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Short communication

Determination of α-tocopherolquinone in human serum samples by liquid chromatography with fluorescence detection and on-line post-column derivatization

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Abstract

A HPLC/fluorescence method with on-line post-column derivatization by a photoreactor was developed, where α-, β + γ- and δ-tocopherolquinone (TQ) are separated on a 250 mm × 4.6 mm RP-18 column. The LOD is about 250 pg for all TQs. In combination with a two-step sample preparation procedure, this method was successfully employed for measurement of α-TQ in human serum samples. Recovery for α-TQ from spiked serum was excellent (99 ± 5%) and results of α-TQ determinations in 111 serum samples are reported. Additionally, possibilities for determination of other TQs in serum and alternative derivatization with a zinc reduction column are discussed.

Keywords: Derivatization, LC; Zinc-reduction column; Photoreactor; Vitamin E quinones; Tocopherolquinones

1. Introduction

Tocopherols (Vitamin E) are considered to be nature's most important lipid-soluble, chain-breaking antioxidants, protecting cell membranes from peroxidative damage. In fulfilling their antioxidant functions, the tocopherols themselves are prone to become irreversibly oxidized. Although numerous research papers have discussed oxidation products of tocopherols generated in model systems in vitro, relatively little attention has been paid so far to the occurrence and fate of such metabolites in vivo.

As tocopherolquinones (TQs, see Fig. 1) have been shown to be likely the candidates for important metabolites of tocopherols in vivo [1–5], we decided to develop a sensitive RPLC method for detection of TQs, especially α-TQ, in extracts of human serum samples.

Due to their strong UV-absorption at about 260 nm the TQs are good targets for UV detection, but sensitivity and selectivity are not satisfactorily for detection of low concentrations of the analytes in complex matrices. On the other hand, they show no significant native fluorescence, so direct use of the usually more selective and often more sensitive fluorescence detection is not applicable. Therefore, we evaluated the application of on-line post-column derivatization of TQs, using either a photoreactor (PR) or a zinc reduction column (Zn), in combination with fluorescence (FLU) detection of the generated reaction products.

Additionally, a sample preparation procedure for serum samples was developed, comprising of a liquid–liquid extraction step and subsequent cleaning of the crude lipid extract by chromatography on a silica gel minicolumn. Results of α-TQ measurements in human serum samples are reported and possibilities for determination of other TQs in serum are discussed.

2. Experimental

2.1. Chemicals

α-TQ was from Acros Organics (KMF Laborchemie, Sankt Augustin, Germany). β-, γ- and δ-TQ were synthesized from the tocopherols by oxidation with $FeCl_3$ and 2,2′-bipyridyl [6]. The reaction products were purified by preparative TLC on silica gel with light petroleum (bp,

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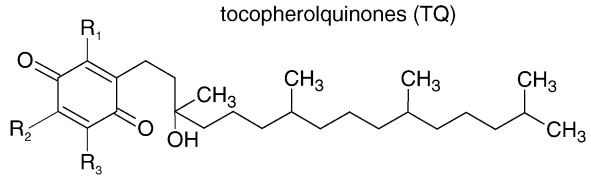

	R_1	R_2	R_3
α-TQ/THQ	CH_3	CH_3	CH_3
β-TQ/THQ	CH_3	H	CH_3
γ-TQ/THQ	H	CH_3	CH_3
δ-TQ/THQ	H	H	CH_3

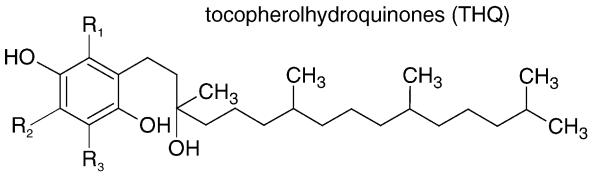

Fig. 1. Structure of tocopherolquinones (TQs) and tocopherolhydroquinones (THQs).

