Journal of Chromatography, 414 (1987) 440–448
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3447

Note

Determination of α-tocopherol and α-tocopherylquinone in small biological samples by high-performance liquid chromatography with electrochemical detection

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(First received July 7th, 1986; revised manuscript received October 13th, 1986)

α-Tocopherol (vitamin E) is a lipophilic phenolic antioxidant which is gaining interest for its role in the prevention of certain disease states and the toxicities of a number of xenobiotics [1]. We have recently shown that during a toxic chemical or oxidative insult to isolated hepatocytes, the cellular content of α-tocopherol decreases dramatically, parallel with a stimulation of lipid peroxidation, and is followed by biochemical expressions of membrane damage and cell injury [2,3]. An elevation in the α-tocopherol content of these cells protects them against the oxidation-associated cytotoxicity. α-Tocopherol has been proposed to protect biological systems against oxidative damage by prevention of membrane lipid peroxidation [4], stabilization of membrane structure, and through the maintenance of the redox balance of intracellular thiols [3]. The mechanism of prevention of lipid peroxidation by α-tocopherol is through the transfer of hydrogen atoms from the tocopherol to the lipid peroxyl radical $ROO^{\cdot}$, thus interrupting propagation of the radical chain reaction [4]. The subsequently formed α-tocopheryl radicals do not further the propagation of the autoxidation reaction, thereby terminating the peroxidative process.

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0378-4347/87/$03.50

Since the depletion of cellular α-tocopherol increases the potential of peroxidative damage to cell membranes, its loss through oxidation to the tocopheryl radical would predispose the cell to chemical or oxidant-induced injury. The α-tocopheryl radical, furthermore, may be oxidized in vitro to α-tocopherylquinone, and probably in vivo [5], via a peroxy ketal intermediate [6]. It has been proposed that α-tocopherol is additionally metabolized in vivo to polymers, conjugates with glucuronide or sulfate [5], and adducts of tocopherol–lipid formed during free radical attack on membrane lipids [6]. The relative contribution of these metabolites to the profile of α-tocopherol metabolism during an oxidative challenge has not been ascertained. Although generally considered an inactive product of α-tocopherol metabolism, a role for α-tocopherylquinone in platelet function has been proposed [7].

In vitro biological models of cytotoxicity generally utilize small samples of tissue, and therefore require an extremely sensitive assay for α-tocopherol and its oxidative metabolites. Previous methods of tocopherol separation by high-performance liquid chromatography (HPLC) coupled with ultraviolet detection for simultaneous quantitation of α-tocopherol and α-tocopherylquinone lacked sufficient sensitivity for small biological samples [8]. In addition, the more sensitive HPLC–fluorescence assay does not detect α-tocopherylquinone. In order to overcome these problems of sensitivity and the presence of interfering substances routinely encountered with hepatocyte systems, this report describes an HPLC system for the simultaneous determination of femtomole quantities of α-, γ-, δ-tocopherol and α-tocopherylquinone in biological tissue using dual-electrode electrochemical detection [LC/electrochemistry (LCEC)].

EXPERIMENTAL

Chemicals

α-Tocopherol and γ-tocopherol were purchased from Kodak (Rochester, NY, U.S.A.); δ-tocopherol was a generous gift of Hoffmann-La Roche (Nutley, NJ, U.S.A.); α-tocopherylquinone was purchased from ICN Biochemicals (Cleveland, OH, U.S.A.); α-tocopheryl succinate was purchased from Sigma (St. Louis, MO, U.S.A.). Sodium perchlorate was purchased from Aldrich (Milwaukee, WI, U.S.A.). All reagents were HPLC grade.

