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Simultaneous determination of α-tocopherol and α-tocopherolquinone by high-performance liquid chromatography and coulometric detection in the redox mode

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Abstract

A simple, selective and highly sensitive assay method for the simultaneous determination of α-tocopherol and α-tocopherolquinone in plasma or erythrocyte membrane by high-performance liquid chromatography (HPLC) with a series of multiple coulometric working electrodes (CWE) was investigated. For good separation of α-tocopherol and α-tocopherolquinone, an MC MEDICAL C_{18} reversed-phase column and a mobile phase consisting of 96% methanol [methanol–HPLC-grade distilled water (96:4, v/v)] with 40 m*M* sodium perchlorate were used. Also, selective, highly sensitive and simultaneous detection of these substances was performed in redox mode using a series of four CWE. In this detection mode, the first, second and third CWE were set at −0.45 V for pre-reaction and to prevent interference, the fourth CWE was used as an electrode for actual measurement with its potential set at +0.40 V against a palladium reference electrode. The detection limits were 50–100 pg. Excellent chromatograms of α-tocopherol and α-tocopherolquinone were obtained within 8 min. The usefulness of reversed-phase HPLC with the redox detection mode was confirmed by application to the determination of the concentrations of α-tocopherol and α-tocopherolquinone in a crude ethanol–hexane extract of rat plasma or erythrocyte membrane. These findings suggest that reversed-phase HPLC with the redox detection mode using a series of four CWE is applicable to study the preventive effect of α-tocopherol on lipid peroxidation.

1. Introduction

α-Tocopherol is a lipophilic phenolic antioxidant. It is known that α-tocopherol protects cells against oxidation-associated cytotoxicity by prevention of membrane lipid peroxidation [1,2], stabilization of the membrane structure [3] and maintenance of the redox balance of intracellular thiols (e.g., glutathione) [4,5]. Assessment of the physiological functions and pharmacological effects of α-tocopherol requires a quantitative assay with high sensitivity, selectivity, reproducibility and reliability for the determination of this substance and its oxidative metabolite in biological samples. High-performance liquid chromatographic (HPLC) techniques have become widely accepted and applied for this purpose [3,6–19]. The combination of ultraviolet [6–10] or fluorimetric detection [11–14] with HPLC separation techniques is currently used for the determination of α-tocopherol, but it is not necessarily satisfactory with regard to sensitivity,

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selectivity and reproducibility for the measurement of this substance in small and crude biological samples. Also, assay methods for α-tocopherol involving HPLC with a single flow-through amperometric electrode as an oxidative detection source have been reported [15–17]. These greatly enhanced the sensitivity and selectivity of this analysis. However, the use of HPLC with dual or multiple electrochemical detectors is required for the simultaneous determination of α-tocopherol and its quinone [3,18,19].

In this study, a simple, selective and highly sensitive assay method for the simultaneous determination of α-tocopherol and α-tocopherolquinone in plasma or etythrocyte membrane by reversed-phase HPLC with a series of multiple coulometric working electrodes was investigated in detail.

2. Experimental

2.1. Chemicals and standard solutions

All reagents were purchased at the highest available purity and used without further purification; α-, β-, γ- and δ-tocopherol were purchased from Eisai (Tokyo, Japan) and α-tocopherolquinone from ICN Biomedicals (Costa Mesa, CA, USA). Analytical-reagent grade chemicals for sample preparation and chromatography were obtained from Wako (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan).

A stock standard solution containing α-, β-, γ- and δ-tocopherol and α-tocopherolquinone was prepared by dissolving 1 mg of each in 1 ml of methanol deoxygenated with nitrogen. Working standard solutions were prepared daily by diluting the stock standard solution with methanol deoxygenated with nitrogen.

