*Journal of Chromatography*, 620 (1993) 268–272
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7069

# Short Communication

# Determination of plasma tocopherols by high-performance liquid chromatography with coulometric detection

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(First received May 7th, 1993; revised manuscript received July 12th, 1993)

ABSTRACT

An HPLC procedure has been developed for tocopherol determination with coulometric detection in human serum samples. Eluent optimization and foreign peak identification (bilirubin) by mass spectrometry are described. An extraction procedure gave yields around 98% with 1.3% coefficient of variation, and the calibration ranged from 0.1 to 200 mg/l with a correlation coefficient of 0.999. The detection limit achieved for vitamin E was 60 pg (3 ng/ml).

## INTRODUCTION

α-Tocopherol (vitamin E), in addition to being an effective antioxidant preventing peroxidation of lipid membranes and having a similar protective action in the tissues [1–3], acts as cancer chemopreventive [4,5].

Tocopherol analysis involves purification and separation by reversed-phase high-performance chromatography followed by UV or fluorimetric detection [6–9] coupled with gradient and isocratic elution [10,11]. An HPLC method coupled with UV detection for α-tocopherol determination in erythrocytes has been proposed [12]. More recently, an HPLC procedure with redox-cycling electrochemical detection allowed detection limits of about 0.05 pmol for tocopherols and their quinones [13]. An HPLC procedure has been developed for α-tocopherol determination by using coulometry detection in human serum samples. Eluent optimization and foreign peak identification (bilirubin) by mass spectrometry (MS) are described.

## EXPERIMENTAL

### *Apparatus and materials*

The HPLC equipment consisted of a Gilson

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0378-4347/93/$06.00 

(Villiers-le-Bel, France) Model 305 pump, a Model 7010 Rheodyne (Cotati, CA, USA) liquid chromatograph injector with a 20-μl sample loop, a Model 401 dilutor and a Model 232 autosampler. An ESA electrochemical detector (Bedford, MA, USA) Model 5100 equipped with a Model 5011 analytical cell and an HP 3393A (Hewlett-Packard, Palo Alto, CA, USA) recorder-integrator were used. The chromatographic column was a 250 × 4 mm LiChrocart Merck (Darmstadt, Germany) reversed-phase column (4.0 μm Superspher 100 RP-18). The mobile phase, after optimization, was 2.5 m*M* $HClO_4$ and 7.5 m*M* $NaClO_4$ in ethanol–methanol (90:10, v/v). Isocratic elutions were performed at a flow-rate of 0.6 ml/min. Prior to use the eluent was filtered through a 0.2-μm membrane filter. A Model RX vortex mixer (VELP Scientifica, Usmate, Italy) was used for sample extractions.

Mass spectrometric experiments were run on a Finnigan-MAT 95 Q (Bremen, Germany) instrument with magnetic, electrostatic and quadrupole analysers mounted in series. Desorption chemical ionization (DCI) [14,15] analyses were executed by loading 1.0 μl of sample or standard solution onto the DCI rhenium filament and allowing it to dry. The filament was subsequently introduced into the ion source through a probe and heated by an electric current at a heating rate of 4000°C/min. Instantaneous vaporization of the sample occurred within an atmosphere of methane (0.5 mbar), acting as the reagent gas under chemical ionization conditions. Negative-ion spectra were recorded. The DCI technique and criteria for selecting proper experimental parameters are discussed in detail elsewhere [16]. The ion source temperature was kept low (50°C); the electron energy was set to 200 eV, the emission current to 0.2 mA and the mass resolution to $m/\Delta m$ = 1200 (10% valley). The magnetic analyser was scanned from $m/z$ 150 to $m/z$ 800 at 0.8 s/decade; ions were detected at the first dynode (20 kV) – electron multiplier detection system.

α-, β-, γ- and δ-Tocopherol were obtained from Sigma (St. Louis, MO, USA). Ethyl acetate, *n*-butanol, $CH_3CN$, propanol, $NaClO_4$ and $HClO_4$ were purchased from Carlo Erba (Milan, Italy), methanol from Riedel-de-Haen (Schering, Selze, Germany) and ethanol from Merck. All reagents were HPLC grade.