40–60 °C)/diethyl ether (1/1 (v/v)) as eluent. The tocopherolhydroquinones (THQs) were synthesized from the TQs by reduction with $NaBH_4$ [7]. Control of identity and purity was done by GC/MS measurements as described elsewhere [8]. *N*-Trimethylsilyl-imidazole (TSIM) was from Macherey-Nagel, Düren, Germany. HPLC solvents as well as α-, β-, γ- and δ-tocopherols (tocopherol-kit, Art. No. 15496) were from Merck (Darmstadt, Germany).

2.2. HPLC conditions

Measurements were performed with a Shimadzu (Kyoto, Japan) LC-10A*vp* series HPLC system with RF-10A*XL* and SPD-10A*vp* detector (λ_{ex} = 294 nm, λ_{em} = 331 nm for FLU detection, λ = 260 nm for UV detection). Separations were achieved on a LiChrosorb-100 (10 μm) RP-18 column (250 mm × 4.6 mm; Knauer, Berlin, Germany) with integrated precolumn. On-line post-column derivatization was done with either a Knauer photoreactor A1026 (6 m knitted Teflon coil, 0.3 mm i.d., λ_{ex} = 254 nm) or a 20 mm × 4.6 mm zinc reduction column (Art. No. CH980661-ZN; Interchim, Montluçon, France). Eluent was pure methanol for experiments with PR, or methanol containing 10 mM zinc chloride, 5 mM sodium acetate and 5 mM acetic acid (according to Gao and Ackman [9]) for experiments with Zn column. Flow rate was 1 ml/min, and injection volume was 50 μl.

2.3. GC/MS conditions

Measurements were done with the GCQ system (ThermoFinnigan, Egelsbach, Germany) using the following GC conditions: Rtx-5MS column (30 m × 0.25 mm i.d., d.f. = 0.25 μm; Restek, Bad Homburg, Germany); oven: 220 °C for 1 min, 5 °C/min to 285 °C, final temperature 285 °C for 6 min; injection port: 275 °C, splitless injection; transferline: 285 °C; He (4.6) flow: 30 cm/s, constant velocity. MS conditions: MS/MS mode, collision energy 1 V, q = 0.450, precursor ion: m/z 293, product ions: m/z 146–300, source temperature: 170 °C, multiplier: 1250 V, acquisition start-time: 3 min. Samples were measured as TMS derivatives after reaction with TSIM for 30 min at room temperature.

2.4. Sample preparation

Human serum samples (500 μl) were extracted with *n*-hexane after precipitation of proteins with 1 ml methanol (containing 0.25 mg/ml BHT). Extraction was performed in a stoppered amber glass tube by vortex mixing (1 min) with 1 × 3 ml and 1 × 2 ml *n*-hexane and centrifugation at 3000 rpm for 5 min to support phase separation after each extraction step. The combined *n*-hexane extract was purified by chromatography on a silica gel minicolumn, made from a Pasteur pipette filled with silica gel 60 F_{254} (Art. No 10757.1000; Merck) to a filling height of 5 cm and an upper layer of 0.5 cm of dry Na_2SO_4. While the first fraction, eluted with 3.5 ml *n*-hexane/diethyl ether (20 + 3 (v/v)), can be used for determination of tocopherols (α, β, γ, and δ) by NPLC as described elsewhere [10], the TQs eluting in the second fraction with 4 ml *n*-hexane/diethyl ether (3 + 2 (v/v)) were determined by RPLC/FLU (PR) as described above. For measurement, the eluate was carefully evaporated and reconstituted in 200 μl methanol (containing 0.025 mg/ml BHT).

3. Results and discussion

3.1. RPLC/FLU (PR)

With the applied HPLC method, α-, β + γ- and δ-TQ were baseline separated on a 250 mm × 4.6 mm RP-18 column in 10 min. To allow measurement of the TQs with fluorescence detection, on-line post-column derivatization with a photoreactor was evaluated. In this approach, the eluent stream carries the separated substances to a knitted open tubular reactor coil in which a photochemical reaction is initiated by a low-pressure UV-lamp. The TQs proved to be good target compounds for this approach. Fig. 2 shows a comparison of the UV- and fluorescence signal detected with and without use of the photoreactor (PR). Without PR (dotted lines), vir-