2.2. HPLC–electrochemical detection system

Apparatus

The HPLC–electrochemical detection system is shown schematically in Fig. 1. The HPLC system consisted of a Model LC-9A solvent-delivery system (Shimadzu, Kyoto, Japan) equipped with an extra damper, a Model 7125 sample injector (Rheodyne, Cotati, CA, USA) with a 100-μl sample-holding loop, an in-line filter unit (0.20-μm graphite filter) (ESA, Bedford, MA, USA) and an MC MEDICAL C_{18} reversed-phase column (80 mm × 4.6 mm I.D., 3-μm particle size) (MC Medical, Tokyo, Japan). A BX-7000A column heater (Ishido, Chiba, Japan) was used to maintain the analytical column at a constant temperature (35°C).

Electrochemical detection was performed with

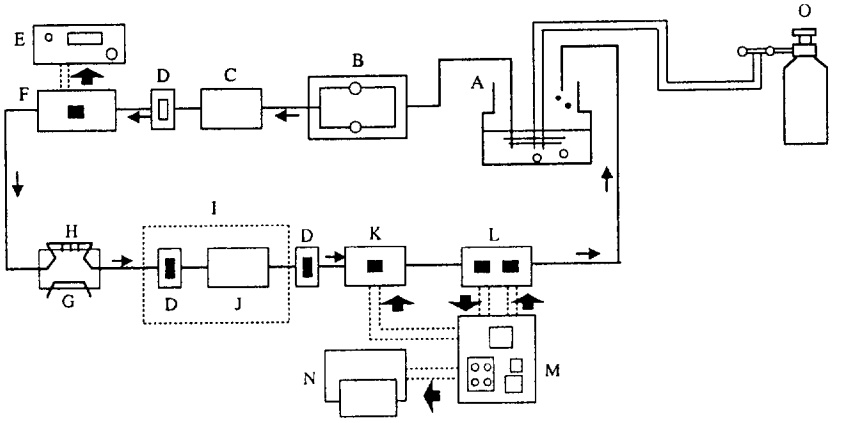

Fig. 1. HPLC–electrochemical detection system for the simultaneous determination of α-tocopherol and α-tocopherolquinone. A = Mobile phase; B = pump; C = damper; D = in-line filter; E = potentiostat; F = Model 5020 guard cell; G = injection valve; H = sampling loop; I = thermostated oven; J = analytical column; K = Model 5021 conditioning cell; L = Model 5011 high-sensitivity analytical cell; M = Model 5100A control module; N = recorder; O = nitrogen.

a coulometric detection system consisting of four coulometric high-efficiency flow-through cells in series. This detection system consisted of a Model 5020 guard cell with a single coulometric working electrode [test electrode 1, T_1 (efficiency 100%)] (ESA, Bedford, MA, USA), a Model 5021 conditioning cell with a single coulometric working electrode [test electrode 2, T_2 (efficiency 100%)] (ESA) [20,21], a Model 5011 high-sensitivity analytical cell containing dual coulometric working electrodes [test electrode 3, T_3 (efficiency 100%), test electrode 4, T_4 (efficiency 70%)] (ESA) [20–24], a potentiostat (MC Medical, Tokyo, Japan) and a Model 5100A control module (ESA). A Model 5020 guard cell (T_1) was placed between the chromatographic pump and the sample injector. Also, the three coulometric working electrodes (T_2, T_3 and T_4) were connected in series by installing a Model 5021 conditioning cell before a Model 5011 high-sensitivity analytical cell. Recording of chromatograms and data analysis were performed with both a chart recorder (Nippon Denshi Kagaku, Tokyo, Japan) and a Model 12 S1C chromatocorder (System Instruments, Tokyo, Japan) connected to T_4.