Chromatography

The HPLC system consisted of two Altex 100A solvent pumps (Beckman Instruments, Fullerton, CA, U.S.A.), a Beckman 421 controller, and a BAS dual-electrode LC-4B electrochemical detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) with the two glassy carbon electrodes in series configuration [9]. As described in Results, the upstream electrode was set for "reduction", potential at −0.7 V, while the downstream electrode was set for "oxidation", potential of +0.6 V. The analytical column was a 3-μm (150 mm×4.6 mm) reversed-phase C_{18} Spherisorb ODS II column (Alltech Assoc., Deerfield, IL, U.S.A.), protected by an Adsorbosphere C_{18} guard column (Alltech Assoc.). The eluting solvent (solvent A) was 96% methanol with sodium perchlorate (50 m*M*) as the supporting electrolyte. The entire system was maintained oxygen-free by

continuous bubbling of solvent A with helium at 50°C, with a 1-h initial warm-up and degassing period, and replacing all PTFE tubing with stainless steel, as recommended by the detector manufacturer. Omission of deoxygenation during use of the series mode resulted in high background current (>50 nA). Background current was minimal during oxidation in the absence of a reductive potential. Deoxygenation of samples was unnecessary with the series mode since the tocopherols were adequately separated from solvent peaks under these conditions.

After equilibration of the system and electrodes with deoxygenated solvent A with a flow-rate of 1 ml/min, the sample was injected onto the column. After elution of α-tocopherol (12 min), 100% methanol (solvent B) was pumped through the column at 1.7 ml/min to wash late eluents from the column. This precaution was unnecessary at low gain since contribution from these compounds was minimal. After 20 min, solvent B was replaced with deoxygenated solvent A (1.0 ml/min) and the electrodes were allowed to re-equilibrate for 5–10 min prior to next sample injection.

Cyclic voltammograms of α-tocopherol and α-tocopherylquinone were obtained with a BAS 100 electrochemical analyzer (Bioanalytical Systems).

Isolated cell preparation

Isolated rat hepatocytes were prepared as described [10], resuspended in Fischer's medium to $2 \cdot 10^6$ cells per ml, and rotated at 37°C. All tocopherol analyses were conducted on viable cells to minimize the contribution of oxidation of α-tocopherol in non-viable cells. Viable cells were separated from medium and non-viable cells by centrifugation through dibutyl phthalate oil into 36% Percoll in Hank's salt solution as described [10]. The number of viable cells per cell pellet was quantitated by analysis of duplicate cell pellets for DNA content [11]. All tocopherol determinations were conducted on cells with greater than 90% viability as determined by the ability to exclude trypan blue [10].

Sample preparation

Viable cell pellets were re-suspended in 0.2 ml of saline to which were added 0.2 ml of ethanol with or without the internal standard in 10 μl of ethanol. This mixture was sonicated for 15 s and extracted with 0.5 ml of hexane by mixing with a vortex mixer for 1 min. From the top hexane layer, 0.4 ml were removed to glass tubes and kept in the dark. The cell pellet–ethanol solution was sonicated and extracted with 0.5 ml of hexane a second time, and 0.4 ml of hexane were removed. The hexane layers were combined, dried under a stream of nitrogen in the dark, and the tocopherols were reconstituted with 0.1–0.5 ml of methanol. For short-term storage (<24 h), samples were gassed with Argon and stored at −20°C, otherwise 10–100 μl of the methanol solution were injected onto the column. Under these conditions, the recovery of α-tocopherol added to the cell pellet in ethanol, and dried under nitrogen prior to extraction, was 78%. Recoveries of γ- and δ-tocopherols and α-tocopherylquinone were 100%.

RESULTS

The HPLC analysis and quantitation of femtomole amounts of tocopherols in biological samples was accomplished with dual-series electrode LCEC. The object

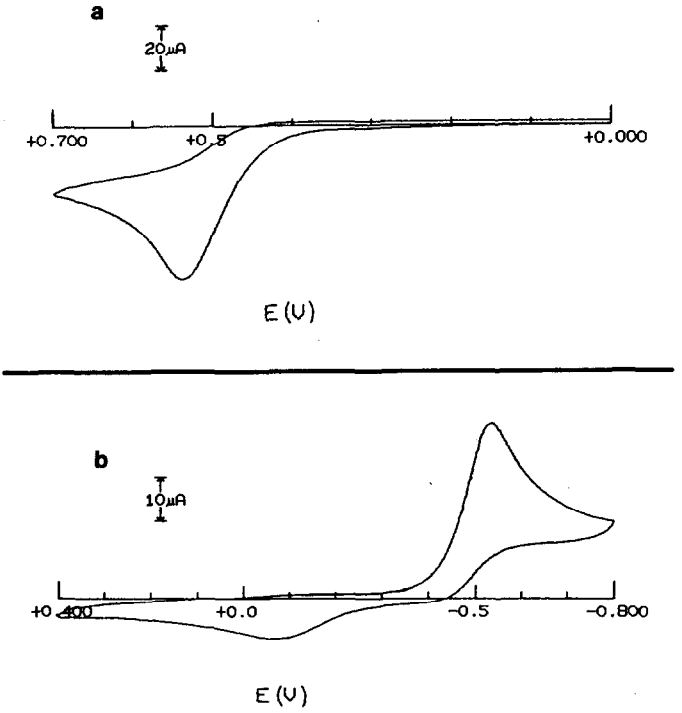
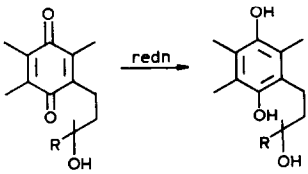


