Quantitative Analysis of the Accumulation of Marine-derived Tocopherol in the Tissue of Mice Fed with Salmon Roe Oil Using HPLC-fluorescence

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Abstract: In this study, we measured the quantity of marine-derived tocopherol (MDT), a monounsaturated vitamin E (VE), stored in the body tissue of mice fed with a diet containing a VE-rich fraction extracted from salmon roe. We first prepared the calibration curves for the MDT concentration using an HPLC-fluorescence system. Ranging from 0.016 to 50 μg/mL, the slope was expressed as first-order equations, with R² values = 0.99. The mice were fed with an AIN-93 based diet containing MDT in doses of 21.4 mg/kg for 4 weeks, and the storage in the heart, lung, liver, stomach, small intestine, large intestine, kidney, pancreas, spleen, testis, skeletal muscle, visceral white adipose tissue (WAT), subcutaneous WAT and brain was quantified. MDT was widely distributed in tissues throughout the whole body, with higher accumulations observed in the adipose tissue, liver and kidney. These results demonstrate means to estimating the MDT concentration in natural products and in the bodies of animals and contribute to the understanding of the physiological functions of MDT in relation to human health.

Key words: α-tocomonoenol, HPLC-fluorescence, marine-derived tocopherol, salmon roe, tissue accumulation, vitamin E

1 INTRODUCTION

Vitamin E (VE) is an essential antioxidant nutrient occurring widely in nature; for example, photosynthetic organisms such as plants and algae synthesize VE. VE prevents the oxidation of lipids and helps to protect the body against reactive oxygen species generated in the body. Owing to its antioxidant properties, VE is world widely used as a food additive and a nutrient supplement and for the treatment of diseases. The VE family consists of eight compounds: α-, β-, γ- and δ-tocopherol and α-, β-, γ- and δ-tocotrienol. α-Tocopherol is predominantly found in natural products and has the highest biological activity of the VE family.

Over the past few decades, VE – found in plant oil and seeds, yet relatively uncommon, has been determined to be a biosynthetic intermediate in the process of reduction to tocopherol from tocotrienol. In 1999 marine-derived tocopherol (MDT) was found in salmon roe and identified as having an unusual methylene unsaturation at the terminus of the phytyl side chain. Following this first report, marine organisms and phytoplankton were also found to contain MDT. Interestingly, the study by Yamamoto et al. showed that the inhibitory effect of MDT on the peroxidation of cholesterol-containing phosphatidylcholine liposomes was stronger than α-tocopherol at 0°C. This activity is considered to be due to a greater rate of diffusion within such viscous lipids, based on the double bond in their side chains. These results suggest that the physical and structural properties of MDT may exert unique and possibly beneficial effects within the human body. Therefore, it is important to understand how MDT is distributed and how it accumulates in the body.

VE homologues have different biological availabilities with α-tocopherol being the predominant form found in natural products and has the highest biological activity of the VE family.

Abbreviations: α-TTP, α-tocopherol transfer protein; FL, fluorescence detector; HPLC, high-performance liquid chromatography; MDT, Marine-derived tocopherol; UV, ultraviolet detector; VE, vitamin E; WAT, white adipose tissue

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tissue due to the presence of the α-tocopherol transfer protein (α-TTP). The α-TTP selectively binds to α-tocopherol in the liver and delivers it to the peripheral tissues through the circulatory system. Of the VE homologues, therefore, the concentration of α-tocopherol is considerably higher than that of the others in the blood and tissues.\(^{8,9}\) On the other hand, although the presence of MDT has been confirmed in human plasma (likely derived from marine foods), the metabolic properties of MDT are still largely unknown.

One study reported an investigation into the accumulation of dietary MDT using mice fed with a VE-concentrated fraction derived from tuna oil\(^{10}\). In this study, the researchers found that MDT has a relatively higher bioavailability in the liver and brain than the other homologues with the exception of α-tocopherol. To obtain more information on the distribution of MDT throughout the body, the present study focused on investigating the storage of MDT in the body tissue of mice. Furthermore, an improvement in the method that we have developed to quantify the accumulation of MDT in the tissue of mice fed with the VE-rich fraction obtained from salmon roe oil.

2 EXPERIMENTAL

2.1 Materials

Salmon roe was purchased from Marutatsu Niwa Foods Co., Ltd. (Hakodate, Japan). Vitamin E was obtained from Mitsubishi-chemical Foods Corporation (Tokyo, Japan). All other chemicals used for lipid extraction, purification and analysis were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2 Preparation of VE-rich fraction from salmon roe and purification of MDT

The salmon roe was homogenized in an electric mixer and the lipid components of the salmon roe were extracted using chloroform-methanol (2:1, v/v). After removing the residue by filtration, the solvent was mixed with a double volume of 0.88% KCl solution to prevent the formation of an intermediate phase. The chloroform phase was collected and concentrated under vacuum using a rotary evaporator. The crude salmon roe oil was then separated using silica gel column chromatography, with hexane-2-propanol (90:10, v/v) as the eluent, and a VE-rich fraction containing triacylglycerol, α-tocopherol and MDT was collected.