Stock solutions 2 g/l of α-tocopherol were prepared in ethanol and δ-tocopherol in $CH_3CN$, the latter immediately diluted in water to 10 mg/l. The reference solutions were stored at −20°C. α-Tocopherol working standard solutions were prepared daily by diluting 1:10 with ethanol, and finally diluted 1:10 by adding to the plasma.

*Sample preparation*

Preliminary analyses of over 100 samples, performed by electrochemical detection, confirmed the total absence of a δ-tocopherol peak but showed the homologues to be electroactive species. δ-Tocopherol has been used as internal standard because of its total absence in human serum [17,18].

Venous blood, transferred into polypropylene tubes (heparin anticoagulant), was centrifuged for 5 min at 3000 *g* (4°C); the clarified plasma was stored at −20°C in plastic tubes. A previously described method [19], using *n*-butanol and ethyl acetate (1:1, v/v), was modified for the extraction procedure; adding $CH_3CN$ to the original mixture gave a better deproteinization. For the analysis 100 μl of δ-tocopherol as internal standard (10 mg/l) were added in a glass tube (100 × 10 mm) to 100 μl of plasma, which was then extracted by adding 1.0 ml of *n*-butanol–ethyl acetate–$CH_3CN$ (1:1:1, v/v/v), vortex mixing for 30 s and centrifuging the organic extract for 5 min at 3000 *g* to remove proteins. A 20-μl portion of this organic phase was injected into the chromatograph.

## RESULTS AND DISCUSSION

The experimental conditions for optimizing the electrochemical oxidation of tocopherols were investigated. The background detector noise was reduced by working at the lower ionic strength consistent with satisfactory separations (see below). The sensitivity was enhanced by working up to +0.6 V, but since this is not a limiting factor the oxidizing potential of +0.35 V for routine determinations was applied in order

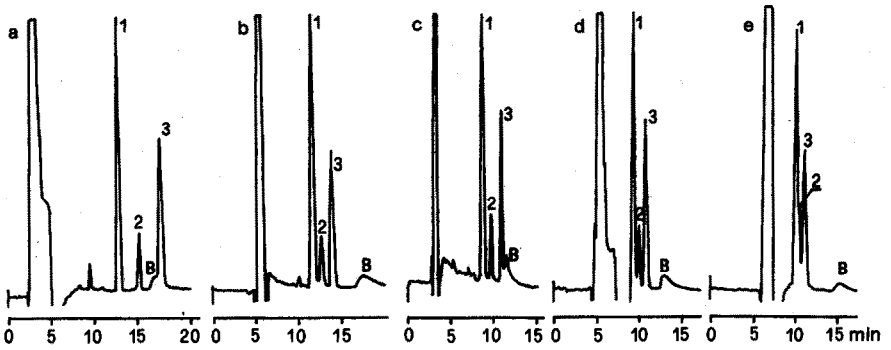


Fig. 1. Retention time of tocopherols and bilirubin as a function of eluent composition: (a) methanol, (b) ethanol, (c) methanol–ethanol (50:50, v/v), (d) methanol–propanol (50:50, v/v), (e) ethanol–propanol (50:50. v/v), 2.5 m*M* $HClO_4$, 7.5 m*M* $NaClO_4$; flow-rate, 0.6 ml/min. Injection volume, 20 μl. Detection: electrochemical oxidation at a potential of +0.35 V. Peaks: 1 = δ-tocopherol; 2 = β- + γ-tocopherol; 3 = α-tocopherol, B = bilirubin.

to reduce background fluctuations and it resulted in clean chromatograms, free from interferences (detection limit of 60 pg of α-tocopherol; signal-to-noise ratio = 3).

