Simultaneous determination of homologues of vitamin E and coenzyme Q and products of α-tocopherol oxidation

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Abstract **A sensitive procedure is described for the simultaneous determination of vitamin E and coenzyme Q homologues and α-tocopherol oxidation products using two-isocratic step high pressure liquid chromatography (HPLC) and electrochemical detection in the oxidative mode. Zinc-catalyzed reduction in a post-column reactor allows the detection of α-tocopherolquinone, epoxy-tocopherolquinone, and ubiquinones. This technique was used to quantify lipophilic antioxidants in the liver tissue of rats treated or not with α-tocopherolquinone and in a plant oil. α-Tocopherolquinone and its epoxide derivatives, formed from α-tocopherol during iron-catalyzed phospholipid peroxidation, were also determined in a liposome suspension. The high selectivity and sensitivity of the coulometric detection system enabled use of low oxidation potentials giving little baseline noise, while a fast isolation procedure and quantitative recoveries of all oxidized and reduced forms made it possible to measure a high ubiquinol/ubiquinone ratio in liver tissue. Administration of α-tocopherolquinone to rats did not alter the antioxidant status of the liver, despite strong accumulation of both this quinone and its reduced form, α-tocopherolhydroquinone. These results indicate the presence of an efficient reductase and suggest that it could contribute to the protection of cellular membranes from oxidative stress.—**Leray, C., M. D. Andriamampandry, M. Freund, C. Gachet, and J-P. Cazenave. **Simultaneous determination of homologues of vitamin E and coenzyme Q and products of α-tocopherol oxidation. *J. Lipid Res.* 1998.** 39: **2099–2105.**

Supplementary key words HPLC • electrochemical detection • Zn catalyst reduction • ubiquinols • ubiquinones • tocopherols • tocotrienols

There is currently considerable interest in the role of vitamin E and ubiquinone compounds (coenzyme Q) in the protection of membrane lipids against oxidative stress. The most important lipid-soluble antioxidant (1), α-tocopherol, yields several oxidation products including epoxy-tocopherolquinone and α-tocopherolquinone (2, 3). Assay of these compounds has been proposed as an index of the oxidation of membrane lipids (3–5). Recent evidence that α-tocopherolhydroquinone is a powerful inhibitor of low density lipoprotein (LDL) oxidation (6), emphasizes the need for its accurate determination in biological samples along with the other forms of vitamin E. Ubiquinones are in cell membranes (7) and in LDL (8) where they can act as efficient antioxidants (8–10).

The simultaneous determination of vitamin E components, ubiquinols and ubiquinones using electrochemical detection alone (11–13) or in combination with UV detection (14, 15) has been reported previously. However, these procedures require a complex coulometric detector with important negative potentials. Recently, a post-column reactor and coulometric detector were used for the parallel detection of α-tocopherol, ubiquinone-10, and ubiquinol-10 (16, 17). In the present study, we describe a simple and sensitive procedure for simultaneous quantification of the most important lipophilic antioxidants related to ubiquinones, α-tocotrienol and α-tocopherol, using a two-step isocratic HPLC method with post-column zinc reduction and single electrode coulometric detection. This methodology was applied to an α-tocotrienol-rich plant oil, to the liver of rats treated or not with α-tocopherolquinone, and to a liposome suspension subjected to metal-catalyzed peroxidation.

MATERIALS AND METHODS

Materials

All solvents and reagents were of high purity. Methanol, ethanol, and hexane (Chromasolve grade), $ZnCl_2$, $FeCl_2$, $FeCl_3$, Na acetate, ascorbic acid and acetic acid were from Merck (Darmstadt, Germany) and $ZnCl_2$ and 200-mesh zinc powder were from Prolabo (Fontenay-sous-Bois, France). Phosphatidylcholine (Type XVI-E), xanthine, xanthine oxidase (Grade III), sodium dodecyl sulfate (SDS), sodium dithionite, butyl hydroxytoluene (BHT), ubiquinone-9 and -10 standards, and tocopherol standards

Abbreviations: BHT, butyl hydroxytoluene; HPLC, high pressure liquid chromatography; LDL, low density lipoprotein; SDS, sodium dodecyl sulfate.

