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Determination of α-tocopherol in different biological samples by GC-MS without derivatization

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A method has been developed to determine the levels of α-tocopherol in human plasma and colon tissue using gas chromatography with mass spectrometric detection. The samples were acquired from 27 patients with Crohn's disease. Samples were deproteinized and α-tocopherol was obtained using a two-step extraction and measured by GC-MS without prior derivatization. The analytical parameters of the method were satisfactory: the intra-day and inter-day coefficients of variation were below 7% and quantitative recoveries range between 92.2-100.5%. The method shows an excellent linear range from 0.6-64.3 μmol/L. The median measured α-tocopherol concentration in human plasma was 21.6 μmol/L and in healthy colon tissue and tissue affected by Crohn's disease was 26.5 nmol/mg and 34.9 nmol/mg, respectively. We did not find any statistically significant correlation or differences between the α-tocopherol content in plasma and tissue. Overall, our method has potential to be easily implemented in special studies for monitoring α-tocopherol as a biomarker of oxidative stress.

Keywords: α-Tocopherol; Crohn's disease; Colon tissue; GC-MS

Introduction

Vitamin E is an antioxidant soluble in lipids and can be divided into two groups, tocopherols and tocotrienols, when each group includes four isoforms (α, β, γ, and δ). Tocopherols, the major form of vitamin E, contain the chromanol ring system and phytyl saturated chain of 16 carbons, whereas tocotrienols have a side chain with three double bonds. Based on the number and positions of methyl groups on the

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ring, tocopherols exist as α-tocopherol (three methyl groups), β- and γ-tocopherol (two methyl groups), and δ-tocopherol (one methyl group) [1]. Naturally occurring tocopherols have the RRR absolute configuration of stereoisomers. All stereoisomers have the same antioxidant effect, but only RRR-α-tocopherol exhibits a high biological activity [2].

Vitamin E belongs among the most important antioxidants protecting the lipid environment against oxidative damage. Its function is to prevent the chain propagation of lipid peroxidation caused by peroxyl radicals. The relative reactivity of tocopherols toward the oxygen radicals decreases with the number of methyl groups in their molecule in the order of $\alpha > \beta = \gamma > \delta$ [3]. Vitamin E can be regenerated by reactions with a reducing agent, such as ascorbate, ubiquinol, or polyphenol [4]. α-Tocopherol is important for the normal morphology of erythrocytes and inhibits platelet aggregation and the oxidation of LDL particles in plasma [5–7]. Due to its ability to eliminate reactive oxygen species (ROS), α-tocopherol plays an important role during chronic inflammation when sustained production of ROS occurred, especially in patients with Crohn's disease [8]. It has been reported that patients with Crohn's disease are at higher risk of oxidative stress from lipid peroxidation and altered antioxidant defences because of a decrease in antioxidant micronutrients contained in the intestinal mucosa and in plasma [9].

Numerous methods have been described for the analysis of α-tocopherol in various biological matrices. In some studies, saponification is used for the removal of neutral lipids from the matrix [10]. However, this step may lead to an α-tocopherol loss. Several gas chromatography (GC) methods coupled with flame ionization or mass spectrometric (MS) detection have been developed [11–14]. In GC-MS analysis, derivatization is predominantly performed to increase the stability of tocopherols. Commonly used derivatization techniques are silylation or acylation [15]. In high-performance liquid chromatography (HPLC), separation of tocopherols is mostly performed in the reversed mode using C_{18}, $C_{30,}$ or pentafluorophenyl stationary phase. For detection of α-tocopherol, fluorometric, electrochemical, or mass spectrometer with ESI or APCI ionization technique are normally used [16–19].

The aim of the study was to develop reliable and sensitive GC-MS method for the determination of α-tocopherol in human plasma and colon tissue.

Materials and method

Chemicals and reagents

The butylated hydroxytoluene (BHT), α-tocopherol (α-TOC) and internal standard (±)-α-tocopherol-D_6 (α-TOC-D_6) were from Sigma Chemical Company (St. Louis, MO, USA). HPLC gradient-grade ethanol and *n*-hexane were purchased from Merck (Darmstadt, Germany).

Instrumentation

Chromatographic analyses were performed with a gas chromatograph coupled to a GCMS-QP2010 Ultra Gas Chromatograph Mass Spectrometer from Shimadzu (Kyoto, Japan). Data were collected and processed with GCMS solution software (Shimadzu, Kyoto, Japan). Spectrophotometric measurements were carried out on a Shimadzu UV-1800 PharmaSpec spectrophotometer. Tissue homogenization was performed with a Precellys 24 Homogenizer (Bertin technologies SAS, Montigny-le-Bretonneux, France).

