Chem. Pharm. Bull. 27(5)1237—1244(1979)

UDC 543.83.06 : 543.544.2.061

Electrochemical Detector for High-Performance Liquid Chromatography. II.[1] Determination of Tocopherols, Ubiquinones and Phylloquinone in Blood

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(Received December 19, 1978)

An electrochemical detector combined with high-performance liquid chromatography (HPLC–ECD) was applied to the analysis of tocopherols (TP), ubiquinones (UQ) and phylloquinone (PQ). *n*-Hexane extracts of these substances from blood were injected into a reversed-phase column (Nucleosil C-18). The minimum detection limits were 50, 150—200 and 100 pg for TP, UQ and PQ, respectively. Blood levels of these substances were in good agreement with those obtained by other methods. The HPLC–ECD method is a simple, sensitive and relatively rapid method, and should be useful for the determination of α-tocopherol, ubiquinone-10 and PQ in biological materials.

Keywords——high-performance liquid chromatography; electrochemical detector; tocopherols; ubiquinones; phylloquinone; blood

Many investigators have developed electrochemical detectors (ECD) using carbon paste electrodes for high-performance liquid chromatography (HPLC). However, carbon paste electrodes cannot be used in non-aqueous solvents because they disintegrate, and this restricts the range of compounds that can be analyzed and the type of chromatography that can be employed. Furthermore, high cathodic residual currents make them unsuitable for the detection of highly reducible compounds.

We have previously described the design and characteristics of an ECD cell using a glassy carbon electrode and its application to the high-performance liquid chromatographic determination of water-soluble biogenic amines in biological materials. Our ECD was designed so as to be suitable for use in non-aqueous solvents. In this paper, we report the application of our ECD to the detection of oxidizable and reducible organic compounds in an organic mobile phase.

We chose electroactive chromanol, benzoquinone and naphthoquinone compounds with lipophilic isoprenoid side-chains as test compounds, that is, tocopherols (TP), ubiquinones (UQ) and phylloquinone (PQ). TP, UQ and PQ are contained in biological tissues and are known to play important roles as physiological lipid antioxidants, carriers in the mitochondrial electron transport system and an anticoagulant vitamin, respectively. These compounds have also been used for the therapy of human diseases. In order to investigate the roles of these substances in animals and humans, it is necessary to establish simple, selective, sensitive and precise methods for their determination.

We have reported HPLC methods for the determination of TP,[3] UQ[4] and PQ[5] in pharmaceutical preparations and biological materials. However, these methods require several

1) Part I: S. Ikenoya, T. Tsuda, Y. Yamano, Y. Yamanishi, K. Yamatsu, M. Ohmae, K. Kawabe, H. Nishino, and T. Kurahashi, *Chem. Pharm. Bull.* (Tokyo), 26, 3530 (1978).
2) Location: *4-6-10, Koishikawa, Bunkyo-ku, Tokyo, 112, Japan.*
3) *a*) K. Abe, Y. Yuguchi, and G. Katsui, *J. Nutr. Sci. Vitaminol.*, **21**, 183 (1975); *d*) K. Abe, M. Ohmae, and G. Katsui, *Vitamins* (Japan), **50**, 453 (1976); *c*) K. Abe and G. Katsui, *ibid.*, **49**, 259 (1976); *d*) K. Ishibashi, K. Abe, M. Ohmae, K. Kawabe, and G. Katsui, *ibid.*, **51**, 415 (1977).
4) *a*) Y. Yamano, S. Ikenoya, T. Tsuda, M. Ohmae, and K. Kawabe, *Yakugaku Zasshi*, **97**, 486 (1977); *b*) K. Abe, K. Ishibashi, M. Ohmae, K. Kawabe, and G. Katsui, *Vitamins* (Japan), **51**, 111 (1977); *c*) K. Abe, K. Ishibashi, M. Ohmae, K. Kawabe, and G. Katsui, *ibid.*, **51**, 119 (1977).
5) *a*) Y. Yamano, S. Ikenoya, M. Anze, M. Ohmae, and K. Kawabe, *Yakugaku Zasshi*, **98**, 774 (1978); *b*) K. Abe, O. Hiroshima, K. Ishibashi, M. Ohmae, and K. Kawabe, *Yakugaku Zasshi*, **99**, 192 (1979).

detectors, *i.e.*, ultraviolet (UV), fluorometric (FD) and chemical reaction-fluorometric detectors (CR–FD) depending on the analytical purpose, which is a disadvantage.