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were from Sigma (Sigma-Aldrich Chimie, Saint Quentin Fallavier, France).

Preparation of standards

Standard solutions of α-, γ- and δ-tocopherol and ubiquinone-9 and -10 were made up in ethanol and their concentrations were determined spectrophotometrically (15). α-Tocopherolquinone was prepared by oxidation of α-tocopherol with $FeCl_3$ (18) and its purity was verified by HPLC using UV detection (λ_{max}: 262 nm, ϵ_M: 19500). Standard solutions of α-tocopherolhydroquinone and ubiquinol-9 and -10 were obtained by sodium dithionite reduction of α-tocopherolquinone and ubiquinone-9 and -10, respectively (14). α-Tocotrienol-rich fractions were purified from wheat germ oil saponified in the presence of BHT, ascorbic acid, and pyrogallol (19). Epoxy-tocopherolquinone (a mixture of 2,3- and 5,6-epoxy-tocopherolquinone) was prepared as previously described (2). Briefly, a liposome suspension containing a mixture of phosphatidylcholine (4 mg) and α-tocopherol (40 μg) in 10 ml of 50 mm Tris-HCl buffer, pH 7, was oxidized by addition of 5 mg xanthine, 0.2 units xanthine oxidase, and 0.2 mg $FeCl_2$. After 1 h at 37°C, oxidation products were extracted in 2 vol of a hexane–2-propanol mixture 3:2 (v/v), evaporated and treated for 30 min with 2 ml ethanol and 1 ml 0.1 N HCl. The final products were extracted in hexane and analyzed by HPLC with UV detection (270 nm). All stock solutions were stored at −80°C and working standards were obtained by diluting appropriate amounts of the stock solutions in a methanol–water mixture 98:2 (v/v) and kept at −20°C in amber glass vials.

Animals

Male Wistar rats (average weight 250 g) fed a standard chow (AO4, UAR, Villemoisson, France) received by gavage 20 mg α-tocopherolquinone dissolved in 0.5 ml olive oil once a day for 4 days. One day after the last gavage, the rats were anesthetized and the liver was removed and rinsed in saline. Aliquots were frozen in liquid nitrogen and stored at −80°C until analysis.

Processing of samples

Liver tissue was finely pulverized in liquid nitrogen and samples were extracted according to a slight modification of the method of Burton, Webb, and Ingold (20). Briefly, 100 mg of powdered liver was dispersed in 1 ml of cold saline solution (0.9% NaCl), 1 ml 2.5% SDS, and 2 ml ethanol containing 1 mg BHT were added and the mixture was vortexed for 15 min. Lipid compounds were recovered by extraction in 2 × 3 ml hexane. After evaporation under nitrogen, the extracts were resuspended in chloroform–methanol 1:3 (v/v) and kept for no longer than 4 h at −20°C before HPLC analysis. The entire procedure was carried out in dim light and in amber glass tubes.

HPLC analysis

The HPLC system consisted of a solvent degaser (GT-103, Gastorr, Prolabo, France), an HPLC pump (PU-980, Jasco, Prolabo) with a ternary gradient unit (LG-980-02, Jasco, Prolabo), a Rheodyne injector (Model 7725), and a 125 × 4 mm LiChrocart Merck reverse-phase column (5 μm LiChrosorb 100 RP-18, Merck). A solid-phase post-column reactor was made by dry packing of zinc particles into a 20 × 4.6 mm stainless steel column under vibration (21). The column eluate was monitored with a Coulochem II electrochemical detector (ESA, Bedford, MA) fitted with a Model 5010 analytical cell, using only the first electrode which was set at a potential of +700 mV. One carbon filter was placed before the injector valve and another between the post-column reactor and the coulometric cell. The electrochemical detector was coupled to an electronic integrator (Datajet, Thermo Separation Products, Les Ulis, France) controlled by WOW software (Thermo Separation Products). Two eluents were used successively at a flow rate of 1 ml/min: for 9 min eluent A containing 5 mm $ZnCl_2$, 2.5 mm Na acetate, and 2.5 mm acetic acid in methanol–water 98:2 (v/v) and for 22 min eluent B containing 7.5 mm $ZnCl_2$, 3.75 mm Na acetate, and 3.75 mm acetic acid in ethanol–methanol–water 49:49:2 (v/v). Eluent A without salt has been previously used to separate tocopherols from cholesterol (22). In order to prevent oxidation of labile components and improve the reduction efficiency of the post-column reactor, the mobile phases were deaerated with a continuous stream of nitrogen bubbles 1 h before use and during each run.