*Eluent*

In order to optimize eluent composition pure solvents and mixtures were evaluated at 2.5 m*M* $HClO_4$ and 0, 2.5, 7.5, 17.5, 47.5 and 97.5 m*M* $NaClO_4$. The tested eluents were methanol, ethanol, methanol–ethanol (50:50), methanol–propanol (50:50) and ethanol–propanol (50:50). Fig. 1 compares the chromatograms obtained for the selected eluents containing 3.5 m*M* $HClO_4$ + 7.5 m*M* $NaClO_4$. It can be seen that a small peak (B) eluted close to α-tocopherol, before it with methanol and after it with all other eluents. With methanol the retention time was too high and the unknown peak B was not resolved for all other explored ionic strengths. Ethanol gave good separation of B but the analysis time was too long. Mixtures of methanol or ethanol with propanol gave lower retention times but reduced resolution for the investigated peaks, and longer retention times and poor resolution were obtained by increasing salt concentration above 10 m*M*. Fig. 2 shows the retention time for the tocopherol and the unknown peak as a function of ionic strength ($HClO_4$ + $NaClO_4$) for a methanol–ethanol (50:50, v/v) mixture. Because lower salt concentration did not improve the separation of peak B, and considering the effect of methanol (Fig. 1) on the retention time of the unknown peak, experiments were performed with methanol–ethanol mixtures of different composition. The best results were obtained with an ethanol–methanol mixture (90:10, v/v) at 2.5 m*M* $HClO_4$ and 7.5 m*M* $NaClO_4$ (Fig. 3).

*Sample extraction*

The extraction procedure and analyses were performed on three kinds of sample: plasma, spiked plasma (10 mg/l concentration of ± α-to-

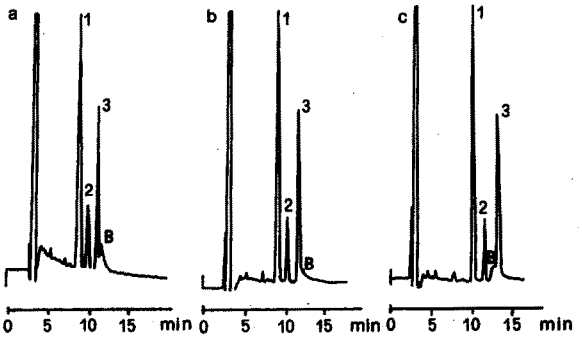


Fig. 2. Retention time of tocopherols and bilirubin as a function of ionic strength for methanol–ethanol (50:50, v/v) eluent at 2.5 m*M* $HClO_4$: (a) 7.5 m*M* $NaClO_4$, (b) 17.5 m*M* $NaClO_4$, (c) 47.5 m*M* $NaClO_4$; flow-rate, 0.6 ml/min. Injection volume, 20 μl. Detection: electrochemical oxidation at a potential of +0.35 V. Peaks: 1 = δ-tocopherol; 2 = β- + γ-tocopherol; 3 = α-tocopherol; B = bilirubin.

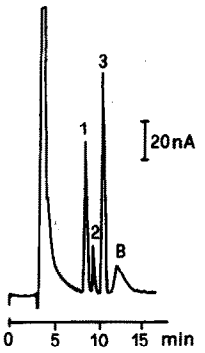


Fig. 3. Separation of tocopherols and bilirubin after eluent optimization. Eluent: methanol–ethanol (10:90, v/v), 2.5 m*M* $HClO_4$, 7.5 m*M* $NaClO_4$; flow-rate, 0.6 ml/min. Detection: electrochemical oxidation at a potential of +0.35 V. Peaks: 1 = $\delta$-tocopherol; 2 = $\beta$- + $\gamma$-tocopherol; 3 = $\alpha$-tocopherol; B = bilirubin.

copherol) and ethanolic standard (10 mg/l). Under these conditions the absolute recovery yield, evaluated by comparison with a direct injection, was 100% (C.V. 1.0) for the standard and 98.5% (C.V. 1.3) for standard additions to serum prior to deproteinization. This means that analyses using a calibration curve give incorrect values. Thus analyses were performed following the standard addition procedure and in all case samples with and without spikes were run in triplicate.