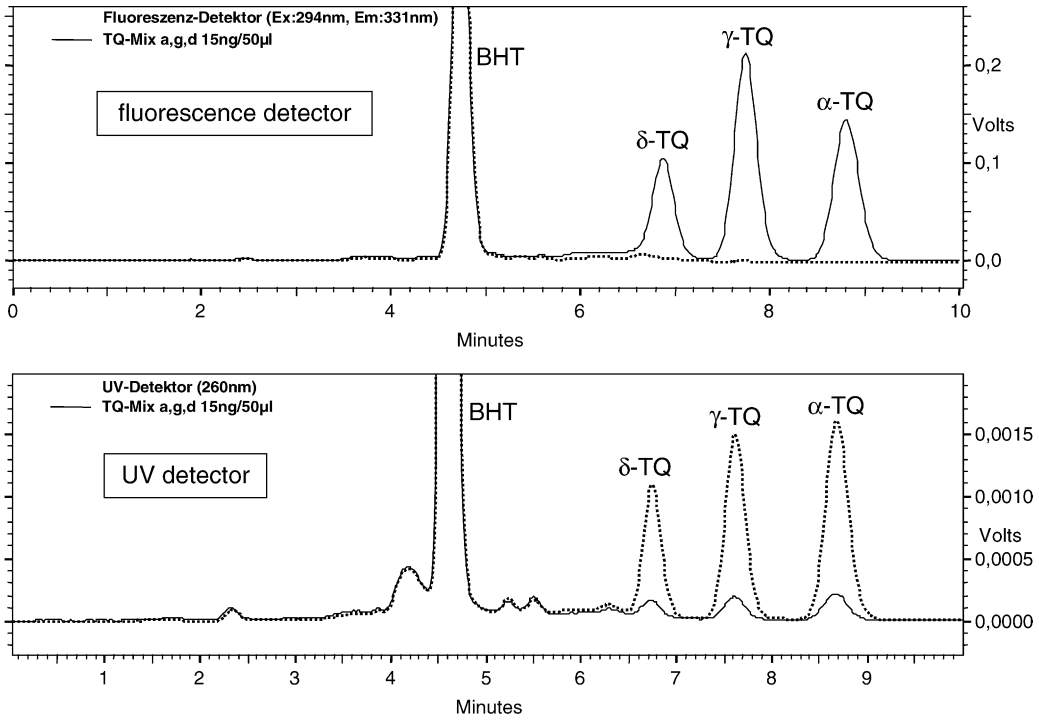

Fig. 2. Comparison of RPLC chromatograms of a TQ-reference mix (α-, γ-, and δ-TQ, 15 ng/50 μl) with FLU and UV detection, with (solid line) or without (dotted line) use of the photoreactor for post-column derivatization.

tually no FLU-signal is detected for the TQs, while a good UV-signal can be seen. On the other hand, with the PR-lamp switched on (solid lines), a strong FLU-signal appears and the UV-signal at 260 nm is heavily reduced. With this method, a limit of detection (LOD, S/N ≥ 3) of about 250 pg (in 50 μl injection volume) is achieved for all TQs and the selectivity of determination is substantially improved in comparison to UV detection.

3.2. RPLC/FLU (Zn)

Alternatively, application of a little zinc reduction column for on-line post-column derivatization was evaluated. A similar approach has already been described by other authors for successful determination of α-TQ in combination with electrochemical detection [11,12]. We employed a 20 mm × 4.6 mm Zn column and FLU detection. The resulting products, most probably the respective THQs (see Fig. 1), showed good detectability with FLU detection. With a fresh Zn column, even lower LODs could be obtained than with the photoreactor method (100 pg compared to 250 pg). However, performance of the Zn column deteriorates rapidly, leading to substantial loss of sensitivity, especially for α-TQ, within short time (after approximately 100 injections). Therefore, we preferred the more stable and reliable RPLC/FLU (PR) method for the serum measurements.