Fig. 1. Cyclic voltammograms of (a) α-tocopherol and (b) α-tocopherylquinone. Solvent was 96% methanol with 0.1 *M* sodium perchlorate as supporting electrolyte.

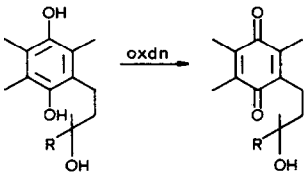
of this technique was to first electrochemically reduce α-tocopherylquinone after the chromatographic separation of the tocopherols, then oxidize the tocopherols and the tocopherylquinone reduction product at the downstream electrode. Similar LCEC schemes have been described for hydroquinone analysis, but with downstream detection of the reductive reaction after upstream oxidation [9], the reverse of that described herein.

Reductive and oxidative potentials for maximal detection of the tocopherols were determined by cyclic and hydrodynamic voltammetry. From the cyclic voltammograms of α-tocopherol and α-tocopherylquinone in 96% methanol with sodium perchlorate as the supporting electrolyte (Fig. 1), the irreversible oxidation of α-tocopherol and the reversible redox couple of α-tocopherylquinone are evident. With an initial anodic scan, oxidation of α-tocopherol occurred as a single peak at +0.53 V, with no reduction peak detectable during the reverse cathodic scan (Fig. 1a). α-Tocopherylquinone, on the other hand, was reduced during an initial cathodic scan at −0.52 V, and exhibited a small oxidation peak upon the reverse anodic scan at −0.1 V. These results are consistent with the orginal reports by Marcus and Hawley [12,13] on α-tocopherol and α-tocopherylquinone electrochemistry in acetonitrile and water with similar supporting electrolyte. The cyclic voltammetry described in those reports and the voltammograms presented herein indicate that the reduction of α-tocopherylquinone at the upstream electrode (−0.7 V) during thin-layer flow-cell electrochemistry produces exclusively the hydroquinone (eqn. 1), which is oxidized at the downstream electrode (+0.6 V) to regenerate α-tocopherylquinone (eqn. 2). Fur-

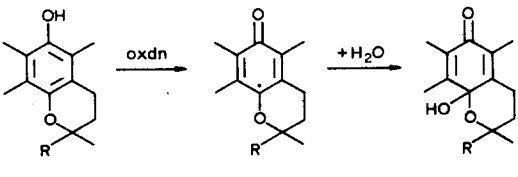
thermore, α-tocopherol is oxidized at the downstream electrode in the presence of water via a carbonium ion intermediate to 8a-hydroxy-α-tocopherone (eqn. 3).



(1)



(2)



(3)

R = $C_{16}H_{33}$

Hydrodynamic voltammograms for α-tocopherol oxidation and α-tocopherylquinone reduction, utilizing the dual-series thin-layer flow cell, are depicted in Fig. 2. The potential of the downstream (detecting) electrode was varied from +0.4 to +1.0 V for oxidation of α-tocopherol, followed by setting the downstream (oxidizing and detecting) electrode at +0.6 V and varying the upstream electrode from 0 to −1.0V for reduction of α-tocopherylquinone. From the cyclic and hydrodynamic voltammograms, a reductive potential of −0.7 V for the upstream electrode and an oxidizing potential of +0.6 V for the downstream (detecting) electrode were chosen for further experiments.