Electrochemical techniques (differential pulse polarography) are thought to be well suited for the analysis of electroactive substances and many reports have been published on the determination of these compounds in pharmaceutical preparations.[6] However, these methods have not been applied for the determination of these compounds in biological materials, because this would require separation from other electroactive substances by complicated methods such as column chromatography. In the HPLC method combined with ECD, TP, UQ and PQ can be separated from other electroactive substances by HPLC, and then detected by controlled potential electrolysis using ECD.

The present paper describes the application of ECD with HPLC for the determination of TP, UQ and PQ in human serum and rat plasma.

Experimental

Apparatus——The HPLC system and ECD cell were similar to those described previously.[1] The reference electrode (Ag/AgCl) was connected with the ECD cell by means of a methylcellulose junction containing 0.1 M $NaClO_4$. For chromatographic separation, a 25 cm × 4.6 mm I.D. stainless steel column was packed with Nucleosil C-18 (Macherey–Nagel Co., 10 μm) using the balanced viscosity packing technique. HPLC measurements were performed at 25 ± 0.1°.

Materials——Retinol (RN), retinyl acetate (RA), β-carotene and ergocalciferol (VD_2) were purchased from Tokyo Kasei Co. Ubiquinone-10 (UQ-10), PQ and menaquinone-4 (MQ) were obtained from Nisshin Chemical and Eisai Co., Ltd., respectively. Ubiquinone-8 (UQ-8), ubiquinone-9 (UQ-9), 2,3,6-trimethyl-5-nonaprenyl-1,4-benzoquinone (TQ-9) and 2,3,6-trimethyl-5-decaprenyl-1,4-benzoquinone (TQ-10) were synthesized according to the method of Rugg *et al.*[7] and purified by column chromatography and recrystallization. α-, β-, γ- and δ-tocopherols (α-, β-, γ- and δ-TP) were obtained from Tama Biochemical Co. Ltd. and purified by thin-layer chromatography. Tocol (TC) was synthesized from hydroquinone and isophytol according to the published method.[8] α-Tocopheryl quinone (α-TQ) was prepared by $AgNO_3$ oxidation of α-TP. All other chemicals were of reagent grade.

Assay Procedure——Tocopherols in Human Serum: Human serum (0.5 ml) was placed in a brown glass centrifuge tube and TC (5 μg) was added as an internal standard. The solution was extracted with *n*-hexane (5 ml) according to the previous report[3] and 4 ml of the *n*-hexane extract was evaporated down under a N_2 stream at 40°. The residue was dissolved in 100 μl of isopropanol and 15 μl of the solution was injected into the HPLC column. The mobile phase was prepared by dissolving 7.0 g of $NaClO_4 \cdot H_2O$ in 999 ml of methanol and mixing with 1.0 ml of pyridine.

Ubiquinones in Human Serum: Human serum (1 ml) was placed in a brown glass centrifuge tube, TQ-10 (5 μg) was added as an internal standard, and then the solution was extracted with *n*-hexane (5 ml) according to the direct extraction method described in the previous report.[4b] The *n*-hexane extract (4 ml) was evaporated down under a N_2 stream. The residue was dissolved in 100 μl of isopropanol and 20 μl of the solution was injected into the HPLC column. The mobile phase was prepared by dissolving 7.0 g of $NaClO_4 \cdot H_2O$ in 999 ml of ethanol and mixing with 1.0 ml of $HClO_4$ (70%) solution, and the solution was then deaerated by argon gas bubbling.

Phylloquinone in Rat Plasma: Adult male Sprague–Dawley rats weighing 220—300 g were used. After a single oral administration of 1.0 mg/kg of PQ, rat blood was withdrawn with a heparinized syringe, transferred into a centrifuge tube and centrifuged. Rat plasma (0.5 ml) was extracted with *n*-hexane (5.8 ml) according to the previous report.[5b] The *n*-hexane extract (4 ml) was evaporated down under a N_2 stream at 40°. The residue was dissolved in 100 μl of isopropanol and 30 μl of the solution was injected into a HPLC column. The mobile phase was prepared by dissolving 7.0 g of $NaClO_4 \cdot H_2O$ in 999 ml of ethanol–methanol (6: 4) and mixing with 1.0 ml of $HClO_4$ (70%) solution, and the solution was then deareated by argon gas bubbling.

