Determination of α -Tocopherol Stereoisomers in Biological Specimens Using Chiral Phase High-Performance Liquid Chromatography

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Summary The α -tocopherol stereoisomers in biological specimens were investigated using high-performance liquid chromatography. All-*rac*- α -Toc acetate was separated into four peaks (peak area ratio: 4:2:1:1) by Chiralpak OP(+) HPLC. 2*R*-isomers constituted the first peak and 2*S*-isomers were separated into three peaks (peak area ratio: 2:1:1). 2-*Ambo*- α -Toc acetate was completely separated into *RRR*- and *SRR*- α -Toc acetate by this method. The present HPLC method was used for the separation of all-*rac*- α -Toc in blood and tissues of rat. The analytical recoveries of *RRR*- and *SRR*- α -Toc acetate added to blood and tissues were 90.5–98.2% for *RRR*- α -Toc and 93.7–100.5% for *SRR*- α -Toc. The distribution of α -Toc stereoisomers in the blood and tissues from rats administered all-*rac*- α -Toc acetate was investigated by HPLC. The concentrations of the 2*R*-isomers in the blood and tissues were markedly higher than the concentrations of the 2*S*-isomers, and the levels of α -Toc stereoisomers showed marked differences between blood and tissues.

Key Words tocopherol, stereoisomer, determination, chiral phase column, high-performance liquid chromatography, blood, tissue

All-*rac*- α -tocopherol (Toc) is the most common vitamin E preparation used as a drug and as a food additive. It consists of an equimolar mixture of eight stereoisomers. Its biological activity is reported to comprise different activities ranging from 100% for *RRR*- α -Toc acetate to 21% for *SSR*- α -Toc acetate by the resorption-gestation test (1). According to these data, the biological activity of the four 2*R*-isomers of the α -tocopherol stereoisomers is generally higher than that of the 2*S*-isomers. Recently, the absorption, transport, and distribution of α -Toc stereoisomers have been investigated using deuterium-substituted *RRR*- and *SRR*-Toc (2,3), and determination of the isomers was performed by the gas chromatography (GC)/mass spectrometry-selected ion monitoring method (4).

The metabolism and biological activity of these stereoisomers is of interest. However, there are few reports concerning the metabolism and biological activity of the stereoisomers. One reason may be that simple analytical methods for these stereoisomers in biological specimens have not been developed.

Yamaguchi *et al.* reported a separation method of all-*rac*- α -Toc acetate of drugs by chiral phase (Chiralpak OT) HPLC (5). Using this method, the eight stereoisomers were separated into only two peaks. In order to investigate absorption, transport and metabolism of these isomers *in vivo*, clear separation of the isomers is required. In 1990, Vecchi *et al.* reported an analytical method combining HPLC and capillary GC for authentic all-*rac*- α -Toc derivatives (6). With this method, the eight α -Toc stereoisomers were separated into two groups of 2R- and 2S-configurations by HPLC, and each of the four isomers in the 2R- and 2S-groups was determined by GC. However, the analysis time was consuming because of the use of two different analytical techniques.

We have established a simple and suitable determination method of α -Toc stereoisomers in biological specimens using chiral phase HPLC. The distribution and metabolism of α -Toc stereoisomers were investigated by this method.

EXPERIMENTAL

Materials. Eisai Co. (Tokyo, Japan) provided a set of *RRR*- α -, β -, γ -, δ -Toc and 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMC) as an internal standard. All-*rac*-(2*RS*,4'*RS*,8'*RS*), 2-*ambo*-(2*RS*,4'*R*,8'*R*), and 2-*epi*-(2*S*,4'*R*,8'*R*) α -Toc acetate were donated by Eisai Co. The purity of these materials was more than 98%, except for 2-*epi*- α -Toc acetate which contained about 10% of *RRR*-Toc acetate.

Animals. Male Fisher 344/DuCrj strain rats (28 days of age) were purchased from Charles River Co. (Japan). The rats were separated into two groups. One group was a vitamin E-deficient AIN-76 diet (produced by the addition of vitamin E stripped corn oil). One kilogram of this diet contains less than 1 mg vitamin E. The other group was fed the AIN-76 diet which contains 100 mg all-*rac*- α -Toc acetate/kg. The groups were fed either diet for 8 weeks.

After 17 h fasting overnight, the rats were sacrificed under anesthesia, and blood and tissues were taken.