Equimolar concentrations of α-, δ-, and γ-tocopherols and α-tocopherylquinone were cleanly separated and detected by the LCEC technique described herein (Fig. 3). Relative response factors were determined for calculation of tocopherol contents in tissue samples with the internal standard method. The limit of detection of the tocopherols was 1.0 fmol, whereas that of α-tocopherylquinone was 10 fmol. The unequaled sensitivity of LCEC for tocopherol quantitation is clearly evident from these data.

The concentrations of α-tocopherol and α-tocopherylquinone in freshly isolated rat hepatocytes were determined (Table I), and found to be in agreement with reports using HPLC–UV detection [8] as well as with our own determinations using UV and fluorescence detection for α-tocopherol [3]. A representative chromatogram of tocopherols from 1.8 mg (wet weight) of freshly isolated liver

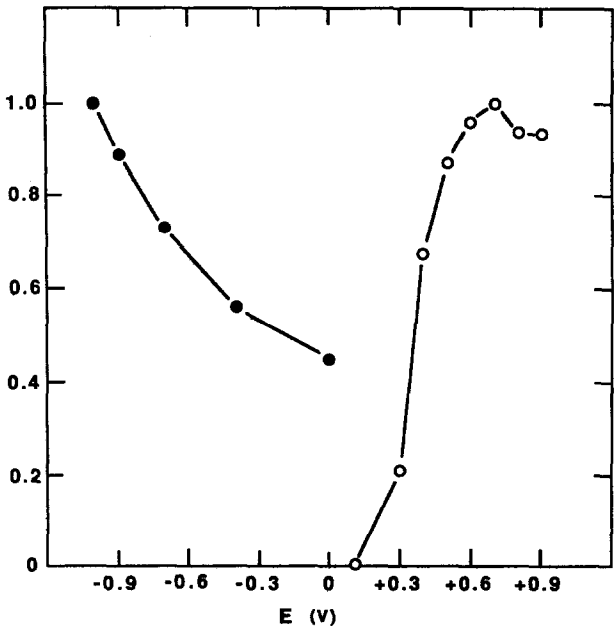


Fig. 2. Hydrodynamic voltammograms of α-tocopherol (○) and α-tocopherylquinone (●). Standard solutions of α-tocopherol and α-tocopherylquinone in ethanol were loaded into the HPLC system and monitored by dual-electrode LCEC. Samples of α-tocopherol (150 pmol) were monitored at the downstream (detecting) electrode with increasing positive potentials from +0.1 to +0.9 V. Setting the downstream electrode at +0.6 V, samples of α-tocopherylquinone (150 pmol) were monitored at the downstream electrode while the upstream electrode was varied from 0 to −0.1 V. Values for the electrochemical response are expressed as relative to the maximal response of 1.0 for each tocopherol.

cells ($2 \cdot 10^5$ cells) is depicted in Fig. 4. α-Tocopherol and α-tocopherylquinone contents were 30 and 0.75 pmol, respectively. The lack of interfering substances, especially at the retention time of the internal standard, δ-tocopherol (compare to Fig. 3), and a steady baseline at high sensitivity are evident, and allow for the quantitation of femtomole amounts of tocopherols in tissue extracts.

We have found that the storage of α-tocopherol extracts in the final methanol phase at −40°C, either after deoxygenation with Argon or supplementation with the antioxidant N,N′-diphenyl-*p*-phenylenediamine (DPPD, 2%), proved to be inadequate to insure its long-term stability in experiments involving oxidative injury to isolated cells (up to 5% loss per day in experiments involving a non-oxidant type of injury, and up to 20% loss per day during incubation of cells with oxidizing chemicals). Cellular α-tocopherol determinations during chemically induced cell injury should therefore be conducted on fresh samples.