6) *a*) S.S. Atsuma and J. Lindoquist, *Analyst*, **98**, 886 (1973); *b*) H.D. McBride and D.H. Evans, *Anal. Chem.*, **45**, 446 (1973); *c*) O. Podlaha, A. Eriksson, and B. Toregard, *J. Am. Oil. Chem. Soc.*, **55**, 530 (1978); *d*) T. Erabi, H. Hiura, and M. Tanaka, *Bull. Chem. Soc. Jpn.*, **48**, 1354 (1975); *e*) J. Lindoquist and S.M. Farroha, *Analyst*, **100**, 377 (1975); *f*) Y. Hayakawa and K. Takamura, *Yakugaku Zasshi*, **95**, 1292 (1975).
7) R. Rugg, U. Gloor, R.N. Goel, G. Ryser, O. Wiss, and O. Isler, *Helv. Chem. Acta*, **43**, 2616 (1960).
8) P. Manalis, D. McHale, J. Green, and S. Marcinkiewicz, *J. Chem. Soc.*, **1958**, 1850.

Results and Discussion

Operating Conditions for HPLC

The optimal conditions for HPLC were established as regards the effect of supporting electrolyte concentration, conductivity of the mobile phase, retention time, peak resolution and sensitivity.

TP compounds in biological materials have been separated by HPLC using a normal phase column and *n*-hexane–isopropyl ether as a mobile phase.[3] However, this mobile phase cannot be used for HPLC with ECD because of limited solubility of the supporting electrolyte. Therefore we used a reversed-phase column with methanol as a mobile phase, in which $NaClO_4$ could be used as a supporting electrolyte. Figure 1A illustrates the plot of logarithmic retention time ($\log k$, $k=(t_R-t_0)/t_0$) for TP against $NaClO_4$ concentration. Horvath and Molnar[9] have demonstrated that increasing salt concentration in the hydrophobic mobile phase increases the capacity factor of neutral solutes and this effect was ascribed to the increasing surface tension of the mobile phase. Our observations were in good agreement with their results. Figure 1B illustrates the dependence of the detector response (peak height) on ionic strength at constant flow rate. Although the detector response increases with concentration of $NaClO_4$ up to 0.05 M, there is a definite decrease in the detector response at salt concentrations higher than 0.05 M, which is ascribed to peak broadening resulting from the increased retention and the decreased diffusion coefficient of the electroactive compounds.

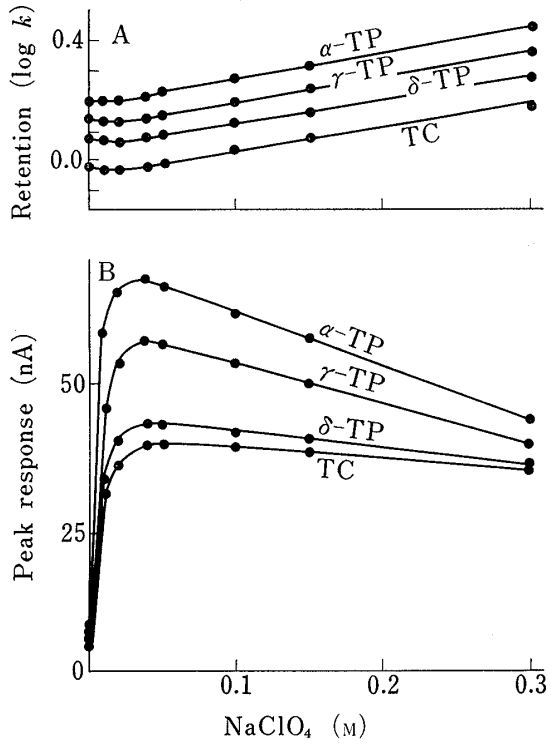

Fig. 1. Dependence of Retention (A) and Peak Response (B) upon $NaClO_4$ Concentration in the Mobile Phase

Column; Nucleosil C-18 (10 μm), 25 cm×4.6 mm I.D.
Mobile phase; methanol.
Applied potential; 0.7 V *vs.* Ag/AgCl.
Flow rate; 0.44 ml/min.
Injected amount(ng): α-TP, 74.6; γ-TP, 47; δ-TP, 34.6; TC, 33.