To purify MDT, column chromatography was used: the VE-rich fraction was applied to the packed column and eluted with a hexane-ethyl acetate (95:5, v/v) mixture to obtain an intermediate phase. The chloroform phase was collected and concentrated under vacuum using a rotary evaporator. The purity of the MDT (>99%) was confirmed by HPLC analysis.

2.3 Preparation of calibration curves for MDT

A calibration curve for the MDT was obtained using the HPLC-UV chromatogram peak area. The HPLC system consisted of a pump (LC-10AD, Shimadzu Corporation, Kyoto, Japan), a triacetyl silyl (C30) column (Inertsil\(®\) 250 \(\times\) 4.6 mm, i.d. 5 \(\mu\)m, GL Sciences Inc., Tokyo, Japan) and a UV detector (SPD-10A, Shimadzu) set to an absorption wavelength of 298 nm. The mobile phase used was a methanol and acetonitrile mixture (60:40, v/v) and the flow rate was 10.0 mL/min. The substance represented by the peak which appeared between the peaks for δ-tocopherol and γ-tocopherol was collected (Figs. 2a and b), and the MDT fraction was dried using a rotary evaporator. The purity of the MDT (>99%) was confirmed by HPLC analysis.
used as the mobile phase, with the flow rate set to 1.0 mL/min.

The MDT was weighed in a glass tube, dissolved in n-hexane and the concentration adjusted to various levels (50, 10, 2.0, 0.40, 0.080, 0.016 μg/mL) using a volumetric flask. δ-tocotrienol was added to all of the dilutions at the concentration of 0.10 μg/mL as an internal standard. The MDT concentration and the ratio of the chromatogram peak areas (MDT/δ-tocotrienol) were plotted on the x-axis and y-axis, respectively. The calibration curve was expressed as a first-order equation to determine the linearity.

2.4 Animal care

Male ddY mice (4 wk old; Japan SLC, Shizuoka, Japan) were used for the experiments. The animals were treated in accordance with the guidelines established by the Institutional Animal Care and Use Committee of the Tokyo University of Marine Science and Technology. All animals were housed in plastic cages with free access to tap water and a basal rodent diet MF (Oriental Yeast, Tokyo, Japan) under controlled conditions of humidity (50 ± 10%), lighting (12:12 h light/dark cycle) and temperature (23 ± 2°C). After acclimatization for a week, the mice were randomly divided into experimental and control groups (n = 5) and given the AIN-93M based diet containing 7% (w/w) of soybean oil or a VE-rich fraction composed of triacylglycerol, α-tocopherol and MDT. Following treatment over a period of 4 weeks, the mice were killed by decapitation and the heart, lung, liver, stomach, small intestine, large intestine, kidney, pancreas, spleen, testis, skeletal muscle, visceral white adipose tissue (WAT), subcutaneous WAT and brain were removed and stored at −40°C.

2.5 VE and MDT content in animal tissues

The total lipid from the epididymal WAT was extracted according to the Folch method with δ-tocotrienol being added to act as an internal standard (IS). Briefly, approximately 100 mg of the sample tissue and 0.1 μg of IS were put into a glass tube and homogenized in PBS. Lipid extraction was then carried out using a chloroform-methanol mixture (2:1, v/v). After being vortexed for 1 min, the mixture was centrifuged at 3,000 × g for 10 min. The bottom lipid layer was collected in a new tube and dried with N2 gas. The lipid samples were diluted with 1 mL of n-hexane for HPLC analysis. The amount of MDT in epididymal WAT was quantitatively analyzed using HPLC-FL, with the prepared calibration curve (Fig. 3a).

With regards to the other tissues, lipid samples for HPLC analysis were prepared in the same way as described above, but without IS. The MDT content was calculated based on the calibration curve constructed using the MDT concentration ranging from 0.016 to 50 μg/mL (Fig. 3b) and the peak areas. The consistency of the measured values obtained using the two methods described was confirmed by comparing the results of subcutaneous WAT (with IS: 2.86 ± 1.60 mg/g tissue, without IS: 2.93 ± 0.28 mg/g tissue).

3 RESULTS AND DISCUSSION

Potent antioxidant activity of the VE contributes to the stability of the cell membrane lipids and plays an important role in maintaining homeostasis in animal body. A significant number of studies have demonstrated the beneficial effects of VE on health; some examples include protection against cancer, as well as hypercholesterolemia, cardiovascular and neurological disease. MDT also exhibits unique antioxidant activity due to its structure, and is likely to be beneficial for human health. To understand the physiological functions it was necessary to study the distribution and accumulation of MDT in the body. Therefore, the major goals of the present study are: (i) to establish a quantitative method (based on a calibration curve) and (ii) to investigate the accumulation of MDT in whole body tissues in mice.

