Comparison of Natural and Synthetic \( \alpha \)-Tocopherol in Rat Plasma by HPLC and Euryale ferox Seed as a Source of Natural \( \alpha \)-Tocopherol

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INTRODUCTION

\( \alpha \)-Tocopherol, vitamin E, has been studied the most among all tocopherol homologues since its discovery in 1992, presents an important determinant antioxidant for breaking radical driven chain reactions\(^1\). It exists eight \( \alpha \)-tocopherol isomeric forms, the predominant isomer derived naturally from plant sources is the natural (RRR) \( \alpha \)-tocopherol and the synthetic (all-rac) \( \alpha \)-tocopherol consists of eight stereo-isomers (RRR, SRR, RRS, RSS, SSR, SSR, RSS and SSS)\(^2,4\). The United States Pharmacopeia (USP) and Medicine (FNB-IOM) agree that all-rac- and RRR-\( \alpha \)-tocopherol can produced equivalent vitamin E activity in human. But nutritionist tend to regard all rac- and RRR-\( \alpha \)-tocopherol as different formulations of the same nutrient recently. The reason is endogenous protein such as enzymes and receptors usually exist only as one stereoisomer\(^2\). From this point, these stereoisomers may have different affinity to enzymes, which result in differences in therapeutic and adverse effect.

Differences in natural and synthetic vitamin E concentrations in humans and rats were studied under equal dosage of RRR- and all-rac-\( \alpha \)-tocopherol acetate. According to these works, \( \alpha \)-tocopherol is the active ingredient in plasma\(^6,7\). Furthermore, synthetic vitamin E is preferentially excreted as 2,5,7,8-tetramethyl-2-(2’-carboxy-ethyl)-6-hydroxychroman (\( \alpha \)-CEHC) in human urine: studies using deuterated \( \alpha \)-tocopherol acetate\(^6\). Within a range of humans metabolic capacity, the acetate ester of the single, natural stereoisomer RRR-\( \alpha \)-tocopherol acetate is 1.36 or 2 times more biologically potent than all-rac-\( \alpha \)-tocopherol acetate\(^8,9\). But out of the range metabolic capacity has not been reported. Although vitamin E is a nutrient, a high dose vitamin E intake can produce harmful effects on humans and rats. Over dose of vitamin E intake through oral administration will decrease the content of high-density lipoprotein and has adverse effects on lipid metabolism, leading poisoning\(^11\). So it is important to know more about the difference between RRR-\( \alpha \)-tocopherol and all-rac-\( \alpha \)-tocopherol.

In the aftermath of some food scandals, people have been concerning about the link between their health and food, which as the source of nutrients, especially natural antioxidants. They are interested in natural antioxidants continues to grow. Because natural antioxidants are presumed to be safe. Furthermore, evidence is accumulating that natural antioxidants inhibit biologically harmful oxidation reactions in the body\(^12\).

The aim of the present work was development and validation of a HPLC method with UV detection for the
measurement of metabolites of natural and synthetic α-tocopherol acetate in rats orally supplemented with large dose on a short- and long-term basis. From this study, we try to know more about whether natural α-tocopherol is safer and more effective than synthetic α-tocopherol. It was also considered for finding natural foods which are rich in α-tocopherol. From the overall perspective, the metabolism of compounds which have same chemical structure but differ in stereoisomer is complex in animal. We need to do further research to investigate what’s the difference between natural and synthetic α-tocopherol in rats.

**EXPERIMENTAL**

DL-α-tocopherol acetate (100062-201009, 97.8 %) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). DL-α-tocopherol (4-47783, 99.9 %) was purchased from Supelco (Bellefonte, USA). Natural α-tocopherol acetate capsule was purchased from Yang Sheng Tang Pharmaceutical Co., Ltd. Synthetic α-tocopherol acetate capsule was purchased from Xing Sha Pharmaceuticals (Xiamen) Co., Ltd. To calculate the administrated dose, the LC analysis of α-tocopherol acetate was modified versions of a previously published method [11].