RESULTS

HPLC separation

Figure 1 shows a typical chromatogram for analysis of a mixed solution of the most common standards using post-column zinc reduction and coulometric detection at an applied potential of +0.7 V. α-Tocopherolquinone and hydroquinone were clearly separated in 6 min, α- and γ-tocopherol between 6 and 9 min, and coenzyme Q components, reduced or oxidized, in the range 16 to 35 min.

Representative chromatograms of liver extracts prepared from control (**Fig. 2A**) and α-tocopherolquinone-treated (Fig. 2B) rats demonstrate the capacity of this HPLC method to separate all the major antioxidants found in rat liver. During the first 9 min, passage of a methanol-based solvent satisfactorily eluted the vitamin E components, including α-tocopherolhydroquinone (retention time 2.6 min) and α-tocopherolquinone (retention time 5.2 min), which were well separated from minor homologues such as α- and β-tocotrienol, detected in trace amounts only in liver extracts from control rats. Rapid change to an ethanol–methanol-based solvent enabled the separation of all ubiquinols and ubiquinones of interest in rat liver within about 20 min. A fluctuation in background current of too great an importance was avoided by using a higher solute concentration in eluent B than in eluent A. After 35 min, no further peaks were detectable even by UV photometry and a 10 min post-run wash-out with eluent A at a flow rate of 1 ml/min was performed before the next injection.

The separation of α- and β- tocotrienol, α-tocopherolquinone, and α-, γ-, and δ-tocopherol from the unsaponifiable fraction of wheat germ oil is shown in **Fig. 3**. Excellent chromatograms enabling the base line separation of all compounds of interest were obtained within 9 min; no ubiquinones could be detected in this vegetable oil. The present HPLC method also allowed easy separation of the most important products formed during the superoxide-dependent oxidation of α-tocopherol in liposomes (**Fig. 4**). In addition to the prominent peaks of the substrate α-tocopherol and its main oxidation product α-tocopherolquinone, a peak corresponding to a mixture of the two epoxyquinone isomers was found between the elution times of α-tocopherolhydroquinone and α-tocopherolquinone, the major component (2,3-epoxy-tocopherolquinone) having a retention time of about 4.3 min. Taking the retention time of α-tocopherol as reference, the rela-

