher than those reported by Lang *et al.*^{16,17)} However, the level of CoQ₁₀ is in agreement with that described in other papers.^{10,18)} The level of CoQ₉H₂ is similar to that already published.^{16,17)} The level of α -TP in normal human serum agrees well with that of previous reports.^{18,20)} The concentrations of these compounds in

human serum did not vary during the day. We also examined whether the levels of CoQ, CoQH₂ and α -TP in human serum are influenced by physical exercise such as running for 30 min. The serum level of those compounds involved in anti-oxidative effects was unchanged (data not shown), even although free radicals are produced and lipid peroxidation is increased during exercise.²³⁾

In conclusion, the present method allows the simultaneous determination of CoQ, CoQH₂ and α -TP in biological samples. Since the detection system avoids the use of multiple detectors, the present method is both convenient and economical in practical use. The times required for sample preparation and analysis are relatively short. Thus, the proposed method is useful for biochemical and physiological studies of CoQ, CoQH₂ and α -TP.

Acknowledgement The authors are grateful to Eisai Co., Ltd. (Tokyo, Japan) for providing the standards of CoQ and menaquinone-8, and also to Messrs. T. Mino, T. Murai, T. Ito and T. Saitoh for their assistances in the study.

REFERENCES

- 1) M. Gutman, *Biochim. Biophys. Acta*, **594**, 53 (1980).
- 2) A. Spisni, G. Sartor, L. Masotti, G. Lenaz, *Membrane Biochem.*, **4**, 149 (1981).
- 3) H. Katsikas, P. J. Quinn, *Eur. J. Biochem.*, **124**, 165 (1982).
- 4) L. Landi, L. Cabrini, A. M. Sechi, P. Pasquali, *Biochem. J.*, **222**, 463 (1984).
- 5) S. Marubayashi, K. Dohi, K. Yamada, T. Kawasaki, *Biochim. Biophys. Acta*, **797**, 1 (1984).
- 6) G. W. Burton, K. U. Ingold, *Science*, **224**, 569 (1984).
- 7) K. Mukai, S. Kikuchi, S. Urano, *Biochim. Biophys. Acta*, **1035**, 77 (1990).
- 8) B. Frei, M. C. Kim, B. N. Ames, *Proc. Natl. Acad. Sci. U.S.A.*,

- 87, 4879 (1990).
- 9) R. F. G. Booth, D. G. Galanopoulou, P. J. Quinn, *Biochem. Intern.*, **5**, 151 (1982).
- 10) T. Okamoto, K. Fukui, M. Nakamoto, T. Kishi, T. Okishio, T. Yamagami, N. Kanamori, H. Kishi, E. Hiraoka, *J. Chromatogr.*, **342**, 35 (1985).
- 11) T. Okamoto, Y. Fukunaga, Y. Ida, T. Kishi, *J. Chromatogr.*, **430**, 11 (1988).
- 12) G. Grossi, A. M. Bargossi, P. L. Fiorella, S. Piazzi, M. Battino, G. P. Bianchi, *J. Chromatogr.*, **593**, 217 (1992).
- 13) K. Katayama, M. Takada, T. Yuzuriha, K. Abe, S. Ikenoya, *Biochem. Biophys. Res. Commun.*, **95**, 971 (1980).
- 14) S. Ikenoya, M. Takada, T. Yuzuriha, K. Abe, K. Katayama, *Chem. Pharm. Bull.*, **29**, 158 (1981).
- 15) O. Hiroshima, S. Ikenoya, T. Naitoh, K. Kusube, M. Ohmae, K. Kawabe, S. Ishikawa, H. Hoshida, T. Kurahashi, *Chem. Pharm. Bull.*, **31**, 3571 (1983).
- 16) J. K. Lang, K. Gohil, L. Packer, *Anal. Biochem.*, **157**, 106 (1986).
- 17) J. K. Lang, L. Packer, *J. Chromatogr.*, **385**, 109 (1987).
- 18) P. O. Edlund, *J. Chromatogr.*, **425**, 87 (1988).
- 19) H. Wakabayashi, M. Nakajima, S. Yamato, K. Shimada, *J. Chromatogr.*, **573**, 154 (1992).
- 20) M. L. Huang, G. J. Burckart, R. Venkataramanan, *J. Chromatogr.*, **380**, 331 (1986).
- 21) M. M. Delgado Zamarreno, A. Sanchez Perez, C. Gomez Perez, J. Hernandez Mendez, *J. Chromatogr.*, **623**, 69 (1992).
- 22) K. Hirauchi, T. Sakano, S. Notsumoto, T. Nagaoka, A. Morimoto, K. Fujimoto, S. Masuda, Y. Suzuki, *J. Chromatogr.*, **497**, 131 (1989).
- 23) Y. Shimomura, M. Suzuki, S. Sugiyama, Y. Hanaki, T. Ozawa, *Biochem. Biophys. Res. Commun.*, **176**, 349 (1991).