3.3. Sample preparation

For isolation of the TQ analytes from the serum matrix, a sample preparation procedure was developed, which combines a simple liquid–liquid extraction of 500 μl serum with *n*-hexane after precipitation of proteins with methanol and a purification of the crude lipid extract by chromatography on a silica gel minicolumn (see Section 2.4). This purification step ensures cleaner extracts, less matrix interferences and a substantially increased lifetime of the RPLC column, since stronger lipophilic compounds—which would otherwise foul the column within short time—are removed with the void volume and first elution step.

Care has to be taken to prevent artifactual generation of TQs from the mother tocopherols during sample preparation, which could be successfully avoided by addition of BHT as antioxidant to the solvents used in the sample preparation procedure. However, it seems that BHT is not able to protect possibly contained THQs in serum samples from oxidation to the corresponding TQs.

Recovery from spiked serum samples was excellent for α-TQ (99 ± 5%, mean ± S.D.), while β-, γ- and δ-TQ were recovered poorly with 28 ± 4, 63 ± 8% and usually <20%, respectively. These experiments were performed by addition of a mixture of α-, β- (or γ-) and δ-TQ to serum samples and measurement of these samples with and without added standards. Mostly, 20 ng of each compound were given to 0.5 ml serum, but nearly identical results were obtained after addition of 10 or 40 ng of TQ reference substances.

Since recoveries were about 100% for all four TQs when the spiking was done *after* the lipid extraction step (but before the silica gel clean-up), the problems encountered are obviously due to insufficient extraction of the compounds from the serum matrix. Several attempts to optimize the extraction

Table 1
Analytical performance data for α-TQ measurement with the RPLC/FLU (PR) system

Linear calibration range[a]	0.5–50 ng/50 μl (R^2 = 0,9994)
Working calibration range[a]	0.5–30 ng/50 μl (0.5, 5, 10, 20 and 30 ng/50 μl)
LOD[a] (S/N ≥ 3)	250 pg/50 μl
Repeatability of serum measurements	
Intra-day[b] (n = 3–5), R.S.D. (%)	2–6
Inter-day[c] (n = 32), R.S.D. (%)	11

[a] For standard solutions in methanol.
[b] For different serum samples.
[c] For a quality control sample (pool serum, c = 39 ng/ml) over a 4 months period.

(e.g. change of extraction solvents, solvent volumes, addition of saturated NaCl solution to the extraction mixture or use of Extrelut-3 columns (Merck) for lipid extraction) failed to improve the recoveries of β-, γ- and δ-TQ. On the other hand, recovery was near 100% when spiked water was used as model matrix instead of serum.

Therefore, we hypothesize, that the recovery problems observed are due to formation of nucleophilic adducts with serum constituents like glutathion or proteins, which does not occur for α-TQ with it's fully methylated quinone ring, but is favored for the only partially methylated β-, γ- and especially δ-TQs. It has already been shown by Jones et al. [13], that at least γ- and δ-TQ, but not α-TQ, are able to form such adducts.

However, since concentrations of the β-, γ- and δ-tocopherols in European human serum samples are typically quite low compared to the dominating and most bioactive α-tocopherol, it seems reasonable to expect, that concentrations of their oxidation products are also very low and of minor importance compared to α-TQ. Therefore, we did not further focus on their determination, but restricted serum measurements to α-TQ. Table 1 gives an overview of analytical performance data for α-TQ measurement.

3.4. Identity confirmation

The identity of the α-TQ-HPLC peak in serum extracts has been confirmed by GC/MS–MS measurements. For that, the α-TQ fraction from HPLC separations (without PR) of several serum extracts was collected and the pooled α-TQ-HPLC fractions were transferred to the GC/MS system after evaporation of the solvent and silylation with TSIM. The retention time and fragmentation pattern of the Peak derived from the serum extracts showed excellent agreement with authentic α-TQ-TMS standard in GC/MS–MS (Fig. 4).

3.5. Application

The developed method has been employed for determination of α-TQ in human serum. Fig. 3 shows a chromatogram from a serum extract. Generally, for serum extracts, use of the RPLC/FLU (PR) method resulted in significantly reduced matrix interferences compared to RPLC/UV measurement.