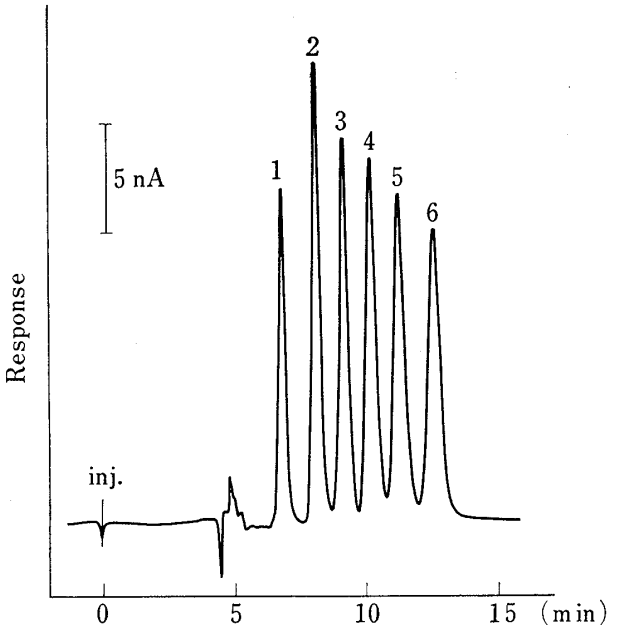

Fig. 2. Chromatogram of Tocopherols, Retinol and Retinyl Acetate

Column; Nucleosil C-18 (10 μm), 25 cm×4.6 mm I.D.
Mobile Phase; 0.1% pyridine–methanol (containing 0.05 M $NaClO_4$)
Applied potential; 0.9 V *vs.* Ag/AgCl.
Flow rate; 0.75 ml/min.
1, RN(8.32);[a] 2, RA(12.0); 3, TC(21.5); 4, δ-TP(20.7); 5, γ-TP(20.2); 6, α-TP(24.3).
a) The injected amount (ng) is shown in parentheses.

9) C. Horvath, W. Melander, and I. Molnar, *J. Chromatogr.*, **125**, 129 (1976).

Parker[10] and Ikenoya *et al.*[11] also reported that hydroquinone and benzanilides were easily oxidized at the anode by the addition of pyridine or a sterically hindered pyridine such as 2,6-lutidine, which causes partial deprotonation of these compounds. Addition of 0.1% pyridine to the mobile phase resulted in approximately 50 mV lower oxidation potential and 10% higher peak response for TP than those in the absence of pyridine. Furthermore, the resolution of the peak was improved. Figure 2 illustrates a typical chromatogram for a mixture of TP, TC, RN and RA. Good baseline resolution and a higher sensitivity were obtained provided that the ionic strength, the amount of pyridine added and the applied potential were controlled carefully. However, β-TP and γ-TP could not be separated by HPLC using a reversed-phase column.

As few application data of HPLC–ECD to reducible compounds have been reported, we carried out fundamental studies on the effect of the mobile phase component on the analysis of reducible compounds.

To obtain electrode stability and a lower background current, it is necessary to remove oxygen dissolved in the mobile phase by deaeration and to apply a lower reduction potential. The applied potentials for α-TQ, UQ, PQ, MQ, TQ-9 and TQ-10 were shifted about 100—150 mV lower by the addition of $HClO_4$ to the mobile phase, which was ascribed to the reduction of a protonated quinone.[12] Increasing concentration of the supporting electrolyte had the same effect on resolution and detector response as that observed for TP (Fig. 3A, B). A typical chromatogram of these quinones is shown in Fig. 4.