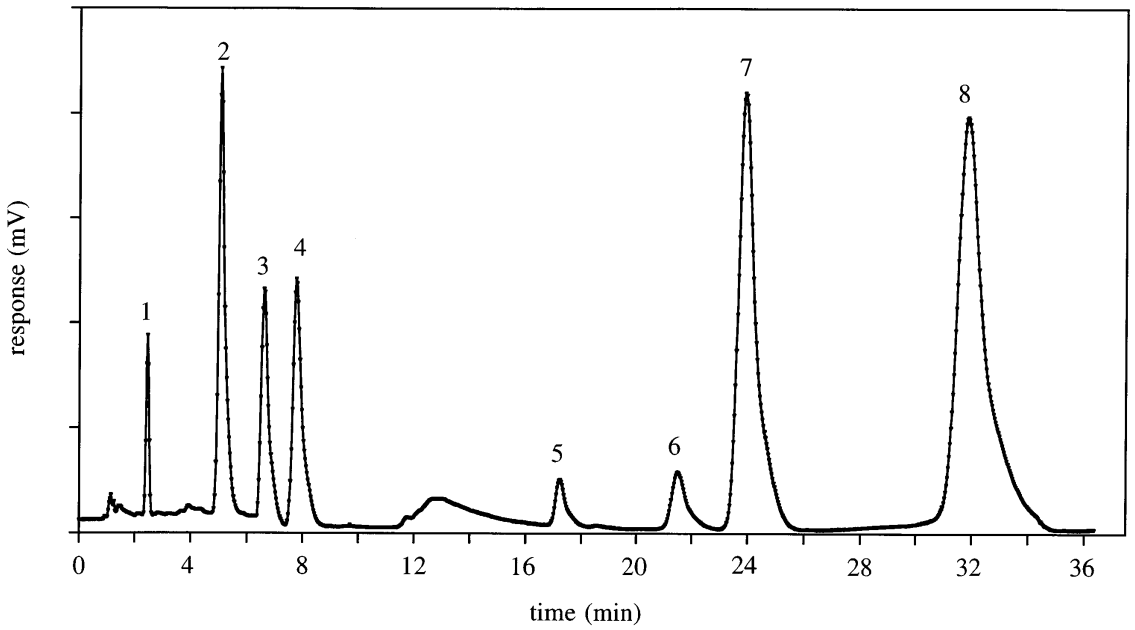

Fig. 1. Chromatogram of a standard mixture of lipophilic antioxidants using post-column zinc reduction and electrochemical detection. The samples, dissolved in 20 μl methanol–water 98:2 (v/v), contained 40 ng α-tocopherolhydroquinone [1], 300 ng α-tocopherolquinone [2], 200 ng γ-tocopherol [3], 100 ng α-tocopherol [4], 10 ng ubiquinol-9 [5], 20 ng ubiquinol-10 [6], 200 ng ubiquinone-9 [7], and 300 ng ubiquinone-10 [8]. The cell potential was +0.7 V and the eluant composition was methanol–water 98:2 (v/v) containing 5 mm $ZnCl_2$, 2.55 mm Na acetate, and 2.5 mm acetic acid for the first 9 min and ethanol–methanol–water 49:49:2 (v/v) containing 7.5 mm $ZnCl_2$, 3.75 mm Na acetate and 3.75 mm acetic acid for the following 27 min.

tive retention times of the compounds detected were: α-tocopherolhydroquinone, 0.33; β-tocotrienol and epoxy-α-tocopherolquinone, 0.51; α-tocotrienol, 0.59; α-tocopherolquinone, 0.65; δ-tocopherol, 0.73; γ-tocopherol, 0.85 and α-tocopherol, 1.

Standard curves

Due to the high sensitivity of coulometric detection, it is possible to work at moderate potentials (+0.5 to +0.7 V), where high selectivity is associated with low background noise. As a practical example, standard curves were established using an oxidation potential of +0.7 V and a current range of 50 nA, because under these conditions the baseline was not too noisy and all compounds showed sufficient response. For the main tocopherol components, linear relationships between the amount of injected tocopherol and peak area were confirmed for the range of interest (10–500 ng), with the regression equations $y = 0.0217\ x + 3.90$ ($r = 0.998$) for α-tocopherol, $y = 0.0529\ x - 1.48$ ($r = 0.999$) for γ-tocopherol and $y = 0.6377\ x + 129.631$ ($r = 0.999$) for δ-tocopherol. The detector response was related to the number of methyl substituants on the chromanol nucleus, more methyl groups leading to a higher detection sensitivity. The regression equations were $y = 0.0431\ x - 1.55$ ($r = 0.997$) for α-tocopherolquinone, $y = 0.0124\ x + 9.30$ ($r = 0.994$) for ubiquinone-9 and $y = 0.0206\ x + 14.30$ ($r = 0.998$) for ubiquinone-10. Despite their similar quinone structures, α-tocopherolquinone and ubiquinone-9 and -10 displayed different linear relationships between sample content and peak area, ubiquinone-9 being the most readily detected compound. Reduced ubiquinols and α-tocopherolhydroquinone gave the same standard curves as their oxidized homologues (data not shown), while no such curves could be established for tocotrienols and epoxy-α-tocopherolquinone as purified compounds were unavailable.