Blood was withdrawn from the neck veins with heparinized syringes and mixed gently in a 15 ml glass tube with 20 μ l of 1% (w/v) EDTA sodium salt solution to 1 ml of whole blood. The blood was centrifuged in the glass tube $1,160 \times g$ for 10 min to obtain plasma. The red blood cells (RBC) in the glass tube were then washed with 0.9% cold saline, and made up to a hematocrit of about 50%. Tissues were stored at -50°C until the analysis of Toc.

Analytical recovery of stereoisomers. This experiment was performed using the blood and tissues of vitamin E-deficient rats.

The extraction of Toc in biological specimens was conducted according to our previous paper (7). That is, 200 μ l of plasma and 400 μ l of RBC suspension (ca.

50% hematocrit) were pipetted into a 10 ml centrifuge tube with a Teflon-coated screw-cap, and about 100 mg of liver or brain or 30 mg of adipose tissue which were cut into small pieces (< ca. 1 mm), accurately measured, was placed in a 10 ml centrifuge tube. Then, 100 μ l of 1% NaCl (w/v) solution was added to the tubes to accelerate the saponification by dispersion of the samples. One milliliter of an ethanolic solution containing 1.5 μ g each of *RRR*- and *SRR*- α -Toc acetate and 6% (w/v) ethanolic pyrogallol solution was added to each sample tube while stirring. After adding 200 μ l of 60% (w/v) KOH solution to each of the sample tubes, the contents were saponified with occasional shaking at 70°C for 30 min. After cooling in ice water, 4.5 ml of 1% NaCl solution was added to the saponified medium, and the medium was shaken vigorously with 3 ml of 10% ethyl acetate in hexane for 1 min. The mixture was centrifuged at $1,160\times g$ for 5 min, and 2 ml of the upper layer was pipetted into a 10 ml centrifuge tube with a Teflon-coated screw-cap. This hexane layer was evaporated under a stream of nitrogen gas at 40°C and acetylated to separate the α -Toc stereoisomers.

Acetylation of the residue was conducted in 20 μ l of dry pyridine with 100 μ l of acetic anhydride at 50°C for 15 min. After cooling to room temperature, 3 ml of 1% NaCl solution was added and the whole medium was extracted with 3 ml hexane. Then, 2 ml of the hexane layer was collected and evaporated under a stream of nitrogen gas at 40°C. The residue was dissolved in 200 μ l of 96% methanol for the determination by HPLC.

Figure 1 shows the acetylation procedure for Toc.

Determination of α -Toc stereoisomers in blood and tissues. Samples of blood and tissues of rats fed a diet containing all-*rac*- α -Toc acetate or a diet without α -Toc for 8 weeks were measured into 10 ml centrifuge tubes for analysis of α -Toc stereoisomers. Then, after addition of 1% NaCl solution and 6% ethanolic pyrogallol and 1 ml of an ethanolic solution containing 1.5 μ g 2,2,5,7,8-pentamethyl-6-hydroxychroman as an internal standard, the samples in the tubes were saponified with 60% KOH solution at 70°C for 60 min. After cooling in ice water, the tocopherols were extracted from whole media with 3 ml of 10% ethyl acetate in hexane as described above. Then, 2 ml of the hexane layer was collected into a 10 ml centrifuge tube with a Teflon screw-cap and evaporated under a stream of nitrogen gas at 40°C. The residue was acetylated to analyze α -Toc stereoisomers.

Chromatographic apparatus and conditions. The HPLC system of Model LC-6AD Series from Shimadzu Manufacturing Co. (Tokyo, Japan) consisted of a SIL-9A automatic injector, CTO-6A column oven, LC-6AD pump, and SPD-6AV UV detector linked to a C-R6A integrator. The analysis of Tocs was performed at 35°C using a Chiralpak OP(+) column (250 $\times$ 4.6 mm I. D., Daicel Chem. Ind., Tokyo, Japan). The mobile phase was methanol-water (96:4, v/v) at a flow-rate of 0.3 ml/min. The detection wavelength for Toc acetate was 284 nm.

Toc extracted (from biological specimens)
 |
 Collection (2ml of n-hexane layer)
 into 10ml centrifuge tube
 |
 Evaporation (stream of N₂)
 dry pyridine 20 μ l
 acetic anhydride 100 μ l
 |
 Acetylation (at 50°C for 15min)
 1% NaCl 2ml
 n-hexane 3ml
 |
 Extraction (vigorous shaking for 1min)
 |
 Centrifugation (at 3,000rpm for 5min)
 |
 Collection (2ml of n-hexane layer)
 |
 Evaporation (stream of N₂)
 96% MeOH 200 μ l
 |
 HPLC

Fig. 1. Acetylation procedure for tocopherols.