Electrochemical detection mode

The principle of the electrochemical detection mode in this HPLC–electrochemical detection system is illustrated in Fig. 2. For the selective, highly sensitive and simultaneous detection of α-tocopherol and α-tocopherolquinone, the redox detection mode [21] was adopted using a series of four coulometric working electrodes. In this detection mode, the first high-efficiency electrode (T_1) was set at the same potential as the second (T_2) and third electrodes (T_3) to prevent interference of electroactive substances in the mobile phase with the reduction reaction of the analyte in T_2 and T_3. T_2 and T_3 were set at potentials corresponding to the top of the reduction current–voltage curve for the analyte, to reduce these substances completely. Also, the fourth electrode (T_4) was set at a potential corresponding to the top of the oxidation current–voltage curve for the analyte, to measure these substances.

Mobile phase

For a good separation of α-tocopherol and α-tocopherolquinone, a mobile phase consisting of 96% methanol [methanol–HPLC-grade dis-

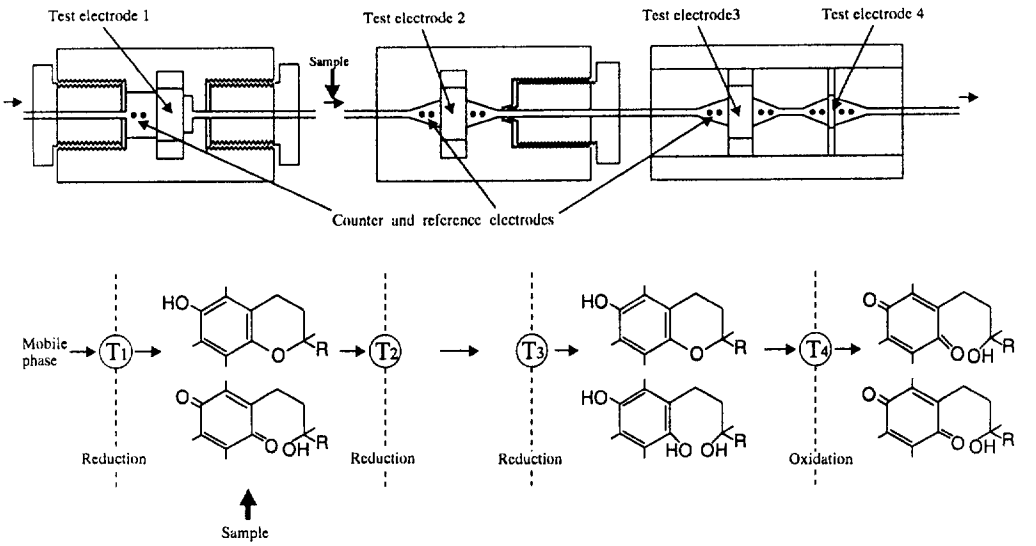

Fig. 2. Electrochemical detection system and mode for the simultaneous determination of α-tocopherol and α-tocopherolquinone. T_1, T_2, T_3 and T_4 = test electrodes 1, 2, 3 and 4, respectively. R = $-CH_2CH_2CH_2CH(CH_3)CH_2CH_2CH_2CH(CH_3)-CH_2CH_2CH_2CH(CH_3)CH_3$.

tilled water (96:4, v/v)] containing 40 m*M* sodium perchlorate as the supporting electrolyte [3] was used. The mobile phase was maintained oxygen-free by continuous bubbling with nitrogen. The mobile phase flow-rate was maintained at 1.0 ml/min. Mobile phase was also circulated in the HPLC–electrochemical detection system and was exchanged when marked changes in the back-current occurred.

2.3. Sample preparation

Male Sprague–Dawley rats (Charles River Japan) weighing 250–280 g were used. Rat venous blood samples were collected in tubes containing dry sodium heparin. Plasma was prepared from fresh heparinized blood by centrifugation at 3000 rpm for 10 min at 4°C. The plasma sample (usually 0.5 ml) was pipetted into a tube containing 30 μl of 0.5 *M* sodium hydrogensulphite and was dexoygenated with nitrogen. Also, an erythrocyte membrane sample was prepared from 0.5 ml of erythrocyte suspension according to the method of Dodge et al. [25] under a stream of nitrogen.