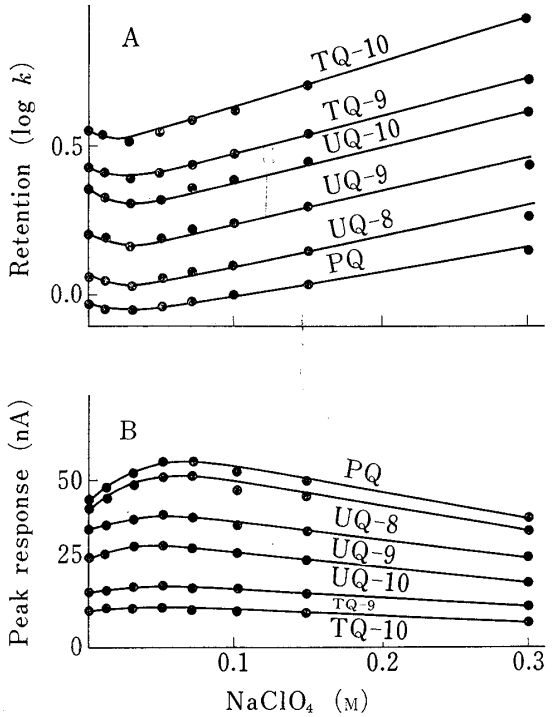

Fig. 3. Dependence of Retention (A) and Response (B) upon $NaClO_4$ Concentration in the Mobile Phase

Column; Nucleosil C-18 (10 μm), 25 cm×4.6 mm I.D.
Mobile phase; $HClO_4$ (70%)–methanol–ethanol (1: 400: 600).
Applied potential; −0.28 V *vs.* Ag/AgCl.
Flow rate; 0.85 ml/min.
Injected amount (ng): PQ, (240); UQ-8(300); UQ-9 (300); UQ-10(300); TQ-9(248); TQ-10(235).

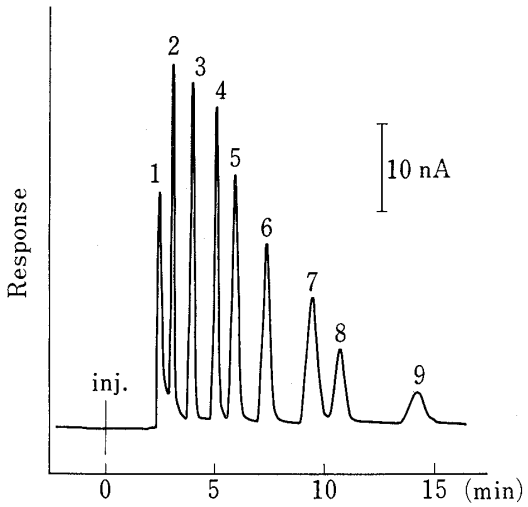

Fig. 4. Chromatogram of Quinone Derivatives

Column; Nucleosil C-18 (10 μm), 25 cm×4.6 mm I.D.
Mobile phase; $HClO_4$ (70%)–methanol–ethanol (1: 500: 500, containing 0.05 M $NaClO_4$).
Applied potential; −0.30 V *vs.* Ag/AgCl.
Flow rate; 1.56 ml/min.
1, solvent; 2, α-TQ(136);[a] 3, MQ(75); 4, PQ(120); 5, UQ-8(150); 6, UQ-9(150); 7, UQ-10(150); 8, TQ-9(124); 9, TQ-10(118).
a) The injected amount(ng) is shown in parentheses.

10) V.D. Parker, *Electrochimica Acta*, **18**, 519 (1973).
11) S. Ikenoya, M. Masui, H. Ohmori, and H. Sayo, *J. Chem. Soc., Perkin II*, **1974**, 571.

The detector responses of oxidizable and reducible compounds were enhanced by increasing the flow rate to 1.2 ml/min. However, the detector responses decreased at flow rates higher than 1.2 ml/min because of the decrease of coulometric yield for the compounds.

The half-wave potential ($E_{1/2}$) obtained by the hydrodynamic technique, the retention time and the detection limit for each of the compounds investigated are shown in Table I. The detection limits of α-, β-, γ- and δ-TP were 50 pg. VD_2 was not detected at +0.7 V *vs.* Ag/AgCl but was detected at +1.0 V *vs.* Ag/AgCl with the same retention time as β- and γ-TP. The detection limits of MQ, PQ, UQ-8, UQ-9 and UQ-10 were 50, 100, 150, 150 and 200 pg, respectively. The linear dynamic range of current for these compounds was approximately 10^3—10^4 (100 pg—1 μg).