Fig. 2. UV-absorption spectrum of all-*rac*-, 2-*ambo*-, 2-*epi*-, and *RRR*- α -tocopheryl acetate and PMC acetate in methanol. ----, all-*rac*- α -Toc acetate; 2-*ambo*- α -Toc acetate; - · - · - 2-*epi*- α -Toc acetate; - - - - *RRR*- α -Toc acetate; —, PMC acetate.

RESULTS AND DISCUSSION

Separation of authentic samples

Figure 2 shows UV-absorption spectrum of all-*rac*-, 2-*ambo*-, 2-*epi*-, and

Fig. 3. Chromatograms of authentic samples of all-*rac*-, 2-*ambo*-, and 2-*epi*- α -tocopheryl acetate. P-1 consists of *RRR*, *RRS*, *RSR*, and *RSS* configuration. P-2 consists of *SSS* and *SSR* configuration. P-3 is *SRR* configuration. P-4 is *SRS* configuration. Peak area ratio of P-1, P-2, P-3, and P-4: 4:2:1:1. Peak area ratio of *RRR* and *SRR* in 2-*ambo*- α -Toc acetate: 1:1. PMC: internal standard.

RRR- α -Toc acetate and PMC acetate in methanol. As shown in Fig. 2, the UV-absorption maximum wavelength of these compounds in methanol was 284 nm.

Figure 3 shows HPLC chromatograms of authentic samples of all-*rac*-, 2-*ambo*-, and 2-*epi*- α -Toc acetate.

All-*rac*- α -Toc acetate was separated into four peaks (peak area ratio: 4:2:1:1) by HPLC under these conditions. After each peak was collected and derived to methyl ether derivatives, the qualitative analysis of α -Toc isomers in each peak was performed by capillary GC (5).

The first peak (P-1) consisted of *RRR*, *RRS*, *RSS*, and *RSR*; the second peak (P-2) consisted of *SSS* and *SSR*; the third peak (P-3) was *SRR*; and the fourth peak (P-4) was *SRS*. 2-*Ambo*- α -Toc acetate was completely separated into two peaks of *RRR*- and *SRR*- α -Toc acetate.

Calibration curves

Calibration curves were prepared to evaluate the quantitative analysis of this method with authentic samples of all-*rac*- α -tocopheryl acetate and 2-*ambo*- α -tocopheryl acetate using 1.5 μ g PMC as the internal standard.

That is, 1 ml each of 1.5, 3.0, 6.0, and 9.0 μ g/ml ethanolic all-*rac*- and 2-*ambo*- α -Toc acetate solutions were saponified with 1.5 μ g PMC, and acetylated again by a similar procedure for the analysis of the samples. The experiment was repeated 4 times.

These standard solutions were analyzed by chiral HPLC. The correlation between the concentration ratio (y) and the peak area ratio (x) to the internal standard of each peak was determined. The results, with reference to P-1, P-2, P-3, and P-4, are the linear regression equations of $y = 2.081x - 0.090$ (correlation coefficient: $r = 1.000$, $p < 0.05$), $y = 1.854x - 0.054$ ($r = 1.000$, $p < 0.05$), $y = 2.082x + 0.004$ ($r = 0.994$, $p < 0.05$), and $y = 1.960x + 0.036$ ($r = 0.964$, $p < 0.05$). However, as both the concentration ratio and the peak area ratio of P-1, P-2, P-3, and P-4 showed an equal ratio of 4:2:1:1, these four regression equations could be represented by the one equation $y = 2.073x + 0.015$ ($r = 0.994$, $p < 0.05$). Also, the results, with reference to *RRR* and *SRR* in 2-*ambo*- α -Toc acetate, are the linear regression equations of $y = 2.096x - 0.033$ ($r = 0.999$, $p < 0.05$) and $y = 1.884x + 0.116$ ($r = 0.999$, $p < 0.05$), respectively. Subsequently, since both the concentration ratio and the peak area ratio of *RRR* and *SRR* in 2-*ambo*- α -Toc acetate showed an equal ratio of 1:1, these two equations could be represented by $y = 1.979x + 0.072$ ($r = 0.997$, $p < 0.05$).

A linear response was obtained over the range used in the analysis. The response of each peak area was directly proportional to the number of stereoisomers in each peak.

