Determination of \( \alpha \)-Tocopherol and \( \alpha \)-Tocopherylquinone in Rat Tissues and Plasma by High-Performance Liquid Chromatography with Electrochemical Detection

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\( \alpha \)-Tocopherol and \( \alpha \)-tocopherylquinone in rat tissues and plasma were determined simultaneously by using high-performance liquid chromatography–electrochemical detection (HPLC-ED) with dual electrodes in the series mode. Biological samples were saponified in the presence of a mixture of butylated hydroxytoluene, ascorbic acid, and pyrogallol and then extracted with hexane. The compounds were separated on a C18 column using a mobile phase containing 95% methanol and 0.05 M sodium perchlorate as the supporting electrolyte. After HPLC separation, \( \alpha \)-tocopherylquinone was first reduced at an upstream electrode at \(-500\) mV. Both \( \alpha \)-tocopherol and the reduction product of \( \alpha \)-tocopherylquinone were then oxidized downstream at \(+600\) mV. Only the downstream electrode current was monitored for the determination. Linearity of the standard curves was obtained over the range 5–30 pmol for \( \alpha \)-tocopherol and \( \alpha \)-tocopherylquinone. Minimum detectable quantities (S/N of 3) were 0.25 pmol for \( \alpha \)-tocopherol and 0.31 pmol for \( \alpha \)-tocopherylquinone. The method was applied to analysis of the contents of \( \alpha \)-tocopherol and \( \alpha \)-tocopherylquinone in rat tissues and plasma. By hyperoxia, the content of \( \alpha \)-tocopherol was decreased remarkably in lung, and in contrast, the contents of \( \alpha \)-tocopherylquinone were increased in all tissues studied with the exception of plasma, though the content of \( \alpha \)-tocopherylquinone in normal rats is quite small. The technique is particularly useful in the quantitation of the oxidation of \( \alpha \)-tocopherol in biological samples.

Key words \( \alpha \)-tocopherol; \( \alpha \)-tocopherylquinone; HPLC-ED; rat tissue, hyperoxia

Tocopherols are potent antioxidants and their presence in biological membranes is believed to represent the major defense system against peroxidative membrane lipids, which are highly susceptible to peroxidative degradation.\(^{1–9}\) Either antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase, or antioxidants such as ascorbic acid, tocopherol, and glutathione are presumed to prevent the oxidative deterioration of physiological function in living tissues. Especially, \( \alpha \)-tocopherol is now accepted to function as a potent physiological chain breaking antioxidant in biomembranes by scavenging the chain-carrying peroxyl radicals, preventing oxidative damage to polyunsaturated lipids. From kinetic studies, it is known that \( \alpha \)-tocopherol can trap one peroxyl radical to generate tocopherol radical, which traps another peroxyl radical to be consumed to \( \alpha \)-tocopherolquinone. Since it has been recognized that \( \alpha \)-tocopherolquinone is detected in several organs, it is important to analyze the content of \( \alpha \)-tocopherolquinone in living tissue for global assessment of antioxidative defense systems. Nevertheless, there have been only a few articles concerning determination of tocopherols and their oxidation products, tocopherolquinones, in biological samples.\(^{10–14}\) One of the causes of uncertainly is artificial oxidation of tocopherols during sample processing. Previously, it has been reported that the addition of a mixture of butylated hydroxytoluene (BHT), ascorbic acid and pyrogallol into biological samples during saponification eliminates the oxidation of \( \alpha \)-tocopherol and \( \alpha \)-tocopherylquinone.\(^ {3}\) Therefore, we used these experimental conditions, and investigated the influence of oxidative stress on the content of \( \alpha \)-tocopherol and \( \alpha \)-tocopherylquinone in several organs.\(^ {3}\)

Conventional methods for simultaneous quantitation of \( \alpha \)-tocopherol and \( \alpha \)-tocopherylquinone involve separation by HPLC with UV detection,\(^ {11}\) however they generally lack sufficient sensitivity for \( \alpha \)-tocopherylquinone. Although fluorescence assay is generally sensitive, \( \alpha \)-tocopherylquinone is not detectable. Previously, we have developed an electrochemical detector (ED) with a new porous carbon coulometric electrode\(^ {15}\) and investigated analysis of neurotransmitters, such as the catecholamines and its metabolites, in biological fluid.\(^ {16–18}\) In the present study, we report the determination of \( \alpha \)-tocopherol and \( \alpha \)-tocopherylquinone in rat tissues and plasma using a series dual-electrode detector which consists of the downstream-oxidative detection of reduction products from an upstream electrode (reductive-oxidative detection). The effect of hyperoxia in rat on contents of \( \alpha \)-tocopherol and \( \alpha \)-tocopherylquinone in homogenates of tissues and plasma were examined by this method. In addition, elimination of oxygen interference during reductive mode HPLC-ED and improvements in detection limits for the compounds were investigated.