The variability (coefficient of variation) for each tocopherol from cellular extracts within a run was 2.1% ($n=8$), while variability between runs was 7.2% ($n=4$ for each run). Recoveries were calculated by addition of tocopherols to hepatocyte pellets (0.15 and 0.6 nmol in 10 μl of ethanol, $n=5$), followed by evaporation of the ethanol under nitrogen, extraction and LCEC analysis of the tocopherols, and correction for endogenous tocopherols. Recoveries were consistently found to be 78% for α-tocopherol and 100% for the other tocopherols, and

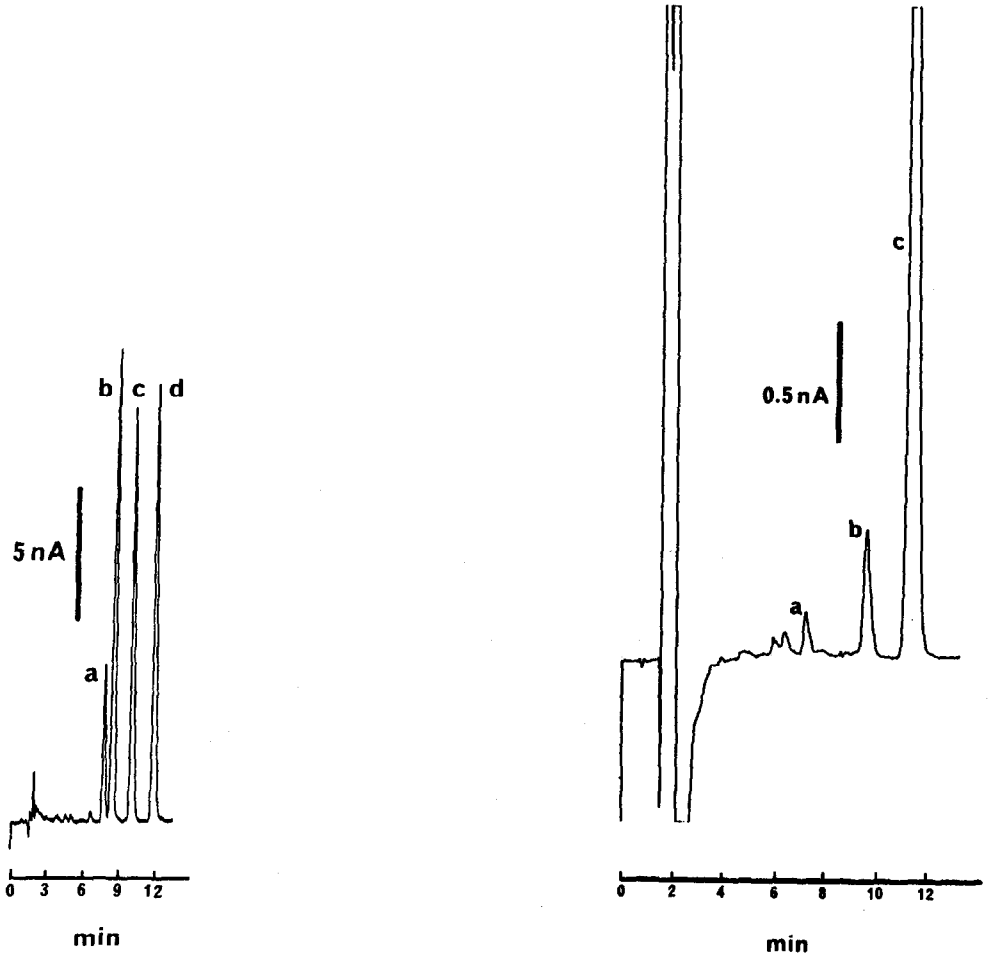


Fig. 3. LCEC profile of a mixture of tocopherols. A sample containing 40 pmol of each tocopherol in 10 μl of ethanol was injected onto the column. The upstream electrode was set at -0.7 V, while the downstream detecting electrode was set at $+0.6$ V. Chart-speed was 0.2 cm/s. Peaks: a = α-tocopherylquinone; b = δ-tocopherol; c = γ-tocopherol; d = α-tocopherol.

Fig. 4. Representative LCEC profile of endogenous tocopherols from isolated rat hepatocytes. Freshly isolated hepatocytes were resuspended in Fischer's medium and incubated for 10 min at 37°C. A 0.75-ml aliquot of viable cells was prepared and assayed for tocopherol contents as described in Experimental. Sensitivity range was 10 nA; chart-speed was 0.5 cm/s. Peaks: a = α-tocopherylquinone (0.5 pmol); b = γ-tocopherol (2.0 pmol); c = α-tocopherol (21.1 pmol). Endogenous δ-tocopherol was not detectable in this sample.

did not necessitate the addition of antioxidants such as butylated hydroxytoluene or pyrogallol during the extraction process as suggested by others [14,15].