Table I. Retention Time (t_R), Half-Wave Potential ($E_{1/2}$) and Detection Limit Values of Various Oxidizable (Ox) and Reducible (Red) Compounds

	Compounds	t_R (min)	$E_{1/2}$ (V *vs.* Ag/AgCl)	Detection limit(pg)[c]
Ox[a]	RN	6.8	0.85	200
	RA	8.2	0.88	200
	TC	9.2	0.68	100
	δ-TP	10.3	0.62	50
	γ-TP	11.5	0.53	50
	β-TP	11.5	0.53	50
	α-TP	12.7	0.45	50
	VD_2	11.5	1.2	2000
	β-Carotene	47.0	0.41	—
	3,4-Benzopyrene	11.5	0.88	100
Red[b]	α-TQ	3.0	−0.31	100
	MQ	3.9	−0.28	50
	PQ	5.0	−0.29	100
	UQ-8	5.8	−0.20	150
	UQ-9	7.3	−0.20	150
	UQ-10	9.3	−0.20	200
	TQ-9	10.6	−0.33	500
	TQ-10	14.2	−0.33	800

a) Measurement conditions were the same as in Fig. 3.
b) Measurement conditions were the same as in Fig. 4.
c) The applied potential for Ox and Red were 0.9 and −0.4 V *vs.* Ag/AgCl. The signal-to-noise ratio was 2.

Determination of TP, UQ and PQ in Blood

TP in human serum were extracted according to our previous report[3] after addition of TC as an internal standard, then determined by HPLC combined with ECD at 0.7 V *vs.* Ag/

Table II. Human Serum Concentration of Tocopherols determined by Two Methods

Sample	HPLC–FD (μg/ml)				HPLC–ECD (μg/ml)		
	α	β	γ	Total	α	β+γ	Total
A	7.48	—	0.89	8.37	7.70	0.99	8.69
B	6.22	—	0.43	6.65	6.59	0.55	7.14
C	8.21	0.96	1.88	11.05	8.00	2.85	10.85
D	9.56	—	1.27	10.85	9.75	1.45	11.20
E	15.14	—	0.94	16.08	15.80	1.15	16.95

12) V.D. Parker, *J.C.S. Chem. Commun.*, **1969**, 716.

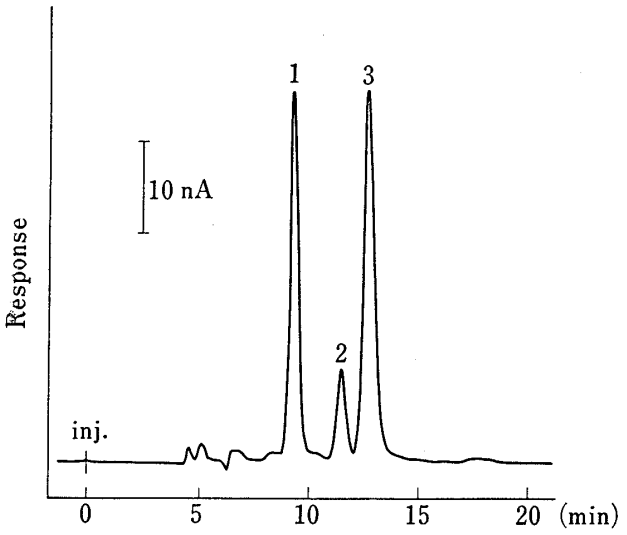

Fig. 5. Chromatogram of Tocopherols in Human Serum

Column; Nucleosil C-18 (10 μm), 25 cm × 4.6 mm I.D.
Mobile phase; 0.1% pyridine–methanol (containing 0.05 M $NaClO_4$).
Applied potential; 0.7 V *vs.* Ag/AgCl.
Flow rate; 0.75 ml/min.
1: TC, 2: β- or γ-TP, 3: α-TP.