The two compounds were determined by LC analysis to be 35.2 % pure RRR- and 43.1 % pure all-rac-tocopherol acetate, respectively. Euryale ferox seeds (20130306) was purchased from Fu Lin Tang Pharmaceutical Co., Ltd.

All solvents HPLC grade quality purchased from Merck (Darmstadt, Germany). Methanol, n-hexane, isopropyl alcohol were purchased from Fengchuan Chemical Co., Ltd (Tianjin, China). Drug-free blank plasma was obtained by sacrificing male rats.

The standard stock solutions of the α-tocopherol acetate and α-tocopherol were prepared to a content of 1.914 and 1.44 mg mL\(^{-1}\) in methanol. The stock solutions were stored at -20 °C.

**Study design:** Male rats (n = 20, weight = 160-180 g), were obtained from Laboratory Animal Centre of Kunming Medical University. All rats were kept in a controlled environment at 23 ± 2 °C and 50 ± 10 % relative humidity of a 12 h dark-light cycle. Food which does not contain vitamin E and water were allowed ad libitum. The rats had free access to water during the experiment. After a 12 h fast prior to the experiment.

Rats were divided into two groups randomly (n = 6) and administrated an oral dose of 800 mg kg\(^{-1}\) a day of natural α-tocopherol acetate and synthetic α-tocopherol acetate, respectively. Plasma samples were collected in a sodium citrate tube via the heart of the rat.

**Sample preparation:** Plasma sample was processed without saponification and therefore 200 μL plasma was transferred to a 5 mL plastic test tube and 140 μL methanol/isopropyl (8:2, v/v) was added, the test tube closed and mixed for 1 min by vortex (Hanuo Instruments Co., Ltd, Shanghai, China).

Then the plasma samples were centrifuged at 10,000 rpm for 10 min (HC-3018R, USTC Zonia Scientific Instruments Co., Ltd, Anhui, China) to facilitate phase separation and an aliquot of the supernatant was transferred to a fresh test tube. The extraction was repeated with an additional 1 mL n-hexane, 2 min vortexed-mixing, centrifugation (12000 rpm, 4 min) and transfer of supernatant. Then the extracts was evaporated to dryness at 55 °C, the residues resuspended in 100 μL methanol and 20 μL immediately injected into the HPLC system.

Euryale ferox seed powder (2 g) was shaken with 20 mL of methanol at ambient temperature and ultrasound for 15 min, three times. Then the mixture filtered through filter paper. The filtrate was then passed through a 0.45 μm filter and 20 μL immediately injected into the HPLC.

**Liquid chromatography:** The chromatography system was performed with a high performance LC (Agilent Technologies series 1200) system comprising quaternary pumps, a diode-array detector, an auto injector (Waldbromn, Germany) and a chromatography workstation. Chromatographic separations were achieved using a Kromasil C\(_18\) reversed-phase column (200 mm × 4.6 mm, 5 μm). The mobile phase was consisted of methanol/water (98:2, v/v) as eluent at a flow rate of 1 mL min\(^{-1}\). All solutions were filtered through a 0.45 μm membrane (Ø 50 mm, 0.22 μm, Shanghai Xingya purifying material factory, China) and degassed prior to use. The column was kept at 40 °C. The channel on the diode-array detector was carried out at 285 nm (α-tocopherol acetate) and 295 nm (α-tocopherol).

**RESULTS AND DISCUSSION**

**Method validation:** Specificity the confirmation of identity for α-tocopherol acetate and DL-α-tocopherol were obtained by comparing the retention time of the calibration solution. In the case the retention times were 20 min for DL-α-tocopherol acetate and 13.4 min for DL-α-tocopherol.