The calibration curve for MDT was prepared using the HPLC-FL system (Fig. 3a). The slope was expressed as a first-order equation with $R^2 = 0.99$. To date, several methods for detecting tocopherols and tocotrienols have been reported. The approaches used for analyzing VE have been established with both normal phase and reversed phase HPLC. Current official methods for analyzing VE are based on normal phase HPLC. However, under normal phase conditions, there is poor chromatographic
resolution between the tocopherol and the tocotrienol, with both compounds having the same structure of chromanol. The reversed-phase column has the advantage of being able to distinguish the difference in degree of unsaturation in the phytyl side chain. Yamamoto et al. and Gotoh et al. achieve a good separation between MDT and an α-type of tocopherol and tocotrienol (found in palm oil and whose chemical structure has one double bond between the 11’ and 12’ carbons of the phytyl side chain) under reversed-phase conditions. For this reason, a C30 column was used instead of a tandem-jointed octadecyl silyl column (used in previous studies). In addition, mixing acetonitrile into the mobile phase solved the previously observed problems of poor resolution between the γ-tocopherol and MDT (Fig. 2). A study carried out by Cunha et al. involved a comparison of three detectors (UV detector, evaporative light scattering detector (ELSD) and FL detector) for their ability to determine the concentrations of tocopherols and tocotrienols. The researchers found that the FL detector obtained the best limit of detection: FL, 0.0002 μg/mL; ELSD, 5.4 μg/mL; and UV, 2.69 μg/mL in α-tocopherol. This result suggests that a FL detector can provide MDT analysis with high sensitivity. Compared to the ECD method used previously, the proposed method provides the advantages of a simpler mobile phase and purifying step. Since the ECD detects signals based on amperometry, the eluent needs a supporting electrolyte (e.g., sodium perchlorate). Thus, a non-aqueous ionic eluent cannot be used for the HPLC-ECD method because of the solubility requirement of the electrolyte, while both normal phase and reversed phase chromatography are available for the HPLC-FL method. In addition, the proposed method can also be used for the purification step because the mobile phase does not contain an electrolyte. With these results, it has become possible to quantify MDT in food materials and in the animal body simply and quickly.

Figure 4 shows the accumulation of MDT in each organ and tissue. Dietary MDT accumulated widely in whole body tissues. Such a wide distribution has been observed with tocotrienols in a 2 weeks feeding trial as tested by Gotoh et al. MDT was not detected in either the lung or the spleen, while significant accumulation was confirmed in this 4 week study. No accumulation of MDT was obtained in control mice fed with diet containing soybean oil. Additionally, it was found that adipose tissue stored the highest amount of MDT of the whole-body tissues. The preferential accumulation in adipose tissue was consistent with previous studies on the distribution of α-tocotrienol in the tissues, which demonstrated that these lipophilic compounds accumulate in the greatest concentration in adipose tissue, although the accumulation process was slow compared to in the other tissues. In the mice fed with the VE-free diet, the concentrations of stored tocopherol...
and tocotrienol depleted quickly in the liver but not in the adipose tissue, due to their slower metabolism in this tissue\textsuperscript{25}. Uchida \textit{et al.} observed that the levels of tocotrienols in adipose tissue are maintained (without any degradation), and the differences typically result in preferential accumulation of tocotrienols over tocopherols. Similar to tocotrienols, MDT may also have the property to be metabolized slowly in adipose tissue, resulting in a high accumulation in comparison to the liver. Interestingly, we found that MDT accumulates in high concentrations in the kidney. Non-\(\alpha\)-tocopherols are likely to be metabolized by hepatic P450 enzymes into water-soluble metabolites, including carboxychromanols with shorter carbon chains\textsuperscript{26}. Recent studies have detected sulfate conjugates of intermediate carboxychromanols and terminal metabolites in the urine. Unconjugated forms of carboxychromanols were also found in the feces of mice fed with \(\gamma\)- or \(\delta\)-tocopherol\textsuperscript{27-29}. Although we did not measure the MDT content of the urine and feces in the present study, our findings indicate the possibility that MDT accumulates in the kidney with its structure intact rather than undergoing metabolism. Further studies involving: (i) comparisons between the accumulation of MDT and other homologues, and (ii) whether other VEs affect the accumulation and metabolic fate of MDT, should be conducted to obtain information about the metabolic properties of MDT. In addition, elucidation of the mechanism of the metabolism of MDT would shed some light on the functionalities of MDT.

4 CONCLUSION

We established a useful tool for the evaluation of MDT in food materials and in the body of an animal by using the reversed-phase HPLC with FL detection. Dietary MDT accumulated in the whole-body tissues in mice. The present results are important for further studies on the physiological functions of MDT.

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