Extraction was based on the procedure of Pascoe et al. [3] The plasma or erythrocyte membrane sample was pipetted into a tube containing 0.5 ml of ethanol deoxygenated with nitrogen and mixed vigorously for 3 min. A 2.5-ml volume of *n*-hexane deoxygenated with nitrogen was then added, followed by re-mixing and centrifugation at 3000 rpm for 5 min at 4°C. The hexane layer was carefully transferred into a new tube and dried under a stream of nitrogen at 40°C. The residue was dissolved in 100 μl of mobile phase solution deoxygenated with nitrogen and a portion of the solution was injected into the HPLC–electrochemical detection system.

2.4. Data analysis

The concentrations of α-tocopherol and α-tocopherolquinone in plasma or erythrocyte membrane were calculated from the calibration graphs, obtained by plotting the peak heights of standard samples prepared by the extraction procedure described above versus the concentrations of standards. The concentrations of α-tocopherol and α-tocopherolquinone in plasma were expressed as ng/ml (mean ± S.D.) and the concentrations in erythrocyte membrane were expressed as ng/ml of packed cells (mean ± S.D.). Also, standard samples were analysed periodically (every five samples) throughout the day to assess the standard stability and the chromatographic consistency.

3. Results

3.1. Applied potential

For the determination of the optimum potentials for each coulometric working electrode (T_1–T_4) in the redox detection mode, current–voltage curves for α-tocopherol and α-tocopherolquinone were investigated.

Reduction current–voltage curves for α-tocopherol and α-tocopherolquinone in coulometric working electrodes of 100% efficiency (T_1, T_2 or T_3) are shown in Fig. 3A. The reduction current response for α-tocopherolquinone was detected below −0.25 V, and a stable maximum response was obtained in the range −0.40 to −0.45 V. On the other hand, a current response for α-tocopherol was not detected below +0.05 V.

The oxidation current–voltage curves in the coulometric working electrode of 70% efficiency (T_4) are shown in Fig. 3B. The oxidation current responses for α-tocopherol were detected over +0.05 V. The maximum current response for α-tocopherol was obtained in the range +0.40 to +0.60 V. However, a current response for α-tocopherolquinone was not detected in the range −0.10 to +0.60 V.

From the above results and the roles of T_1, T_2 and T_3 in the redox detection mode using a series of four coulometric working electrodes, the potentials of T_1, T_2 and T_3 were set at −0.45 V. Further, the oxidation current–voltage curves for α-tocopherol and α-tocopherolquinone and the change in the back-current in T_4 after setting the potentials of T_1, T_2 and T_3 at −0.45 V were

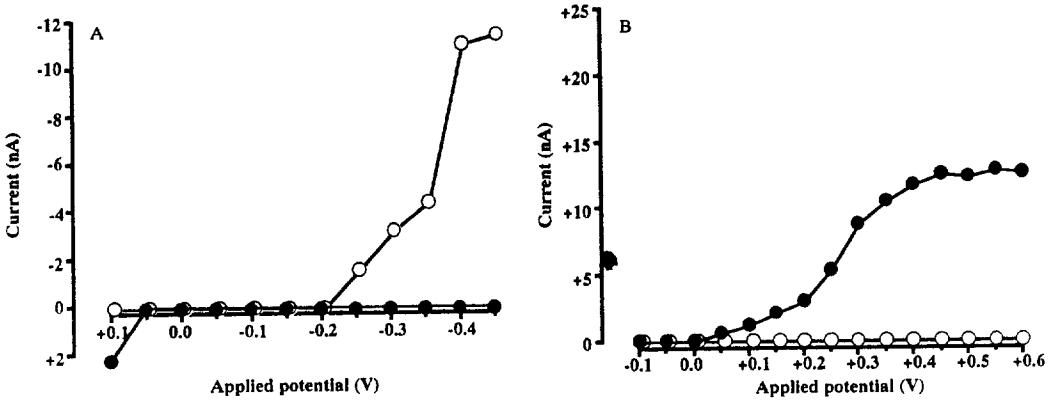

Fig. 3. Relationship between applied potential and reaction current in the coulometric working electrode. (A) Reduction current–voltage curves for (●) α-tocopherol and (○) α-tocopherolquinone in coulometric working electrode of 100% efficiency; (B) oxidation current–voltage curves for (●) α-tocopherol and (○) α-tocopherolquinone in coulometric working electrode of 70% efficiency.