A linear relationship between peak-height ratio ($\alpha$-TP/$\delta$-TP) and the $\alpha$-TP concentration was found for a plasma sample ($y = 1.289 + 0.158\,x$; $r = 0.9995$; $n = 30$ different standard additions; actual concentration 8.51 mg/l; spikes range 0.625–200 mg/l). Consecutive dilutions of the sample ($n = 36$) enabled us to verify the linearity between concentrations of 0.1 and 200 mg/l with a correlation coefficient of 0.999.

*Foreign peak*

A detailed investigation was made in order to identify the nature of compound B. Taking into account the nature of the analysed samples and the behaviour of unknown compounds we suspected the presence of bilirubin and two different approaches were followed in order to verify this assumption. A standard sample of bilirubin was processed in the same way as samples and chromatograms showed a peak at the same retention time as the unknown peak. To confirm this result, fractions of analysed samples were collected at the retention time of the unknown peak. This HPLC fraction was treated with KCl to prevent possible interference by perchlorates and was immediately subjected to DCI–MS analysis in order to determine the molecular ion of the unknown substance.

*Mass spectrum*

Fig. 4 shows the resulting negative-ion mass spectrum of the collected HPLC fractions upon background subtraction. The base peak corresponds to the molecular ion of bilirubin, thus confirming the identity of the foreign substance. The mass spectrum of Fig. 4 was also compared with that obtained from a standard solution of bilirubin (1 mg/l), to which $ClO_4^-$ and then KCl were also added: the two spectra were identical. The positive-ion spectra were also recorded, but the sensitivity for bilirubin was in this case much lower. In contrast, very neat negative-ion spectra could be obtained from as little as 1 ng of substance, provided that the interference of perchlorates was removed. The molecular ion [$M^-$] arises from a process of electron capture, which produces no fragmentation.

*Reproducibility and sample stability*

To evaluate the reproducibility of the proce-

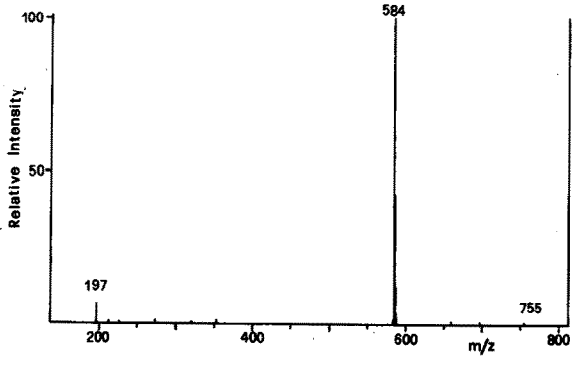


Fig. 4. Negative-ion DCI mass spectrum of HPLC fraction, upon background substraction: molecular ion of bilirubin (MW 584.65).

dure and chromatographic analysis the following experiments were performed.

A sample of plasma was extracted and divided into ten subsamples. The analysis gave a mean value of 11.65 mg/l with S.D. 0.09 mg/l and coefficient of variation (C.V.) of 0.83%.

A second plasma sample was divided into ten subsamples. The subsamples, after extraction, were analysed over a day, every 2 h, and gave 11.63 mg/l concentration with S.D. 0.15 mg/l and C.V. of 1.3%.

The sample stability was evaluated as follows. Samples of the same plasma were processed immediately or frozen and extracted and analysed every week. Concentrations of 9.07 mg/l with S.D. = 0.21 mg/l and values of 2.32% C.V. were obtained over a 2-month period.

The method, applied to 500 samples of human plasma from males and females of various ages receiving no therapy, gave results in good agreement with literature data, 6–20 mg/l. The method is very sensitive as only 100 $\mu$l of plasma are utilized and only 20 $\mu$l of extract are employed for HPLC analysis; sample size is not a limiting factor and a detection limit of 60 pg for vitamin E was achieved.

## ACKNOWLEDGEMENTS

Financial support from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST, Rome) and from the Italian National Research Council (CNR, Rome) is gratefully acknowledged.

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