Levels of α-TQ in fresh serum samples of healthy volunteers (n = 14) with no intake of Vitamin E supplements were 41 ± 8 ng/ml (mean ± S.D., range: 30–61 ng/ml) and reached 0.33 ± 0.14% (range: 0.13–0.62%) of the α-tocopherol concentrations in these samples. In contrast, α-TQ levels measured in a subgroup (n = 97) of serum samples from the German National Health Survey (representative sample of German inhabitant population, see [14] for description) were 148 ± 114 ng/ml (range: 62–857 ng/ml), while α-TQ/α-tocopherol ratios were 0.74 ± 0.41% (range: 0.17–2.34%). As these samples were stored already for longer periods at a temperature of −40 °C without addition of antioxidants, it cannot be fully excluded, that the higher α-TQ levels

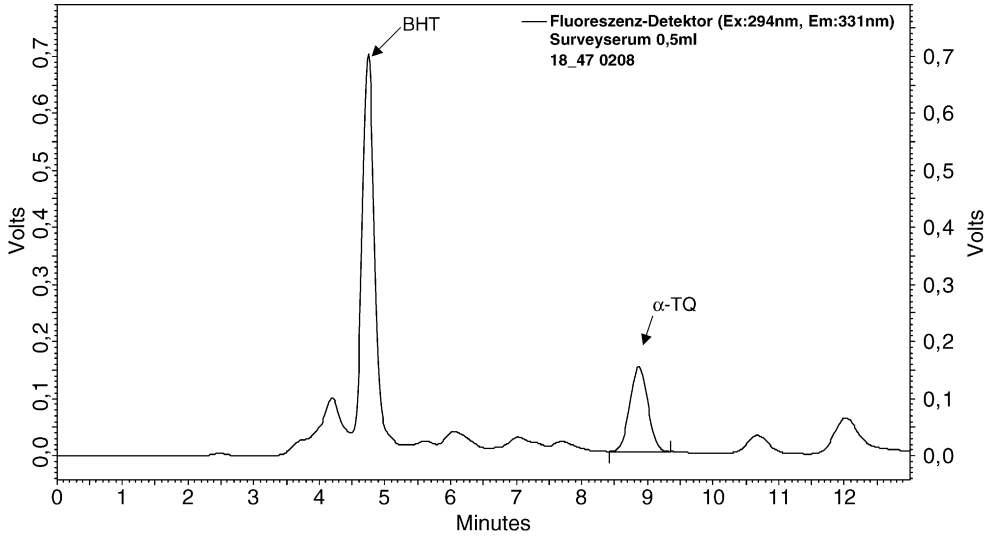

Fig. 3. RPLC/FLU (PR) chromatogram of a human serum extract.