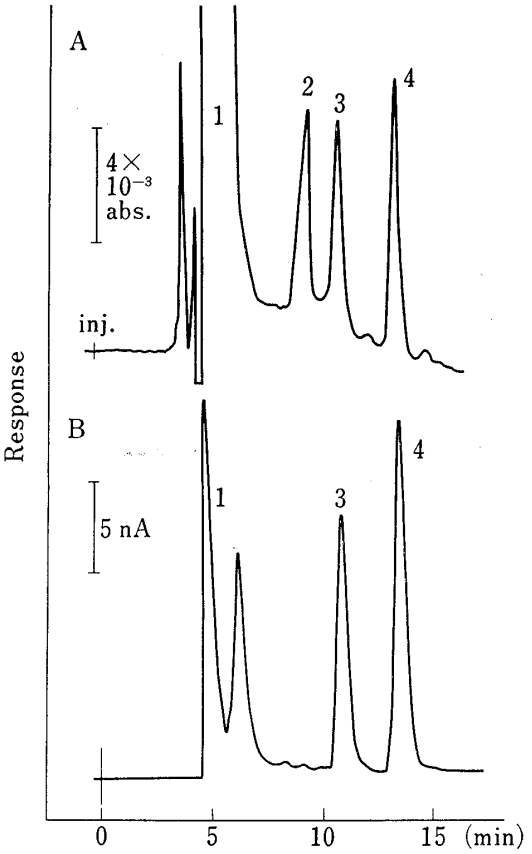

Fig. 6. Chromatograms of Ubiquinone-10 in Human Serum

Column; Nucleosil C-18 (10 μm), 25 cm × 4.6 mm I.D.
Mobile phase; $HClO_4$ (70%)–ethanol (1: 999, containing 0.05 M $NaClO_4$).
Applied potential; −0.30 V *vs.* Ag/AgCl.
Flow rate; 0.55 ml/min.
1: unknown, 2: unknown, 3: UQ-10, 4: TQ-10.
A, HPLC-UV (275 nm).
B, HPLC-ECD.

AgCl. Figure 5 shows a chromatogram of TP in human serum. The calibration curves of peak height ratio against weight ratio of TP to TC were linear from 3 ng to 100 ng. The recoveries of TP throughout the whole procedure were 99.6, 99.2 and 99.4% for α-TP, γ-TP and δ-TP, respectively. The coefficients of variation peak height were 2.0, 2.5 and 2.5% for 8.25 μg/ml of α-TP, 1.1 μg/ml of γ-TP and 0.9 μg/ml of δ-TP in human serum, respectively. The results for the determination of TP are given in Table II along with the data obtained by the HPLC–FD method[3c] using a normal phase column. No significant difference can be seen between the TP levels in human serum obtained by the two methods. Although β-TP and γ-TP could not be separated by a reversed-phase column, this is not important for pharmaco-kinetic studies of TP in humans, because only α-TP is usually administered clinically. The detection limit of TP was two to three times higher than that obtained by the HPLC–FD method.

For the determination of UQ in biological materials, the HPLC–UV method which has been developed by us[4b] seems to be simpler, more sensitive and more specific than other conventional methods. However, in the HPLC–UV method, the greatest care must be taken in the choice of the column and mobile phase to avoid interference due to β-carotene-like substances and other lipids.

UQ in human serum were also extracted according to our previous paper[4b,c] after addition of TQ-10 as an internal standard. Figure 6A, B shows chromatograms of UQ-10 in human serum detected by UV and ECD. Even though the ECD was connected to the outlet of the UV detector, no decrease in resolution or sensitivity was observed. However, the determination of UQ-10 by the HPLC–UV method using a Nucleosil C-18 column was disturbed by the peaks of solvent and β-carotene-like substances, which could be separated from the UQ-10 peak using a Permaphase ODS column. The calibration curves of peak height ratio against

weight ratio of UQ to TQ-10 were linear from 2 ng to 100 ng. The recoveries of UQ throughout the whole procedure were 99.3, 99.3 and 99.5% for UQ-8, UQ-9 and UQ-10, respectively. The coefficient of variation of the peak height was 1.9% for 0.58 μg/ml of UQ-10 in human serum. The results for the determination of UQ-10 in human serum are given in Table III, along with the data for the HPLC–UV method using a Permaphase ODS column. No significant difference can be seen between the two methods. Also, no UQ other than UQ-10 was observed in human serum. For the determination of UQ-10, the HPLC–ECD method was superior to the HPLC–UV method by a factor of ten to twenty as regards sensitivity.