Preparation of the standards

The stock solution of α-tocopherol (≈ 0.5 g/L; 1224 μmol/L) was prepared in ethanol. The internal standard was made by dissolving 2 mg of α-tocopherol-D_6 in 100 mL of ethanol. Stock solutions were stored at –80 °C for a maximum of 3 months. Work solutions of α-tocopherol and internal standard were prepared fresh daily, and their concentrations verified spectrophotometrically using molar absorbance concentration of α-tocopherol = A/3265 (mol) at 292 nm [20]. Calibration solutions were prepared from the stock solution of α-tocopherol diluted with ethanol. The concentrations of the working standards made in the respective sets were in the range from 0.6 to 64.3 μmol/L.

Patients and samples

Samples of peripheral venous blood and colon tissue were obtained from a group of patients with Crohn's disease (n = 27, 14 women with the median age of 34.0 years and 13 men with the median age of 35.5 years). The blood was collected in plastic tubes with K_3EDTA (Vacuette Detection Tube, No. 455036, Greiner Labortechnik Co., Kremsmüster, Austria). The plasma was separated from the blood cells by centrifugation (1700 × g, 15 min, 8 °C), immediately transferred into a well-capped 1.5 mL polypropylene (PP) tube containing BHT (about 500 μL of BHT, 1 g/L, in n-hexane, was pipetted into the tube, followed by evaporation of organic solvent to dryness under a stream of nitrogen at room temperature), and stored at −80 °C. A total of two colon tissue samples were obtained from each patient; one sample of pathological tissue being affected by Crohn's disease and the other of healthy tissue. A thin slice was cut from frozen tissue (weighing between 50 and 100 mg), placed in a Precellys® CK-14 homogenization tube (Bertin Corp., Rockville, MD, USA), and deionized water added (as 1.25 mL). After incubation (5 min at −20 °C), the tubes were placed into a homogenizer (Precellys® 24) and the tissue homogenized in two cycles for 20 s at speed 6500 r.p.m. with 30 s pause between homogenization cycles. The homogenate was transferred into a well-capped

1.5 mL PP tube with BHT and stored at −80 °C. Written informed consent was obtained from all the participants before starting the study, when the Hospital Committee on Human Research of University Hospital in Hradec Kralove approved the study (No. 201706 S12P) being carried out according to the Declaration of Helsinki.

Sample preparation

Isotopically labelled α-tocopherol-D_6 was used as an internal standard. An optimized procedure for sample preparation was used according to Kanďár et al. [17]. For analysis, 50 µL of the sample (plasma or tissue) or of the standard was pipetted into a well-capped 1.5 mL PP tube, 200 µL of ethanol containing internal standard (≈ 4.5 µmol/L) and BHT (1 g/L) were added and the content vigorously mixed on a vortex. To enhance deproteination, the mixture was incubated for 5 min. at −20 °C and then, 200 µL of deionized water and 500 µL of *n*-hexane were added. So treated mixture was vortexed for 5 min and centrifuged (1970 × g, 5 min), followed by transferring the upper organic layer into a glass tube. The hexane extraction was repeated, and the combined extracts evaporated to dryness, again under a stream of nitrogen at room temperature. The dried residue was re-suspended in 100 µL of acetonitrile, vortexed for a few seconds and then transferred into a cramped amber vial with a glass insert.

For recovery experiments, 10 µL of α-tocopherol at different concentrations was added to 90 µl of plasma or tissue. The next steps were the same as those for sample preparation.

Chromatographic method

The GC separation of α-tocopherol was performed on an HP-5 capillary column (30 m length, 0.32 mm internal diameter, 0.25 µm film thickness) coated with 5% phenyl and 95% methylpolysiloxane (Agilent® J&W; Folsom, CA, USA). The temperature of the injector was adjusted to 300 °C and the inlet split set to a ratio of 10:1. A programmed temperature ramp was applied according to the following sequences: the initial temperature at 200 °C for 1 min, then increased to 300 °C at 20 °C/min and held for 5 min. Helium was used as a carrier gas with a flow rate of 1.39 mL/min. The time of analysis was 11 min, and the injection volume of the sample was 1 µL. The quadrupole mass spectrometer with ionization by electron impact (70 eV) was used for the detection when the GC-MS interface temperature was set at 280 °C. The MS worked in the SIM mode monitoring the characteristic ions *m/z* 430 for α-TOC and 436 for α-TOC-D_6.

