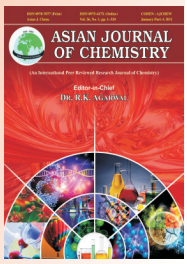
Asian Journal of Chemistry; Vol. 26, No. 16 (2014), 5279-5282

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.17197



Comparison of Natural and Synthetic α-Tocopherol in Rat Plasma by HPLC and *Euryale ferox* Seed as a Source of Natural α-Tocopherol

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Received: 11 February 2014; *Accepted*: 15 April 2014; *Published online*: 28 July 2014; AJC-15678

A validated HPLC with UV method was developed to evaluate the difference between natural (RRR) α-tocopherol and synthetic (all-rac) α-tocopherol in rat plasma by an intragastric administrated does of α-tocopherol acetate. The direct extraction of analytes in two steps with methanol/isopropanol (8:2, v/v) and *n*-hexane. This method employed a Kromasil C_{18} column and methanol/water (98:2, v/v) as mobile phase, at a flow rate of 1 mL min^{-1}. The limits of detection (LOD) were 0.12 μg mL^{-1} for DL-α-tocopherol acetate and 0.1 μg mL^{-1} for DL-α-tocopherol, respectively. The recoveries of the method were near 95 %. Intra-assay and inter-assay precision were, respectively 2.3-5.5 % and 2.1-7.9 %. The method was employed to evaluate natural (RRR) and synthetic (all rac) α-tocopherol in rat plasma. Meanwhile, the α-tocopherol content in *Euryale ferox* seed was measured. The results obtained from this study demonstrated that natural α-tocopherol was less accumulated than synthetic α-tocopherol and safe for long term use. After measurement, *Euryale ferox* seed is a potentially rich source of natural tocopherols and could be used as antioxidants in food for nutraceutical purpose .

Keywords: α-tocopherol, Vitamin E, HPLC, Rat plasma, *Euryale ferox* seed.

INTRODUCTION

α-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 α-tocopherol isomeric forms, the predominant isomer derived naturally from plant sources is the natural (RRR) α-tocopherol and the synthetic (all-rac) α-tocopherol consists of eight stereo-isomers (RRR, SRR, RRS, RSS, RSR, SSR, RSS and SSS)[2-4]. The United States Pharmacopeia (USP) and Medicine (FNB-IOM) agree that all-rac- and RRR-α-tocopherol can produced equivalent vitamin E activity in human. But nutritionist tend to regard all rac- and RRR-α-tocopherol as different formulations of the same nutrient recently. The reason is endogenous protein such as enzymes and receptors usually exist only as one stereo-isomer[5]. 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-α-tocopherol acetate. According to these works, α-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 (α-CEHC) in human urine: studies using deuterated α-tocopherol acetate[8]. Within a range of humans metabolic capacity, the acetate ester of the single, natural stereoisomer RRR-α-tocopherol acetate is 1.36 or 2 times more biologically potent than all-rac-α-tocopherol acetate[9,10]. 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 viamin 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-α-tocopherol and all-rac-α-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[13]. 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 Zonkia Scientific Instruments Co., Ltd, Anhui, China) to facilitate phase separation and an alioquot 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 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 (Waldbronn, 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[14]. 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
LINEARITY RANGE OF REGRESSION EQUATION, LOD, LOQ OF THE PROPOSED METHOD

Compound	Range (µg mL^{-1})	Regression equation	LOD (µg mL^{-1})	LOQ (µg mL^{-1})
DL-α-tocopherol acetate	0.38-76.56	$y = 9.102x + 0.529$; $R^2 = 0.9955$	0.12	0.36
DL-α-tocopherol	0.36-1440	$y = 0.6996x + 33.574$; $R^2 = 0.9934$	0.1	0.3

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[15]. 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[16]. It has been shown that to be an effective antioxidant, α-tocopherol acetate must first be converted to free α-tocopherol[17].

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[18]. During the last two decades, efforts to understand how dietary vitamin E is transported to the tissues have focused on α-tocopherol trans port[19-21]. 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

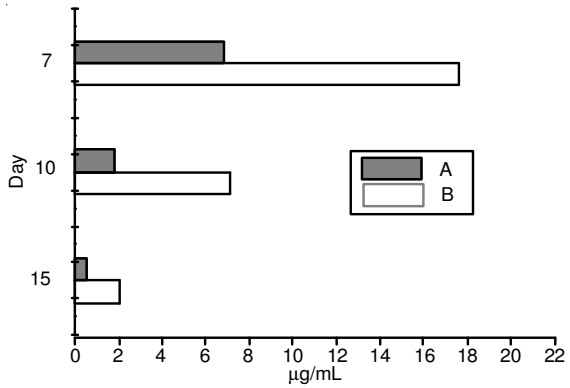

Fig. 1. Plasma concentrations of RRR (A) and all-rac (B) α-tocopherol measured by HPLC method. Results are means ± SD of three measurements run in parallel