**Linearity:** Calibration curves were studied in the following concentration ranges: 0.382-76.56 μg mL\(^{-1}\) for DL-α-tocopherol acetate and 0.36-1440 μg mL\(^{-1}\) for DL-α-tocopherol with the determinant of the regression (R\(^2\)) reaching a good level. For practical purposes, LOD and LOQ can be determined, respectively as 3 and 10 times the SD of repeated (at least 6) measurements of a blank matrix or a sample with low content of the analyte [12]. The limit of detection (LOD) obtained was sufficient for routine clinical application (Table-1).

**Precision:** The precision was evaluated by the inter- and intra-assay repetition method. Intra-assay and Inter-assay repeatability of α-tocopherol acetate were assessed by six successive replicate determinations of three plasma samples at concentrations corresponding to 0.382 μg mL\(^{-1}\), 19.14 and 38.28 μg mL\(^{-1}\) of DL-α-tocopherol acetate, respectively. While

**TABLE-1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Range (μg mL(^{-1}))</th>
<th>Regression equation</th>
<th>LOD (μg mL(^{-1}))</th>
<th>LOQ (μg mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-α-tocopherol acetate</td>
<td>0.38-76.56</td>
<td>y = 9.102x + 0.529; R(^2) = 0.9955</td>
<td>0.12</td>
<td>0.36</td>
</tr>
<tr>
<td>DL-α-tocopherol</td>
<td>0.36-1440</td>
<td>y = 0.6996x + 33.574; R(^2) = 0.9934</td>
<td>0.1</td>
<td>0.3</td>
</tr>
</tbody>
</table>
for DL-α-tocopherol, the three samples at concentrations corresponding to 18 µg mL⁻¹, 36 and 1440 µg mL⁻¹. Precision can be considered very good and this result is mainly due to the simple treatment of samples.

**Stability and accuracy:** Recovery was evaluated by analyzing samples after adding known amounts of spiked samples. Then it was tested for three levels and replicated five times for each level. Average recovery was calculated by comparing mean values of replicates with theoretical concentrations of each replicate and the results are shown in Tables 2 and 3. The stability of DL-α-tocopherol acetate and DL-α-tocopherol were evaluated by determining a sample repeatedly at 0, 6, 12 and 24 h with a represents means of per concentration level in each day (RSD) < 6 % (n = 6). Working solutions of DL-α-tocopherol acetate and DL-α-tocopherol and their standards were found to be stable for 7 days at -20 °C which RSD < 9 % (n = 6).

**Animal experiments:** It is well-recognized that vitamin E is a major lipid soluble antioxidant in humans and that it plays an important role in protecting cell membranes. Both natural α-tocopherol and synthetic α-tocopherol contribute towards the antioxidant defense system. However, synthetic α-tocopherol was less sufficient to deliver the amount of antioxidants required to reduce oxidative stress. It has been shown that to be an effective antioxidant, α-tocopherol acetate must first be converted to free α-tocopherol.

From this work, we determined the concentrations of natural and synthetic α-tocopherol in rat plasma. Based on the measurements of chromatogram peak areas of natural and synthetic α-tocopherol in the rat plasma, we estimated the plasma concentrations of each sample (Figs. 1 and 2).

The result showed that synthetic α-tocopherol is more likely to accumulate in plasma and may cause chronic poisoning when out of the range metabolic capacity. During the last two decades, efforts to understand how dietary vitamin E is transported to the tissues have focused on α-tocopherol transport. α-Tocopherol transfer protein (TTP) has been identified to mediate α-tocopherol secretion into the plasma while other tocopherol-binding proteins seem to play a less important role. α-Tocopherol selectively binds to α-tocopherol.