Recovery, reproducibility, and detection limits

The recovery of α-tocopherol, α-tocopherolquinone, and ubiquinone-9 and -10 was checked using the method of extraction described above by addition of known amounts of the standards to liver homogenates and was greater than 95% for all standards throughout the process. In further experiments, the reproducibility of the procedure was evaluated by analyzing four samples from a single batch of powdered liver from one rat. Results were excellent for tocopherols and oxidized compounds with coefficients of variation ranging from 3 to 5%, but somewhat poorer for α-tocopherolhydroquinone and ubiquinols with coefficients of variation of 8% and 13%, respectively. Using the technical conditions described in the Methods section, the limits of coulometric detection were about 0.3 pmol (100 pg) for α-tocopherolquinone, α-tocopherolhydroquinone, and α-tocopherol, 0.6 pmol (500 pg) for ubiquinols, and 1 pmol (800 pg) for ubiquinones.

Stability of antioxidants in extracts

The stability of α-tocopherol, α-tocopherolhydroquinone, and ubiquinols (mainly ubiquinol-9) in liver homogenates before extraction and in methanol–water

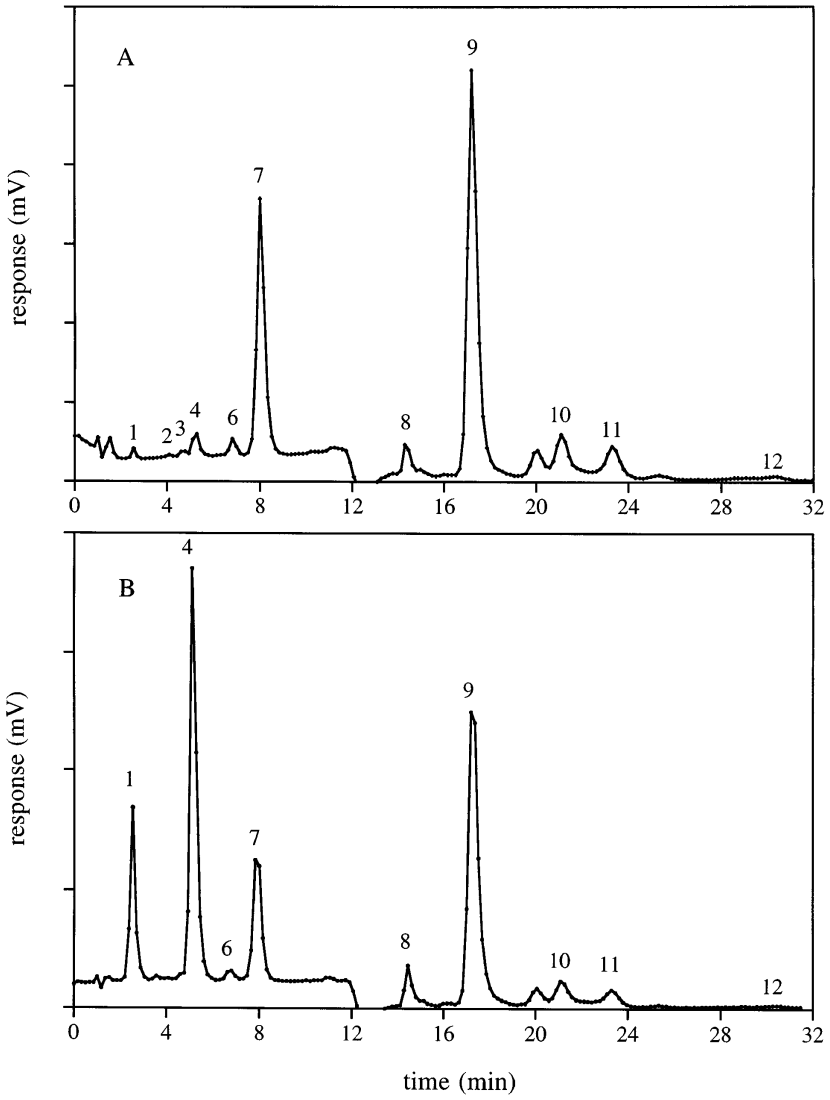

Fig. 2. Chromatograms of lipophilic antioxidants in liver extracts from control rats (A) and from rats treated with α-tocopherolquinone (B). Typical profiles using post-column zinc reduction and electrochemical detection. Peak identification: α-tocopherolhydroquinone [1], β-tocotrienol [2], α-tocotrienol [3], α-tocopherolquinone [4], γ-tocopherol [6], α-tocopherol [7], ubiquinol-8 [8], ubiquinol-9 [9], ubiquinol-10 [10], ubiquinone-9 [11], ubiquinone-10 [12].