investigated for determination of the optimum applied potential of T_4 used as an electrode for actual measurement of the analyte.

Oxidation current response for α-tocopherol were detected over 0.00 V. The stable current response for α-tocopherol was obtained in the range +0.40 to +0.50 V, followed by an increase in this response. Also, a current response for α-tocopherolquinone was detected in the range −0.10 to +0.60 V, and a stable maximum response was obtained in the range +0.35 to +0.60 V (Fig. 4).

The change in the back-current in T_4 is shown in Fig. 5. The back-current in T_4 was gradually increased with increase in applied potential, followed by a marked increase on the current in the range +0.50 to +0.60 V. From these results,

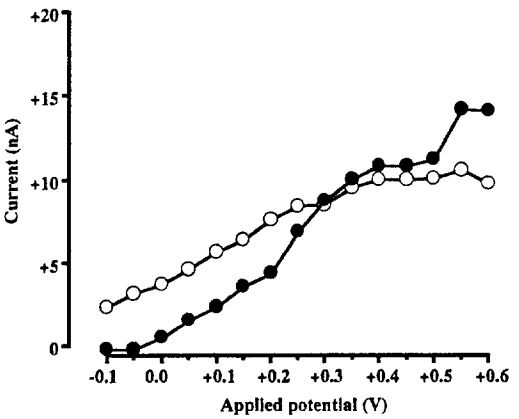

Fig. 4. Relationship between applied oxidative potential and reaction current in detector (T_4, coulometric working electrode of 70% efficiency) of the redox detection system. ● = α-Tocopherol; ○ = α-tocopherolquinone.

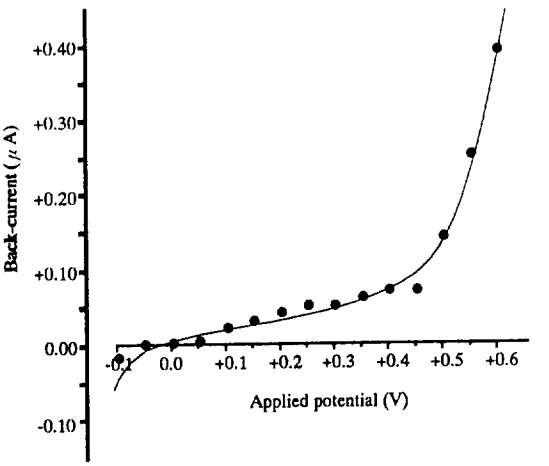

Fig. 5. Change in the back-current in detector (T_4, coulometric working electrode of 70% efficiency) of the redox detection system.

the optimum applied potentials for T_1, T_2, T_3 and T_4 in the redox detection mode were −0.45, −0.45, −0.45 and +0.40 V, respectively.

3.2. Chromatograms

Representative chromatograms obtained by injections of standard solution and ethanol–hexane extract of rat plasma or erythrocyte membrane into the HPLC system with the redox detection mode are shown in Fig. 6. Excellent chromatograms of α-tocopherol and α-tocopherolquinone were obtained within 8 min. Also, the peaks of β + γ− and δ-tocopherols were observed on these chromatograms. The retention times for α-tocopherol and α-tocopherolquinone peaks were 7.2 and 4.5 min, respectively. Further, the large "void volume" signal and baseline drift typically observed in the chromatogram after injection of ethanol–hexane extracts were markedly reduced.