Table III. Human Serum Concentration of Ubiquinone-10 determined by Two Methods

Sample	HPLC–UV (μg/ml)	HPLC–ECD (μg/ml)
F	0.73	0.68
G	0.58	0.54
H	1.33	1.45
I	0.61	0.59
J	0.60	0.59

The determination of extremely small quantities of PQ in biological materials has only been reported by us;[5b] we separated PQ in plasma by HPLC and detected it by fluorometry after reaction with $NaBH_4$ (HPLC–CR–FD). Figure. 7 shows a chromatogram of rat plasma after oral administration of PQ. PQ was extracted according to our previous report[5b] and the PQ concentration was estimated from a calibration curve of peak height against concentration of PQ. The calibration plot was linear from 1 ng to 60 ng. The recovery of PQ throughout the whole procedure was 99.5%. The coefficient of variation of the peak height was 2.6% for 210 ng/ml of PQ in rat plasma. The validity of this method

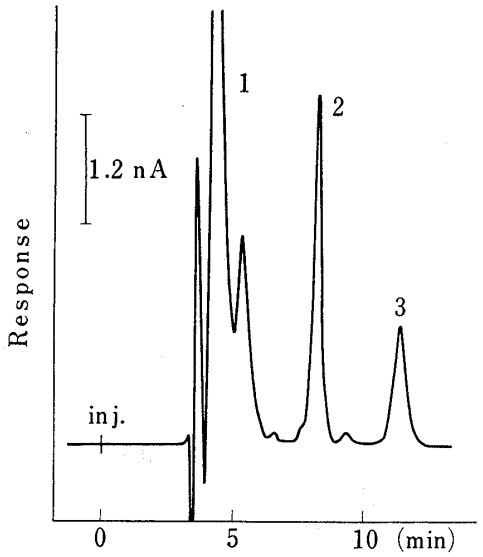

Fig. 7. Chromatogram of orally Administrated Phylloquinone in Rat Plasma

Column; Nuclecsil C-18 (10 m), 25 cm × 4.6 mm I.D.
Mobile phase; $HClO_4$ (70%)–methanol–ethanol (1:400: 600, containing 0.05 M $NaClO_4$).
Applied potential; −0.3 V *vs.* Ag/AgCl.
Flow rate; 0.95 ml/min.
1: unknown, 2: PQ, 3: UQ-9.

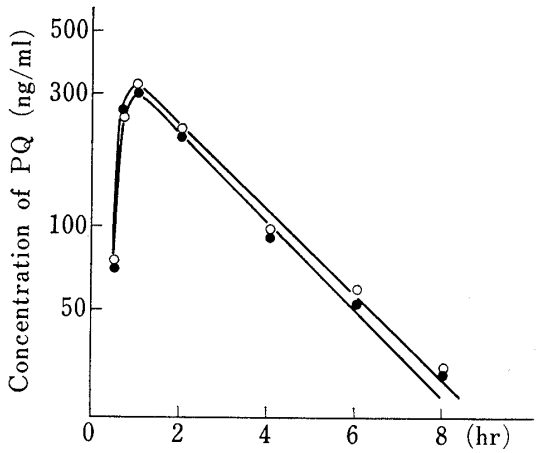

Fig. 8. Concentration of Phylloquinone in Plasma after Oral Administration of 1 mg/kg to Rats

—○—: HPLC-ECD.
—●—: HPLC-CR-FD.

was judged by comparison of the plasma PQ profile with that obtained by the HPLC–CR–FD method. Figure 8 shows the profiles from 0 to 8 hr as measured by both methods. As can be seen in Fig. 8, the values of apparent first-order elimination rate constant ($-0.33\ hr^{-1}$) and the half-life of elimination (2.1 hr) of PQ as determined by the two methods were in good agreement.

As mentioned above, the proposed methods are simple, highly sensitive and specific. For example, we showed here that TP, UQ and PQ in blood could be determined by HPLC–ECD. Future research will focus on clinical monitoring.

Also, to further demonstrate the usefulness of the HPLC–ECD method we are developing a technique for the determination of nonelectroactive compounds by labeling with electroactive compounds; these applications will be reported elsewhere.

Acknowledgements The authors wish to thank Dr. M. Tomoeda, Director, Research and Development Division, for encouragement and support of this research.