Recoveries of *RRR*- and *SRR*- α -Toc added to blood and tissues

A 3.0 μ g sample of 2-*ambo*- α -Toc acetate (containing 1.5 μ g each of *RRR*- and *SRR*- α -Toc acetate) was added to blood and tissue samples of a vitamin E-deficient

Table 1. Analytical recoveries of *RRR*- and *SRR*- α -tocopheryl acetate added to blood and tissues.

	<i>RRR</i> (%)	<i>SRR</i> (%)
Plasma	90.5 $\pm$ 6.6	97.3 $\pm$ 2.0
RBC	91.3 $\pm$ 6.1	100.5 $\pm$ 8.3
Adipose tissue	98.2 $\pm$ 3.2	93.7 $\pm$ 3.9
Brain	96.0 $\pm$ 1.5	96.2 $\pm$ 4.9
Liver	95.4 $\pm$ 3.6	97.7 $\pm$ 5.2

1.5 μ g of *RRR* and/or *SRR*- α -tocopheryl acetate was added to samples. Each value represents $M \pm SD$ of 4 determinations.

Fig. 4. Chromatograms of all-*rac*- and 2-*ambo*- α -tocopheryl acetate added to RBC of vitamin E-deficient rat. A: Chromatogram of all-*rac*- α -Toc acetate added to RBC. B: Chromatogram of 2-*ambo*- α -Toc acetate added to RBC. C: Chromatogram of RBC of vitamin E-deficient rat.

Fig. 5. Chromatograms of all-*rac*- and 2-*ambo*- α -tocopheryl acetate added to plasma of vitamin E-deficient rat. A: Chromatogram of all-*rac*- α -Toc acetate added to plasma. B: Chromatogram of 2-*ambo*- α -Toc acetate added to plasma. C: Chromatogram of plasma of vitamin E-deficient rat.

rat, and the analytical recoveries of *RRR*- and *SRR*- α -Toc were determined by this method.

Table 1 shows the analytical recoveries of *RRR*- and *SRR*- α -Toc acetate. The analytical recoveries of these isomers were above 90%. Figures 4 and 5 show the HPLC chromatograms of all-*rac*- and 2-*ambo*- α -Toc acetate added to plasma and RBC. The measured recoveries of P-1, P-2, P-3, and P-4 of all-*rac*- α -Toc acetate added to these samples showed satisfactory results.

Influence of RRR- β -, γ -, and δ -Toc acetate

Figure 6 shows the HPLC chromatograms of *RRR*- α -, β -, γ -, and δ -Toc acetate under the same conditions. If β -, γ -, and δ -Toc were contained in the samples, β - and δ -Toc interfered with P-1, and γ -Toc interfered with P-2. However, in the blood and tissues of rats fed a diet including β -, γ -, and δ -Toc, these congeners were not detected.

It is therefore concluded that our established method is useful for animal

Fig. 6. Chromatograms of *RRR*- α -, β -, γ -, and δ -tocopheryl acetate.

studies using a semisynthetic diet, but in the case of human blood containing γ -Toc, previous separation of α -Toc must be performed to remove those congeners by the reversed phase or straight phase HPLC method. We are now testing a new method using pre HPLC for elimination of γ -Toc from human blood samples.

Distribution of α -Toc stereoisomers in blood and tissues

Table 2 shows the concentration of α -Toc stereoisomers in the blood and tissues of rats fed a diet containing 100 mg all-*rac*- α -Toc acetate per kg of diet. α -Tocopherols with 2*S* configurations were detected only in tissues, but not in plasma or RBC. The reason why the 2*S*-isomers of α -Toc were not detected in blood from rats starved for 17 h overnight was presumed to be due to the faster metabolic turnover of 2*S*-isomers than 2*R*-isomers (2,8,9). Both 2*S*- and 2*R*-isomers were detected in plasma obtained from nonstarved animals. The concentration of 2*R*-isomers of α -Toc in tissues was that of 2*S*-isomers. Furthermore, the 2*S*-isomers of α -Toc were not detected in adipose tissue, probably because the concentration was below the detection limit of this method. Ingold *et al.* previously reported (2,3,9) that the ratio of *RRR*/*SRR* in rat brain was 5 times that in a diet. However, the ratio of *RRR*/*SRR* in the other tissues was less than 3 times that in a diet. Also, *RRR*- α -Toc was preferentially excreted from the liver in very low

Table 2. Distribution of α -tocopherol stereoisomers in blood and tissues of rats fed a diet containing 100 mg all-*rac*- α -tocopheryl acetate.