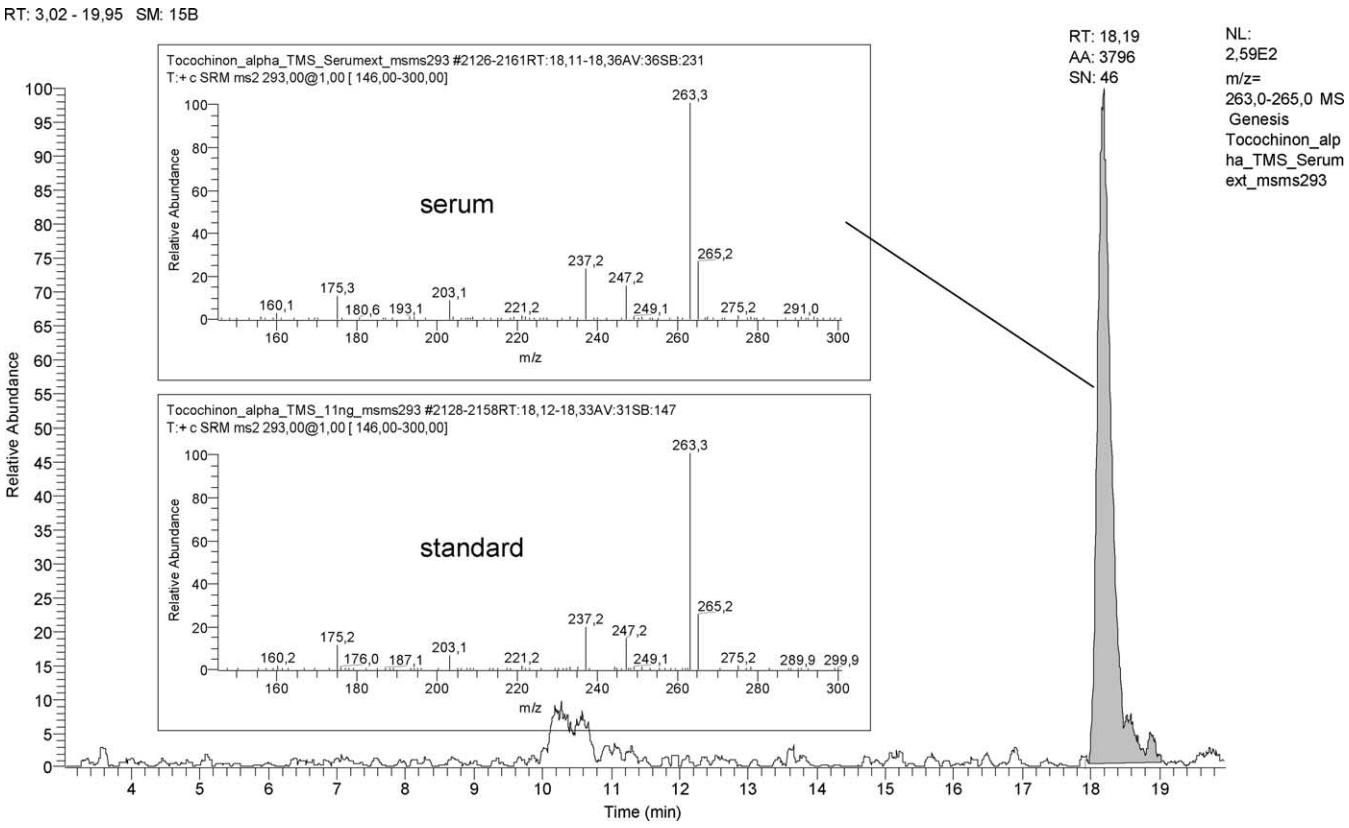

Fig. 4. GC/MS–MS measurement of the collected α-TQ-HPLC peak from pooled serum extracts; daughter ion scan of *m/z* 293 from the peak at 18.19 min in the silylated serum extract (upper MS spectrum) showed excellent agreement of retention and fragmentation with authentic α-TQ-TMS standard (lower MS spectrum).

observed are at least partly an effect of α-tocopherol oxidation during storage. On the other hand, storage experiments with fresh serum over a period of 6 months demonstrated good stability of α-TQ levels under these conditions. Therefore, it is likely, that the broader range of α-TQ values in the survey samples mainly reflects the higher variation in health status and serum tocopherol levels of the survey participants. The study group included 32 persons, which had reported intake of tocopherol supplements within the last seven days before blood sampling and a control group of 65 persons who had not taken any medication. The α-TQ levels and α-TQ/α-tocopherol ratios measured for both groups were not significantly different (data not shown). Age was between 18 and 75 years, 48 persons were female and 49 were male. To our best knowledge, this is the largest and most heterogeneous study group for α-TQ determinations reported in literature so far and the α-TQ and α-TQ/α-tocopherol values measured are in good agreement with data given by other authors for human serum or plasma [15–17].

4. Conclusions

A sensitive and reliable RPLC method with fluorescence detection and on-line post-column derivatization by a photoreactor has been developed for determination of tocopherol oxidation products, the α-, β + γ- and δ-tocopherolquinones. In combination with a two-step sample preparation procedure, this method has been successfully employed for measurement of α-TQ in human serum.

However, detection of tocopherolquinones is not only of interest in serum samples, but also in other biological fluids and tissues as well as in samples like pharmaceutical Vitamin E preparations, plant oils and food or technical products stabilized with tocopherols, allowing either insight into metabolic processes for the biological samples or estimation of oxidative degradation processes for the other matrices.

Therefore, after suitable adaptation of the sample preparation procedure, the described method for determination of tocopherolquinones offers a wide range of further application areas.

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