extracts before injection was determined at 0°C on ice. Concentrations of α-tocopherol and ubiquinols remained unchanged in aqueous liver homogenates kept for 3 h on ice, whereas concentrations of α-tocopherolhydroquinone did not change during the first hour but decreased by about 23% per hour over 3 h (n = 3), with a concomitant increase in α-tocopherolquinone. When methanol–water liver extracts were kept on ice, α-tocopherol and ubiquinols were stable but α-tocopherolhydroquinone was progressively oxidized with increasing storage time (2.5% per hour, n = 3). Drying of the extracts under a nitrogen stream and their redissolution in the same solvent mixture led to a further decrease in α-tocopherolhydroquinone (19.5%, n = 3) and a parallel increase in α-tocopherolquinone. This loss could be minimized by redissolving the dry extract in a nitrogen deaerated solvent (13%, n = 3). On the other hand, BHT, thioctic acid and ascorbic palmitate were inefficient in preventing loss of the reduced compound from the lipid extract.

Concentrations of lipophilic antioxidants in rat liver

As shown in **Table 1**, four tocopherol compounds and four coenzyme Q homologues could be simultaneously determined in rat liver. α-Tocopherol and ubiquinol-9 represented the major lipophilic antioxidants and it is worthy of note that the concentrations of ubiquinols were much greater than those of their respective ubiquinones. Ingestion of α-tocopherolquinone enriched the liver tissue in α-tocopherolquinone and α-tocopherolhydroquinone while leaving the other constituents unchanged. Finally, the small amounts of tocotrienols (Fig. 2A) and