Extraction and LCEC analysis of samples of α-tocopherol (0.15 nmol) showed no presence of detectable α-tocopherylquinone. From these data, it is suggested that the α-tocopherylquinone detected in samples of freshly isolated hepatocytes is not an artefact of this system.

TABLE I

CONCENTRATION OF TOCOPHEROLS IN FRESHLY ISOLATED RAT HEPATOCYTES

Samples of freshly isolated hepatocytes ($1.5 \cdot 10^6$ cells) were prepared, and the tocopherols determined by LCEC, as described in Experimental. Tocopherols were quantitated by use of external standard curves. Values are means ± standard errors of the means ($n=4$).

Compound	Concentration (pmol per 10^6 cells)
α-Tocopherylquinone	2.5±0.8
δ-Tocopherol	1.2±0.6
γ-Tocopherol	15.1±1.1
α-Tocopherol	155.0±12.0

DISCUSSION

The quantitation of metabolites of α-tocopherol during a toxic insult to biological systems is essential to the development of a clear picture of the fate of α-tocopherol during its role as a cellular protective agent. We have demonstrated that oxidative injury to isolated hepatocytes, both chemical oxidant and non-oxidant-induced, depletes cellular α-tocopherol [2,3]. 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. In support of this, evidence has been presented for the regeneration of α-tocopherol from the tocopheryl radical via ascorbic acid hydrogen transfer [16], and via a glutathione redox system-dependent protein [17–19]. In hepatocytes exposed to an oxidative challenge, the deletion of α-tocopherol and its apparent lack of regeneration [2,3] suggest the further metabolism of the tocopheryl radical to α-tocopherylquinone or other oxidative metabolites. The fate of α-tocopherol during these injurious events is unclear and is presently under investigation with both cellular and non-cellular systems in our laboratory.

The applicability of electrochemistry for the analysis of tocopherol redox behaviour was first demonstrated prior to the commercial development of thin-layer electrodes for HPLC usage, and helped define α-tocopherol oxidation pathways [12,13]. More recently, the α-tocopherol content of erythrocytes and liver tissue has been determined by electrochemical techniques after HPLC separation with a single glassy carbon flow-through electrode as an oxidizing/detection source [20–22]. As described herein, however, the simultaneous detection of α-tocopherol and α-tocopherylquinone necessitated the use of dual electrodes set in a series configuration, the first upstream electrode for reduction of the quinone to the hydroquinone, the second downstream electrode for oxidation and detection of the tocopherols and the hydroquinone. In addition to this series configuration for the electrodes, the simultaneous electrochemical determination of oxidizable and reducible compounds, and compounds amenable to redox cycling, may also be performed by parallel configuration of the electrodes. In this technique, the HPLC effluent passes both electrodes simultaneously, with one electrode serving

as an oxidation/detection source and the other as a reduction/detection source for responsive compounds [23]. In spite of the minor difficulties encountered with LCEC parallel electrode configuration, such as the possible necessity of high electric potentials and low flow-rates, it may also prove applicable to tocopherol analysis.

The LCEC assay described herein is simple and the analysis is extremely sensitive, allowing the simultaneous detection of femtomole quantities of tocopherols in biological tissues, highly desirable for small samples. Quantitation of tocopherols in biological samples such as liver tissue may be standardized by the use of δ-tocopherol as an internal standard. Interference from endogenous levels of δ-tocopherol in isolated hepatocytes is minimal and frequently non-detectable, but may become apparent with highly sensitive detection parameters. The quantitation of α-tocopherol and α-tocopherylquinone in less than 1 mg of hepatocytes ($1.2 \cdot 10^5$ cells) with this technique agrees with our own determinatinons with large cell volumes using UV and fluorescent detection after HPLC separation [3].

ACKNOWLEDGEMENTS

The authors wish to acknowledge the expert technical assistance of Marda K. Brown. This research was funded in part by USPHS Grant ES-07060.

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