Experimental

Chemicals \( \alpha , \beta , \gamma , \text{and} \delta \)-Tocopherols were purchased from Eisai (Tokyo, Japan); \( \alpha \)-tocopherylquinone was purchased from ICN Biochemicals (Cleveland, OH, U.S.A.). Sodium perchlorate was purchased from Wako Pure Chemical Industries (Tokyo, Japan). Methanol was HPLC grade. Water was distilled and passed through a Milli-Q purification system (Millipore, Bedford, MA, U.S.A.). All other chemicals were of analytical-reagent grade.

Processing of Biological Samples Animals: Six male Wistar rats of age of 12 months old were used in this experiment. To induce oxidative stress, three rats were subjected to hyperoxia by maintaining them in an atmosphere of 100% oxygen gas with flow-rate of 1 ml/min for 48 h at 20°C. The other three rats were used as a control.

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Dedicated to the memory of Dr. Kyousuke Tsuda.
α-Tocopherol

![α-Tocopherol Diagram]

α-Tocopherolquinone

![α-Tocopherolquinone Diagram]

Fig. 1. Electrochemical Reactions of α-Tocopherol and α-Tocopherylquinone at the Electrode

The sample preparation of tissue extracts and plasma were as follows: 0.5 g of fresh rat tissues were accurately weighed and homogenized in 2 ml of 0.1 M Tris buffer (pH 7.3). For plasma sample preparation, 2 ml of rat plasma was obtained from heparinized venous blood by centrifugation. Each sample was placed under vortex-mixing into tubes containing 2 ml of 0.025 w/v% butylated hydroxytoluene (BHT) in ethanol, 0.2 ml of 15 w/v% ascorbic acid, 0.2 ml of 25 w/v% pyrogallol, and 100 pmol of δ-tocopherol (an internal standard). The samples were saponified at 60°C for 30 min with 1 ml of 10 w/v% potassium hydroxide solution. The tubes were cooled and 2 ml of 0.9% NaCl and 2 ml of hexane–ethyl acetate (9:1) containing 0.025% BHT were added. Tocopherols and tocopherylquinone were extracted into the hexane phase by vortexing for 1 min. The samples were centrifuged for 10 min at 3000 rpm. The hexane phase was separated and evaporated to dryness under nitrogen gas. The residue was redissolved in 0.5 ml of 95% methanol containing 50 mM sodium perchlorate and filtered prior to injection. All the biological samples were analyzed within 24 h.

HPLC Conditions The HPLC system consisted of a Hitachi Model L-6200 intelligent pump, a dual electrode electrochemical detector (original made) and a D-2500 data processor. The electrodes of the coulometric ED consisted of a working electrode made of glassy carbon (3.0 mm diameter×3.0 mm thickness; Kurarashi Giken, Kyoto, Japan) in a series mode, an Ag/AgCl reference electrode and a counter electrode.

The analytical column was a reversed-phase C18 Neo-pack ODS2 column (5 μm, 150×4.6 mm i.d., Niharu Kogyo, Tokyo, Japan). The mobile phase was 95% methanol containing 50 mM sodium perchlorate as the supporting electrolyte, and it was continuously bubbled with helium gas to maintain an oxygen-free state. In the series mode, the upstream electrodes was set at −500 mV, while the downstream electrode was set at +600 mV.

Results and Discussion

Hydrodynamic Voltamograms The reduction of α-tocopherylquinone at the upstream electrode during flow-cell electrochemistry produced exclusively the hydroquinone, which was oxidized at the downstream electrode to regenerate α-tocopherylquinone (Fig. 1). α-Tocopherol is oxidized at the downstream electrode in the presence of water via a carbocation intermediate to 8a-hydroxyl-α-tocopherol.11 Hydrodynamic voltammograms for oxidation of α-tocopherol and reduction of α-tocopherylquinone, using the dual-series flow cell, are shown in Fig. 2. The potential of the downstream electrode was varied from +100 to +700 mV for oxidation of α-tocopherol. Then by setting the downstream electrode at +600 mV, the potential of the upstream electrode was varied from −300 to −500 mV for reduction of α-tocopherolquinone. From the hydrodynamic voltammograms, a reductive potential of −500 mV for the upstream electrode and an oxidizing potential of +600 mV for the downstream electrode were chosen for further experiments.

