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Original Article

Methods for efficient analysis of tocopherols, tocotrienols and their metabolites in animal samples with HPLC-EC

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ABSTRACT

Tocopherols and tocotrienols, collectively known as vitamin E, have received a great deal of attention because of their interesting biological activities. In the present study, we reexamined and improved previous methods of sample preparation and the conditions of high-performance liquid chromatography for more accurate quantification of tocopherols, tocotrienols and their major chain-degradation metabolites. For the analysis of serum tocopherols/tocotrienols, we reconfirmed our method of mixing serum with ethanol followed by hexane extraction. For the analysis of tissue samples, we improved our methods by extracting tocopherols/tocotrienols directly from tissue homogenate with hexane. For the analysis of total amounts (conjugated and unconjugated forms) of side-chain degradation metabolites, the samples need to be deconjugated by incubating with β-glucuronidase and sulfatase; serum samples can be directly used for the incubation, whereas for tissue homogenates a pre-deproteination step is needed. The present methods are sensitive, convenient and are suitable for the determination of different forms of vitamin E and their metabolites in animal and human studies. Results from the analysis of serum, liver, kidney, lung and urine samples from mice that had been treated with mixtures of tocotrienols and tocopherols are presented as examples.

Abbreviations: α-T, β-T, γ-T and δ-T, α-, β-, γ- and δ-tocopherol, respectively; CEHC, carboxyethyl hydroxychroman; CMBHC, carboxymethylbutyl hydroxychroman; CMHHC, carboxymethylhexyl hydroxychroman; CDMOHC, carboxydimethyloctyl hydroxychroman; -CEHenHC, -carboxymethylhexenyl hydroxychroman; CDMDHC, carboxydimethyldecyl hydroxychroman; HTMTdHC, hydroxytrimethyltridecyl hydroxychroman; CTMDodHC, carboxytrimethyldodecyl hydroxychroman; HPLC, high-performance liquid chromatography; CEAS, Coulochem Electrode Array System; MS, mass spectroscopy.

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1. Introduction

Tocopherols and tocotrienols are synthesized by plants to serve as free radical scavengers (i.e., chain-breaking antioxidants) and are an important group of dietary antioxidants, known as vitamin E [1]. These two groups of compounds have the common structural feature of containing a chromanol ring and a hydrophobic side-chain of 16 carbons. Depending on the pattern of methylation of the chromanol ring, these compounds exist as α-, β-, γ- and δ-tocopherols (α-, β-, γ- and δ-T) each with a saturated side chain, or as α-, β-, γ- and δ-tocotrienols (α-, β-, γ- and δ-T3) each with a side chain containing three double bonds in the side chain. Tocopherols are by far the most important vitamin E forms in terms of abundance in the human diet, tissue levels and antioxidant activities. α-T is the major form of vitamin E found in blood and tissues and is commonly considered as "the vitamin E", even though γ-T is the most abundant tocopherol form found in the major sources of tocopherols in our diet – oils from corn, soybean, canola and other vegetables and nuts [1,2].

All vitamin E forms are absorbed together with other types of lipids as chylomicrons into the lymphatic system and then taken up by the liver. In the liver, α-T is preferably transferred via α-tocopherol transfer protein (α-TTP) to very low density lipoproteins, which enters into blood and then nonhepatic tissues. γ-T and δ-T are not efficiently transported to blood because α-TTP has a low affinity for γ-T and an even lower affinity for δ-T. Tocotrienols, especially γ-T3 and δ-T3 are also not efficiently transferred from the liver to the blood. As a consequence, most of the absorbed γ-T, δ-T, tocotrienols and some of α-T are metabolized via a side-chain degradation pathway. The degradation is initiated with ω-oxidation followed by cycles of β-oxidation of the side chain, to generate a series of carboxychromanol metabolites, such as the terminal metabolites carboxyethyl hydroxychromons (CEHCs) and their precursors, carboxymethylbutyl hydroxychromons (CMBHCs) [1–7]. Using γ-T and γ-T3 as examples, the structures of these compounds and their common metabolic pathways are shown in Fig. 1. Although tocotrienols have three double bonds in the side chain, they can be metabolized to produce the same common short-chain metabolites as tocopherols. The conversion to CMBHC from its precursor, γ-carboxymethylhexenyl hydroxychroman (CMHenHC), has been shown to be catalyzed by the auxiliary enzymes 2,4-dienoyl-CoA reductase and 3,2-enoyl-CoA isomerase [8].