3.3. Accuracy of measurement

In the redox detection mode employed in HPLC with a series of multiple coulometric working electrodes, a linear and close correlation ($r = 0.9936$–0.9986) between the current responses of α-tocopherol and α-tocopherolquinone and their amounts in the range 25 pg–5 μg (fifteen concentrations) was observed. Also, the detection limits for α-tocopherol and α-tocopherolquinone were 50 and 100 pg, respectively, at a signal-to-noise ratio of 2.

The recoveries of α-tocopherol and α-tocopherolquinone added to plasma or erythrocyte membrane samples prior to extraction were 98.3 ± 1.2% (mean ± S.D., $n = 5$) (plasma) and

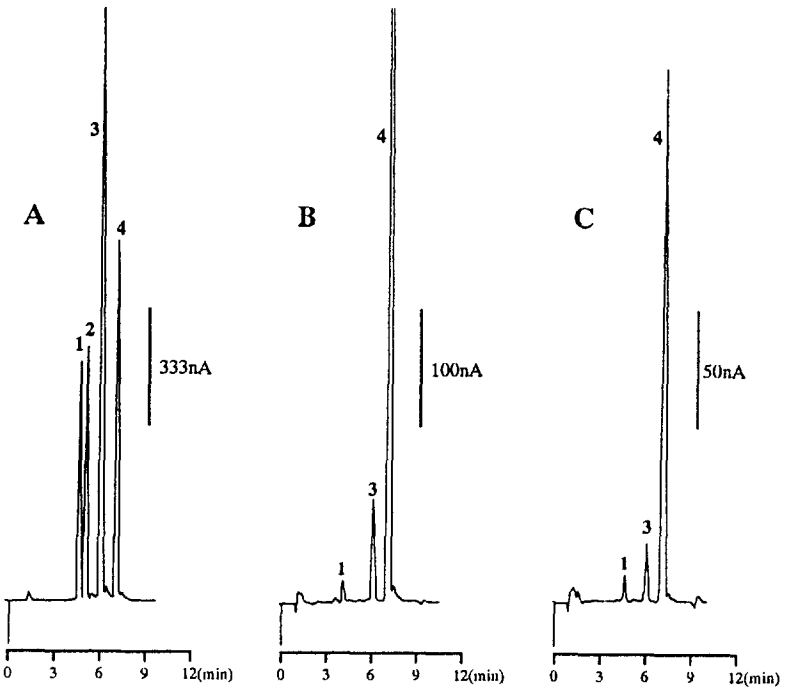

Fig. 6. Representative chromatograms obtained by injection of ethanol–hexane extract of rat plasma or erythrocyte membrane. (A) Standard (500 ng); (B) rat plasma; (C) rat erythrocyte membrane. Peaks: 1 = α-tocopherolquinone; 2 = δ-tocopherol; 3 = β + γ-tocopherol; 4 = α-tocopherol. Chromatographic conditions: analytical column, MC MEDICAL C_{18} reversed-phase column (80 mm × 4.6 mm I.D.); maintenance temperature of analytical column, 35°C; flow-rate, 1 ml/min; mobile phase, 96% methanol [methanol–HPLC-grade distilled water (96:4, v/v)] with 40 m*M* sodium perchlorate. Applied potential: test electrode 1, −0.45 V; test electrode 2, −0.45 V; test electrode 3, −0.45 V; test electrode 4, +0.40 V.

91.5 ± 2.8% (erythrocyte membrane) for α-tocopherol and 96.8 ± 3.1% (plasma) and 89.2 ± 2.4% (erythrocyte membrane) for α-tocopherolquinone.

3.4. Concentrations of α-tocopherol and α-tocopherolquinone in rat plasma and erythrocyte membrane

The concentrations of α-tocopherol in plasma and erythrocyte membrane were 4397 ± 230 ng/ml and 2390 ± 245 ng/ml packed cell (mean ± S.D., $n = 5$), respectively. The α-tocopherolquinone concentrations were 23 ± 5 ng/ml (plasma) and 154 ± 61 ng/ml packed cell (erythrocyte membrane).