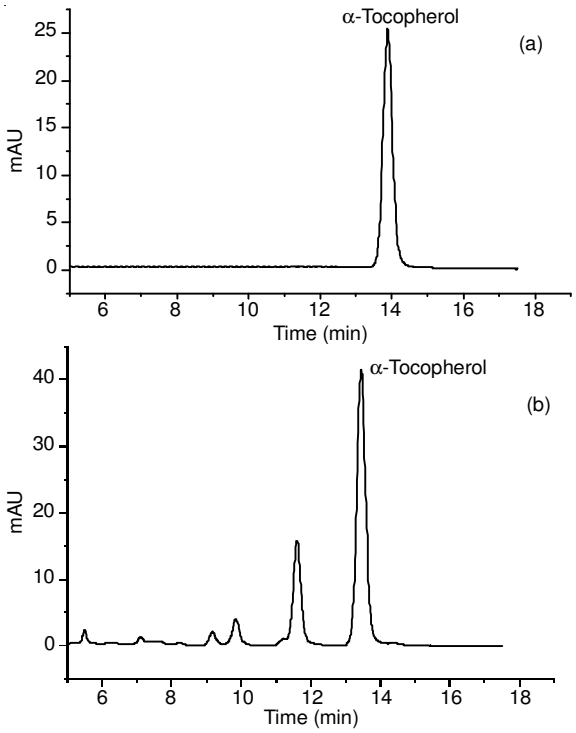

Fig. 2. (a) Typical chromatogram of DL-α-tocopherol standard. (b) Chromatogram of tocopherols in the sample of *Euryale ferox* seed

TABLE-2
INTRA-PRECISIONS AND ACCURACIES OF THE SAMPLES (n = 6)

Sample	Spiked concentration (μg mL^{-1})	Measured concentration (μg mL^{-1})	RSD (%) (n = 6)	Recovery (%)
DL-α-tocopherol acetate	0.382	0.379	3.2	96.2
	19.14	18.96	3.9	99.0
	76.56	77.10	2.4	100.7
DL-α-tocopherol	18.0	17.52	4.3	97.3
	36.0	35.26	5.5	97.9
	1440	1414	2.3	98.2

TABLE-3
INTER- PRECISIONS AND ACCURACIES OF THE SAMPLES (n = 6)

Sample	Spiked concentration (μg mL^{-1})	Measured concentration (μg mL^{-1})	RSD (%) (n = 6)	Recovery (%)
DL-α-tocopherol acetate	0.382	0.361	4.1	94.5
	19.14	18.15	3.5	97.8
	76.56	77.50	2.9	101.2
DL-α-tocopherol	18.0	17.36	7.6	96.4
	36.0	34.89	5.7	96.9
	1440	1405	2.1	97.7

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[22]. It is noted that the affinity of α-tocopherol transfer protein for the stereoisomers of α-tocopherol is less than RRR-α-tocopherol[23,24]. 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[13]. From the previously research, we know that it has antioxidant capacity[25]. In this work, three 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} [26]. People could take *Euryale ferox* seed which cooked into porridge and soup to supply natural α-tocopherol.

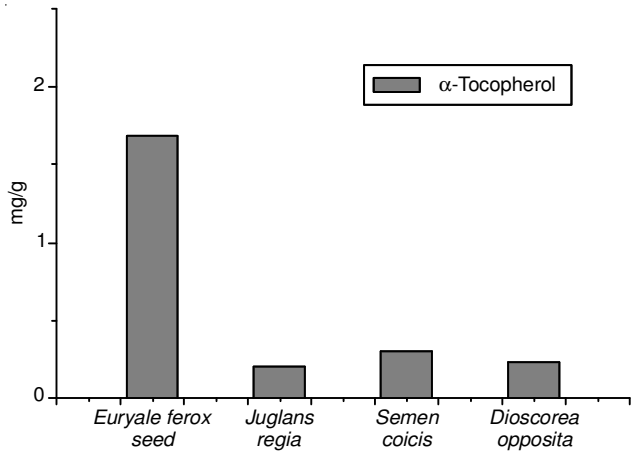

Fig. 3. Comparison of the content of α-tocopherol in *Euryale ferox* seed, *Juglans regia*, *Semen coicis* and *Dioscorea opposita*

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

This work was supported by the National Natural Science Foundation of China (No. 31260399) and Yunnan Innovation Grant (No. 2011CI078).

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