Results and discussion

The conventional method for the determination of α-tocopherol by GC-MS in different matrixes utilizes its derivatization to silyl derivative [11]. The reason for this is the increasing volatility of α-tocopherol, which is important for gas chromatography. Trimethylsilylation also increases the sensitivity of the method and is compatible with MS detection. The most commonly used derivatizing agents are *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) or MSTFA with trimethylchlorosilane (TMCS) in pyridine [15]. Moreover, the saponification step is performed prior to analysis. Saponification is used for the removal of neutral lipids from complex matrixes, such as breast milk or platelets. Such a process takes place in the presence of potassium hydroxide and at elevated temperature; usually, in the range of 60–80 °C [1,10]. However, these procedures are time-consuming and may lead to possible losses of analytes. Our GC-MS method for determination of α-tocopherol without saponification and derivatization steps is sensitive and reliable, showing good precision and recovery.

For the GC-MS method, the temperature gradient applied to the analytical column was optimized to achieve the shortest analysis time with the highest sensitivity. The best results were obtained for the conditions described in the section "Chromatographic methods". The results of precision and recovery of α-tocopherol GC analysis in plasma and tissue samples are shown in Table 1.

Table 1 Precision and recovery of α-tocopherol in plasma and tissue samples

Parameter	Plasma	Tissue
Intra-day precision [μmol/L]	21.5 ± 0.6	2.9 ± 0.1
CV [%]	3.0	3.4
Inter-day precision [μmol/L]	23.5 ± 0.5	2.7 ± 0.2
CV [%]	2.1	6.2
Recovery [%]	97.1–100.5	92.2–96.1

CV, coefficient of variance

To determine the intra-day precision, the pooled sample of plasma or tissue was analysed ten times in the same day under the same conditions. The inter-day precision was evaluated by making the analysis in three replicates of pooled plasma and tissue samples within six days. The coefficients of variation were below 7%. The spike recoveries ranged between 97.1–100.5% for the plasma and 92.2–96.1% for the tissue sample. The standards for the calibration curve were prepared in a neat matrix because of natural occurrence of α-tocopherol in human plasma. An eight-point calibration curve was constructed by plotting the ratio of the peak area of the analyte to that of deuterium-labelled IS against analyte

concentration. Calibration was performed every day before measuring samples and the respective curve was linear over the whole tested range of 0.6–64.3 μmol/L. The lowest concentration of α-tocopherol that could be quantified with acceptable accuracy and precision was 0.26 μmol/L. The limit of detection calculated from calibration curves as $3.3*S_a/b$ (S_a = standard deviation of intercept; b = mean slope of calibration curve) resulted in 0.08 μmol/L.

The newly developed GC-MS method was used for the measurements of α-tocopherol in 27 samples of plasma and in 54 tissue samples from patients with Crohn's disease. The samples were prepared and analysed according to the protocol, which is given in the Experimental section. Data were expressed as median with interquartile range (IQR – the difference between the upper quartile and the lower quartile), which can be seen in Table 2.

Table 2 Concentration of α-tocopherol in plasma and tissue

α-tocopherol	Median (IQR)
Concentration in plasma [μmol/L]; n = 27	21.6 (9.9)
Concentration in tissue [nmol/mg]; n = 54	29.4 (42.1)
Concentration in healthy tissue [nmol/mg]; n = 27	26.5 (36.2)
Concentration in pathological tissue [nmol/mg]; n = 27	34.9 (48.7)

IQR, interquartile range

The median concentration of α-tocopherol in plasma was 21.6 μmol/L (IQR = 9.9, n = 27). The obtained data are in the reference value range and are similar to previously published concentrations in plasma samples [2,19]. The concentration of α-tocopherol in tissue determined by GC-MS was 29.4 nmol/mg (IQR = 42.1, n = 54). The relation between plasma and tissue α-tocopherol concentrations was examined by correlation analysis. A p-value <0.05 was considered statistically significant. In Fig. 1 and 2 the Scatter plots are presented, confirming that there is no significant correlation between the α-tocopherol concentration in colon tissue and plasma. Also, the difference in α-tocopherol concentration between the healthy colon tissue and tissue affected by Crohn's disease was analysed by the Mann-Whitney U test. In this case, we did not find any statistically significant difference (p-value = 0.2913) between healthy and pathological tissues.