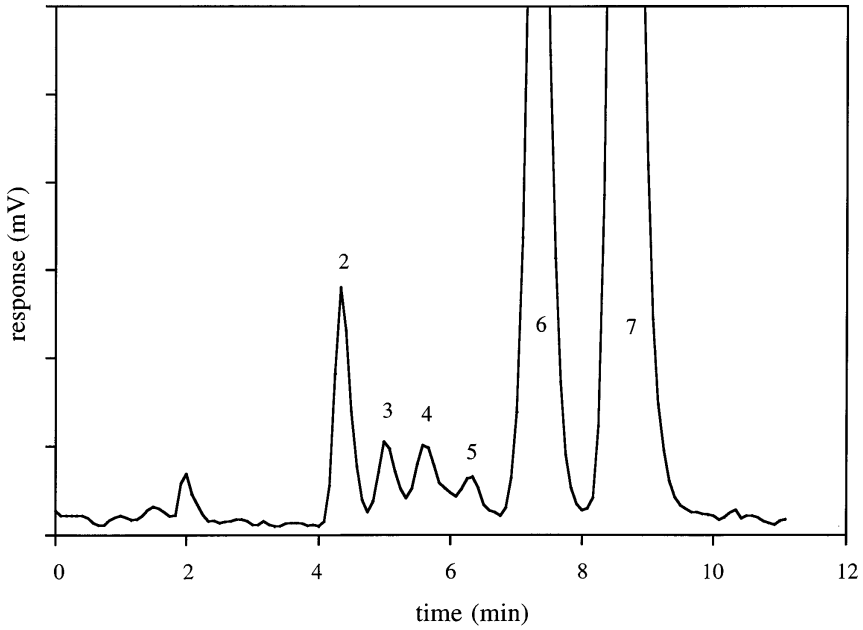

Fig. 3. Chromatogram of lipophilic antioxidants in an unsaponifiable fraction of wheat germ oil, a typical profile using post-column zinc reduction and electrochemical detection. Peak identification as in Fig. 2 with δ-tocopherol [5].

ubiquinol-8 (Figs. 2A and B) detected in chromatograms of liver extracts could not be quantified due to a lack of appropriate standards.

DISCUSSION

Using the simple and rapid method described in this paper, it was possible to define the distribution on the one hand of all the important lipophilic antioxidants in rat liver and on the other hand of the vitamin E homologues in a vegetable oil. The same HPLC technique offers for the first time a simultaneous quantification of both α-tocopherol and its principal oxidative end products, α-tocopherolquinone and -hydroquinone and epoxytocopherolquinone. These end products were recently analyzed using a stable dilution capillary GC–MS method (3). It is also worthy of note that the reduction of these epoxides in a simple zinc reactor, as previously described for the oxide of vitamin K (21), enables their detection at low levels during the trapping of peroxyl radicals generated by xanthine oxidase.

The proposed HPLC technique is easy to manipulate, inexpensive, and yields in a short space of time a direct evaluation of the complex antioxidant functions of tocopherols in tissues. Using mixed standard solutions and UV detection, we could demonstrate that α-tocopherolquinone and oxidized coenzyme Q were completely reduced in the on-line zinc reactor and therefore would be efficiently detected in the electrochemical cell in the oxidative mode. Although use of reduction columns has been previously proposed for the analysis of oxidized coenzyme Q (16, 17), to the best of our knowledge, no such method has been described for the rapid and sensitive detection of α-tocopherolquinone and its epoxide forms. The zinc reduction column used in our system allowed 2 weeks of successive runs with satisfactory reproducibility and background noise, before replacement of the zinc filling. The HPLC pump was left at low flow rate (0.2 ml/min) between runs and overnight with very slow nitrogen sparging in the solvent bottles.

In order to ensure reliable analysis of the lipophilic antioxidants of interest, long extraction procedures and saponification should be avoided as these may increase the risk of oxidation of reduced forms. The present procedure optimizes these requirements by rapid extraction of vitamin E and coenzyme Q homologues in a single step based on hexane/ethanol partition, as has been shown to be the most efficient method (14, 20).

The main problem encountered in sample preparation and analysis was, as expected, the possible interconversion of α-tocopherolquinone and -hydroquinone in the biological extracts (3). In the present work, we found that although relatively stable in a methanol-based solvent, α-tocopherolhydroquinone was oxidized to some extent during evaporation of the extracts even under a nitrogen stream. It was possible to minimize this interconversion by using an excess of BHT in the extraction medium and by analyzing samples immediately after their preparation. On the other hand, the efficiency of the method proposed here is demonstrated by the good reproducibility and high value (8.4) of the ubiquinol/ubiquinone ratio obtained in this work, which is in agreement with a value of 7.1 recently reported in rats (16) and in contrast to pre-