Separation of Tocopherols α-, δ-Tocopherol and α-tocopherylquinone were well separated and determined by the dual-series electrode HPLC-ED. Figure 3 shows a chromatogram of a standard mixture containing 25 pmol each of α-tocopherol, β-tocopherol, δ-tocopherol (used as the internal standard in the assay of biological samples), γ-tocopherol, and α-tocopherylquinone. After HPLC separation, α-tocopherylquinone was first reduced at an upstream electrode at −500 mV. Both tocopherols and α-tocopherylquinone were then detected at the downstream electrode at +600 mV.

Calibration Curves Linear relationships between the amounts and the peak area ratio (α-tocopherol, or α-tocopherylquinone/δ-tocopherol: R) were obtained over the range 5—30 pmol as indicated by the following equations:

α-tocopherol; R/10^−2=1.25x+1.16 (r=0.999)

α-tocopherylquinone; R/10^−2=1.06x−0.79 (r=1.000)
The precision of the method was established from 5 assays. The variability (coefficients of variation) of the retention times were less than 1% and those of peak areas were less than 4%. Minimum detectable quantities (S/N of 3) were 0.25 pmol for α-tocopherol, 0.33 pmol for β-tocopherol, 0.12 pmol for γ-tocopherol, 0.26 pmol for δ-tocopherol and 0.31 pmol for α-tocopherylquinone. The recoveries of the analytical procedure, calculated by addition of α-tocopherol, δ-tocopherol and α-tocopherylquinone to tissues (25 pmol, n = 5), were 86.4%, 100% and 94.2%, respectively. UV-monitoring is also possible for the determination of α-tocopherol and α-tocopherylquinone. α-Tocopherol can be quantitated at 295 nm and α-tocopherylquinone at 265 nm. In our laboratory, electrochemical detection is at least 100 times more sensitive than UV detection for tocopherols and α-tocopherylquinone.

**Determination of α-Tocopherol and α-Tocopherylquinone in Rat Tissues and Plasma**

There have been some controversies regarding the presence of tocopherylquinones in biological organs and tissues.\(^\text{19,20}\) To shed light on the problem and to study the effects of oxidative stress, the contents of α-tocopherol and α-tocopherylquinone in brain, heart, lung, liver, kidney and plasma of rats were measured by the present method. Tocopherols in biological samples are very susceptible to oxidation during sample processing. Previously, addition of a mixture of BHT, ascorbate and pyrogallol was reported to eliminate the loss of α-tocopherol and α-tocopherylquinone during the assay.\(^\text{13}\) To prevent oxidation during the extraction procedure, samples were saponified in the presence of a mixture of BHT, pyrogallol, and ascorbic acid and then extracted with a mixture of hexane and ethyl acetate. Extraction and HPLC-ED analysis of samples of α-tocopherol (0.15 nmol) showed no presence of detectable α-tocopherylquinone, and endogenous δ-tocopherol was not detectable in this sample. From these results, it is suggested that the α-tocopherylquinone detected in samples of tissues and plasma is not an artifact of this sys-
were increased in brain and heart is not clear. The α-tocopherol content of mammalian tissue is primarily maintained by dietary intake, but it has been hypothesized to be maintained during an oxidative challenge by redox cycling to regenerate the parent compound.

The ratio of α-tocopherol and α-tocopherylquinone concentrations were increased under oxidative stress with the exception of plasma as shown in Table 1. It is known that losses of tocopherol and tocopherylquinone during sample processing are greater with plasma than with other biological samples. This oxidative loss of tocopherol may be due to the presence of endogenous iron and heme compounds in red cells. The catalytic effect of iron in oxidation reaction and hematin in epoxidation reactions of lipids are well known. Information on oxidation of tocopherol and production of quinone will be useful in understanding the mechanism of membrane damage resulting from oxidative reactions. The ratio of α-tocopherol and α-tocopherylquinone concentrations might serve as a useful indicator of the redox state of tissues and organs.

**Conclusion**

α-Tocopherol and α-tocopherylquinone in rat tissues and plasma were well separated and determined by HPLC-ED. The influence of oxidative stress on contents of α-tocopherol and α-tocopherylquinone were observed in homogenates of rat tissues. The high selectivity of the electrochemical detection for easily oxidizable compounds such as tocopherols makes it possible to determine these compounds even in highly complex mixtures, i.e., liver extracts. The method described herein is simple and very sensitive, allowing the simultaneous detection of tocopherols in biological tissues, a feature highly desirable for small samples.

**References and Notes**