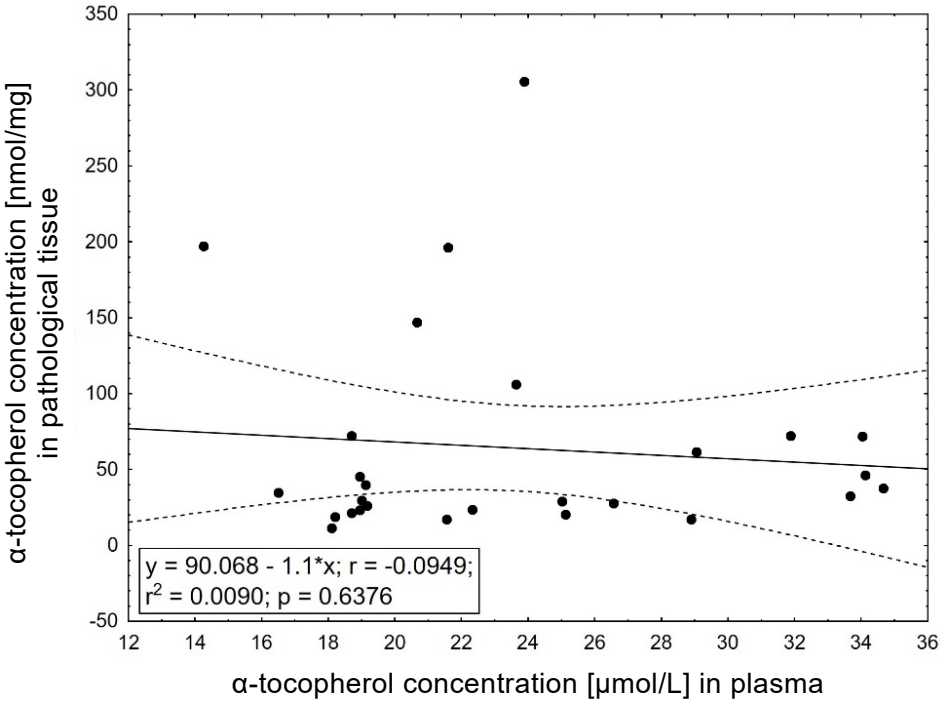

Fig. 1 Scatter plot showing correlation of α-tocopherol concentration in plasma and pathological tissue

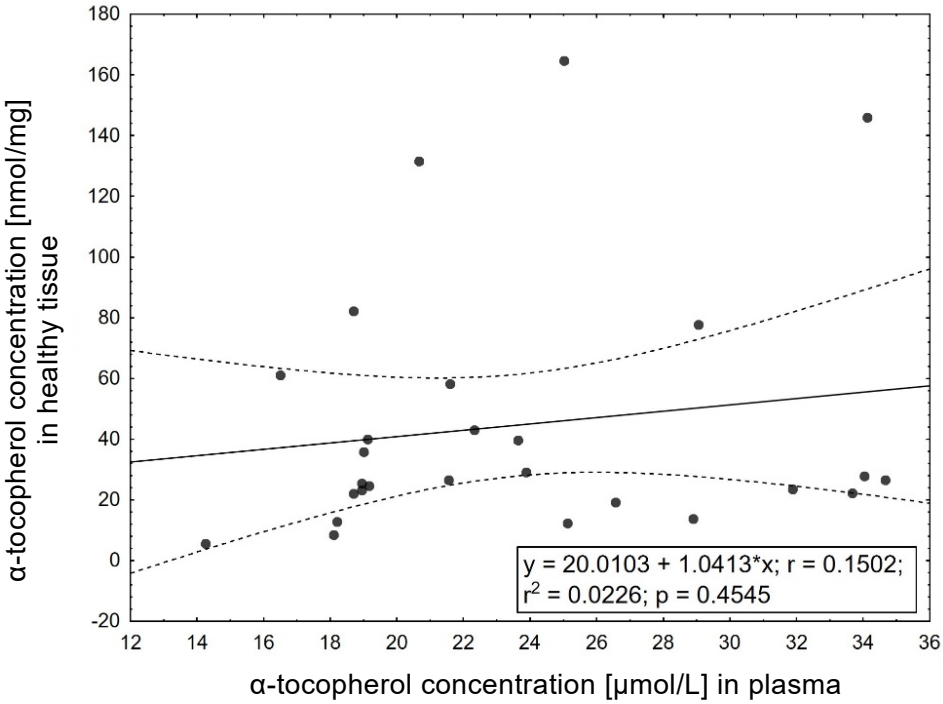

Fig. 2 Scatter plot showing correlation of α-tocopherol concentration in plasma and healthy tissue

Conclusions

A GC-MS method for the determination of α-tocopherol levels in human plasma and tissue has been developed and experimentally proved as reliable and sufficiently sensitive. Due to its simplicity, low cost of analysis, and no need of derivatization, the method is suitable e.g. for studies allowing one to monitor α-tocopherol as a biomarker of oxidative stress.

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