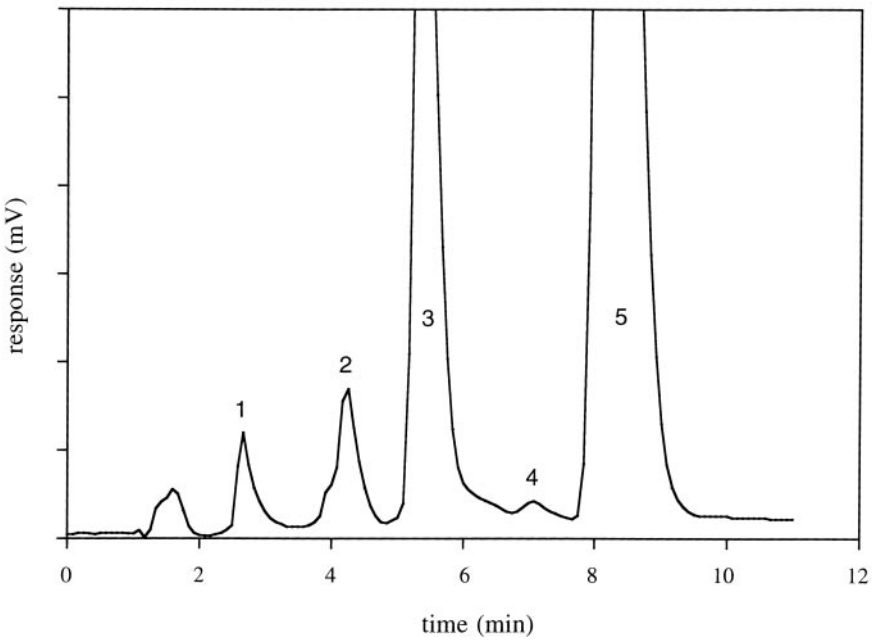

Fig. 4. Chromatogram of products of α-tocopherol oxidation in phosphatidylcholine liposomes incubated with xanthine, xanthine oxidase, and $FeCl_2$ for 60 min at 37°C, a typical profile using post-column zinc reduction and electrochemical detection. Peak identification: α-tocopherolhydroquinone [1], epoxy-α-tocopherolquinone [2], α-tocopherolquinone [3], γ-tocopherol [4], α-tocopherol [5].

vious reports of considerably lower levels of 2.9 in the rat (23) or 0.9 in the mouse (15).

Our chromatographic conditions enabled accurate determination of α-tocopherolquinone and -hydroquinone in the liver of rats fed a standard chow containing the recommended amount of vitamin E (30 mg/kg food). The low concentration of α-tocopherolquinone (1.5 nmol/g) in liver is in agreement with earlier observations (24). In addition, while there now exist reports of the reduction of α-tocopherolquinone to its hydroquinone form in isolated rat hepatocytes and rat liver homogenates (25), this transformation is described here for the first time in rats receiving α-tocopherolquinone by oral ingestion.

In conclusion, the present method allows the simultaneous determination of ubiquinones, ubiquinols, vitamin E homologues, and in particular α-tocopherol and nearly all its oxidation products in animal and plant samples. The time required for a complete analysis is relatively short, even taking into account the recommended preparation of samples immediately before runs. A combined post-column reduction and electrochemical detection system avoids multiple detectors and unstable electrochemical reduction while permitting rapid and economical routine use. Hence this technique should be of value for biochemical studies of the relationships between oxidative stress and lipophilic antioxidants and even for direct evaluation of the antioxidant functions of α-tocopherol in tissues as it allows estimation of its oxidation products.

TABLE 1. Lipophilic antioxidants in liver tissue of control and tocopherolquinone-treated rats

	Control	Treated	*P*
	nmol/g	*nmol/g*	
α-Tocopherol hydroquinone	0.2 ± 0.0	12.7 ± 1.2	<0.001
α-Tocopherol quinone	1.5 ± 0.5	30.9 ± 4.9	<0.001
γ-Tocopherol	1.0 ± 0.3	1.3 ± 0.4	n.s
α-Tocopherol	19.4 ± 4.9	17.5 ± 4.2	n.s
Ubiquinol-9	25.1 ± 7.6	23.6 ± 3.8	n.s
Ubiquinol-10	2.5 ± 1.0	1.9 ± 0.3	n.s
Ubiquinone-9	3.0 ± 1.3	2.1 ± 0.7	n.s
Ubiquinone-10	0.3 ± 0.1	0.3 ± 0.1	n.s

Data are given as mean ± SE (n = 5). Statistical comparisons of the means were carried out using the Student's *t*-test; n.s., not significant.

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