**TABLE 2**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spiked concentration (µg mL⁻¹)</th>
<th>Measured concentration (µg mL⁻¹)</th>
<th>RSD (%) (n = 6)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-α-tocopherol acetate</td>
<td>0.382</td>
<td>0.379</td>
<td>3.2</td>
<td>96.2</td>
</tr>
<tr>
<td></td>
<td>19.14</td>
<td>18.96</td>
<td>3.9</td>
<td>99.0</td>
</tr>
<tr>
<td></td>
<td>76.56</td>
<td>77.10</td>
<td>2.4</td>
<td>100.7</td>
</tr>
<tr>
<td>DL-α-tocopherol</td>
<td>18.0</td>
<td>17.52</td>
<td>4.3</td>
<td>97.3</td>
</tr>
<tr>
<td></td>
<td>36.0</td>
<td>35.26</td>
<td>5.5</td>
<td>97.9</td>
</tr>
<tr>
<td></td>
<td>1440</td>
<td>1405</td>
<td>2.3</td>
<td>98.2</td>
</tr>
</tbody>
</table>

**TABLE 3**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spiked concentration (µg mL⁻¹)</th>
<th>Measured concentration (µg mL⁻¹)</th>
<th>RSD (%) (n = 6)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-α-tocopherol acetate</td>
<td>0.382</td>
<td>0.361</td>
<td>4.1</td>
<td>94.5</td>
</tr>
<tr>
<td></td>
<td>19.14</td>
<td>18.15</td>
<td>3.5</td>
<td>97.8</td>
</tr>
<tr>
<td></td>
<td>76.56</td>
<td>77.50</td>
<td>2.9</td>
<td>101.2</td>
</tr>
<tr>
<td>DL-α-tocopherol</td>
<td>18.0</td>
<td>17.36</td>
<td>7.6</td>
<td>96.4</td>
</tr>
<tr>
<td></td>
<td>36.0</td>
<td>34.89</td>
<td>5.7</td>
<td>96.9</td>
</tr>
<tr>
<td></td>
<td>1440</td>
<td>1405</td>
<td>2.1</td>
<td>97.7</td>
</tr>
</tbody>
</table>
transfer protein. In 2000, the FNB-IOM estimated the relative vitamin E activity of all-rac- and RRR-α-tocopherol in humans by extrapolating from the relative affinity of α-tocopherol transfer protein for the stereoisomers of α-tocopherol. It is noted that the affinity of α-tocopherol transfer protein for the stereoisomers of α-tocopherol is less than RRR-α- tocopherol. So all-rac-α-tocopherol can’t be transported to tissue effectively. Maybe it is the reason that the concentration of all-rac-α-tocopherol in rat plasma is higher than RRR-α-tocopherol in the study.

Euryale ferox seed: Euryale ferox seed, the seed of Gordon Euryale, is one of famous and special grain in China and at same time it is an effective Chinese medicinal used to treat diarrhea and emission. Overall, other species of Chinese medical food were also studied, namely Juglans regia, Semen coicis and Dioscorea opposita.

The result showed that the level of α-tocopherol content in Euryale ferox seed had spiked to 1.68 mg g⁻¹, which is about ten times of three others species (Fig. 3). Overall, Euryale ferox seed possessed highly α-tocopherol. The Recommended Daily Intake (RDI) of vitamin E set by European Union Council Directive no 90/496 is 10 mg day⁻¹. People could take Euryale ferox seed which cooked into porridge and soup to supply natural α-tocopherol.

Fig. 3. Comparison of the content of α-tocopherol in Euryale ferox seed, Juglans regia, Semen coicis and Dioscorea opposita

Conclusion

The difference between natural and synthetic α-tocopherol in rat plasma was measured by an orally administrated dose of α-tocopherol acetate with the reversed-phase liquid chromatographic method. Our finding showed that natural α-tocopherol was less accumulated, easier to metabolized into active substances and more effectively deliver its beneficial biological effects. Therefore, we had better obtain α-tocopherol from foods which rich in natural tocopherols. Euryale ferox seed was found to have high tocopherols content and could be used as health foods. It is a new, natural and economic source of dietary antioxidant, which can be used in the prevention of diseases caused by free radical.

ACKNOWLEDGEMENTS

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