Metabolites of tocopherols and tocotrienols can undergo glucuronidation and sulfation; both free and conjugated forms have been found in tissues and body fluids [6–13]. For example, the sulfate- and glucuronide-conjugates of γ- and δ-tocopherol side-chain degradation metabolites were found at high concentrations in the plasma and tissues of rats fed diet containing γ-T or γ-T3 [6,9]. It has been suggested that the urinary excretion of tocopherol side-chain degradation metabolites in individuals can be an indicator of adequate or excess tocopherol intake [14]. Recent results have demonstrated the anti-inflammatory activities of long-chain metabolites of γ-T [2]. Blood and tissue levels of different forms of vitamin E and their metabolites are essential data for understanding the biological fates and activities of these compounds. This is especially true for the γ- and δ-forms of tocopherols and tocotrienols, which have recently received a great deal of attention for their beneficial health effects [15–24]. Therefore, it is important to establish sensitive and convenient methods for quantifying different forms of tocopherols, tocotrienols and their metabolites in tissues and body fluids of animals and humans.

Previously, we used a sensitive high performance liquid chromatography (HPLC) method, coupled with a Coulochem Electrode Array System (CEAS) or mass spectrometry (MS), to measure different forms of vitamin E and their metabolites in mouse and human samples and identified 18 tocopherol-derived and 24 tocotrienol-derived side-chain degradation metabolites in mouse fecal samples [25]. Short-chain degradation metabolites, such as γ- and δ-forms of CEHCs and CMBHCs were measured in urine, serum and liver samples after mild enzyme hydrolysis of the conjugated metabolites with glucuronidase and sulfatase [20,22,25]. The reported extraction method and HPLC conditions have the advantage of detecting multiple compounds simultaneously. However, the different polarities of tocopherols/tocotrienols and their metabolites of varied chain lengths resulted in different extraction efficiencies and affected the accuracy of quantification. The different methods of sample preparation before the hydrolysis of the conjugated metabolites also affected the results.

In this study, we examined the different experimental conditions for sample preparation as well as the conditions for HPLC analysis and improved the methods. The improved methods for the analysis of the α-, γ- and δ-forms of tocotrienols, tocopherols and their metabolites are reported herein.

2. Materials and methods

2.1. Materials

All tocopherols were purified to >99.5% purity using a flash chromatography (Teledyne Technologies, CA, USA) from commercial sources: α-T and δ-T from Sigma–Aldrich Co. (St. Louis, MO, USA) and a γ-T-rich mixture of tocopherols (γ-TmT) from Cognis Co. (Kankakee, IL, USA). Standards of α-, γ-, δ-CEHCs and α-CMBHC of >95.0% purity were purchased from Cayman Co. (Ann Arbor, MI, USA). α-, γ- and δ-tocotrienol standards of >95.0% purity were generously provided by Davos Life Science (Synapse, Singapore). Lithium acetate, acetic acid, trifluoroacetic acid and sulfatase (S9626, containing 30 U glucuronidase activity per unit of sulfatase) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Acetonitrile, hexane, ethyl acetate, ethanol and methanol were purchased from VWR (Philadelphia, PA, USA). All aqueous solutions were made with nanopure water. Mobile phases were filtered through 0.22 μm nylon membrane and degassed under vacuum prior to use.

2.2. Animal and dietary treatment

All animal experiments were conducted under the protocol no. 02-027 approved by Rutgers University Institutional

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