4. Discussion

A selective, highly sensitive, reproducible and reliable assay method for the simultaneous determination of α-tocopherol and its oxidative metabolite is necessary to study in detail the physiological or pharmacological characteristics of α-tocopherol, a lipophilic phenolic antioxidant.

Quantitative HPLC with ultraviolet [6–10], fluorimetric [11–14] or single flow-through amperometric detection [15–17] is currently used for the determination of α-tocopherol in biological samples. However, certain limitations and areas for improvement have become apparent through experience with developing and applying these HPLC systems. These assay methods have one or more of the following problems and shortcomings: (i) lack of sensitivity and selectivity, (ii) lack of reproducibility and stability, (iii) requirement for large sample volumes, (iv) complicated sample preparation and clean-up and (v) inability to detect simultaneously α-tocopherol and its oxidative metabolite.

In this study, we investigated a simple assay method for the selective, highly sensitive and simultaneous determination of α-tocopherol and α-tocopherolquinone by injection of a crude ethanol–hexane extract of plasma or erythrocyte membrane into the HPLC system with the redox detection mode using a series of four coulometric working electrodes. The results obtained suggest that the separation of α-tocopherolquinone and three fractions of tocopherols (α−, β + γ− and δ−) is possible by using the combination of an MC MEDICAL C_{18} reversed-phase column and a mobile phase consisting of 96% methanol [methanol–HPLC-grade distilled water (96:4, v/v)] with 40 m*M* sodium perchlorate as the supporting electrolyte [3]. However, it is impossible to separate β- and γ-tocopherols.

In the redox detection mode employing a series of four coulometric working electrodes, the first high-efficiency coulometric working electrode was set at −0.45 V to prevent interference of electroactive substances in the mobile phase with the reduction reaction at subsequent electrodes. The second and third coulometric working electrodes were set at −0.45 V to reduce the quinone type of analyte to the hydroquinone type. That is, α-tocopherolquinone eluted from the column is reduced electrochemically to α-tocopherol. Also, this improves the selectivity by eliminating the detection of non-reversible substances at the subsequent electrode operated in the oxidative mode. Hence substances that can be completely reduced at −0.45 V and cannot be subsequently oxidized do not appear in the analysis with the recording electrode set at +0.40 V. Furthermore, reduction of the column effluent greatly reduced baseline drift and virtually eliminated the large void currents routinely observed following injections of crude ethanol–hexane extracts into an HPLC apparatus. The fourth electrode was set at +0.40 V for the actual measurement of the analyte.

The results obtained suggest that HPLC with the redox detection mode using a series of four coulometric working electrodes, assembled on the basis of the experimental results, has several advantages: (i) the highly sensitive and simultaneous determination of α-tocopherol and α-tocopherolquinine is possible with detection limits at the low picogram level; (ii) quantitative analysis with selectivity, reproducibility and stability is possible owing to a significant reduction in the "void volume" signal, the virtual elimination of interference from non-reversible

substances and the stabilization of the baseline signal; (iii) the measurement of α-tocopherol and α-tocopherolquinone in crude ethanol–hexane extracts prepared by a simple extraction technique is possible. This greatly improves the analysis efficiency, because it eliminates errors in sample preparation procedures. The usefulness of HPLC with the redox detection mode was confirmed by its application to the simultaneous determination of α-tocopherol and α-tocopherolquinone in rat plasma and erythrocyte membrane.

In conclusion, reversed-phase HPLC with the redox detection mode using a series of four coulometric working electrodes is useful for the selective, highly sensitive and simultaneous determination of α-tocopherol and α-tocopherolquinone in crude biological samples. In addition, this HPLC system is applicable to studying the preventive effect of α-tocopherol on lipid peroxidation.

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