Samples (<i>n</i> = 5)	Peak No.	M $\pm$ SD (μ g/g)	Conc. ratio (%)
Plasma ¹	P-1	2.90 $\pm$ 0.14	100.0
	P-2	ND	—
	P-3	ND	—
	P-4	ND	—
RBC ²	P-1	5.06 $\pm$ 1.45	100.0
	P-2	ND	—
	P-3	ND	—
	P-4	ND	—
Brain	P-1	13.7 $\pm$ 1.68	84.4
	P-2	2.27 $\pm$ 0.59	14.0
	P-3	0.02 $\pm$ 0.04	0.1
	P-4	0.24 $\pm$ 0.15	1.5
Liver (<i>n</i> = 4)	P-1	14.2 $\pm$ 1.53	84.9
	P-2	0.17 $\pm$ 0.04	1.0
	P-3	0.59 $\pm$ 0.69	3.5
	P-4	1.77 $\pm$ 0.29	10.6
Adrenal glands	P-1	122 $\pm$ 19.1	93.7
	P-2	2.09 $\pm$ 0.83	1.6
	P-3	1.73 $\pm$ 0.31	1.3
	P-4	4.41 $\pm$ 2.03	3.4
Adipose tissue (<i>n</i> = 4)	P-1	13.9 $\pm$ 2.90	100.0
	P-2	ND	—
	P-3	ND	—
	P-4	ND	—

ND, under detection limit; *n*, number of samples. ¹ μ g/ml; ² μ g/ml packed cells.

density lipoproteins and transported to other tissues. α -Toc binding protein is present in the liver cytosol but not in other tissues. Consequently, they presumed that the ratio of *RRR/SRR* in most tissues parallels the ratio of *RRR/SRR* in plasma.

Our data indicate that tissues have a high preference for the *2R*-isomers of α -Toc, and have some preference for *SRS* among the four *2R*-isomers, with the exception of the brain. The decreasing order of concentration of (*SSS*+*SSR*), *SRR*, and *SRS* is (*SSS*+*SSSR*)/2 > *SRS* > *SRR*, and in the liver and adrenal glands is *SRS* > *SRR* > (*SSS*+*SSR*)/2. It was presumed that the difference in distribution between brain and other tissues is due to the blood-brain barrier. The P-1 peak contains four stereoisomers of *2R* configurations (*RRR*, *RRS*, *RSR*, and *RSS*), and P-2 contains two stereoisomers of *2S* configurations (*SSS* and *SSR*). Therefore, it was not possible to compare the concentration of *RRR*, *RRS*, *RSR*, and *RSS*, or *SSS* and *SSR* in the samples by this method.

Fig. 7. Chromatograms of extracts from blood and tissues of rats fed diet containing 100mg all-*rac*- α -tocopheryl acetate per kg of diet. (1) Standard solution of all-*rac*- α -Toc acetate (10 μ g/ml) and PMCs (5 μ g/ml); (2) RBC; (3) plasma; (4) brain; (5) liver; (6) adrenal glands; (7) adipose tissue.

Figure 7 shows the chromatograms of α -Toc stereoisomers in blood and tissues.

CONCLUSION

Synthetic all-*rac*- α -Toc is composed of an equimolar mixture of eight stereoisomers. We investigated the determination method of the stereoisomers in biological specimens using chiral phase HPLC. The determination limit of the stereoisomers in samples with this method was about 10 ng. The recoveries of *RRR*- and *SRR*- α -Toc acetate added to biological specimens were satisfactory.

All-*rac*- α -Toc stereoisomers in the blood and tissues of rats were separated into four peaks of (*RRR* + *RSR* + *RRS* + *RSS*), (*SSS* + *SSR*), *SRR*, and *SRS*, acetylation derivatives were produced. The concentration of 2*R*-isomers of α -Toc in the blood and tissues of rats fed a diet containing all-*rac*- α -Toc acetate for 8 weeks was significantly higher than the concentration of 2*S*-isomers. The distribution of α -Toc having 2*S* configurations in the liver and adrenal glands was in the order *SRS* > *SRR* > *SSS*, *SSR*. It is presumed that the metabolic turnover of 2*S*-stereoisomers in the blood is faster than that of 2*R*-stereoisomers. It is suggested that the biological activities of the 2*R*-stereoisomers of α -Toc are higher than those of the 2*S*-stereoisomers, and that each stereoisomer of α -Toc has different biological activity.

REFERENCES

- 1) Weiser, H., and Vecchi, M. (1982): Stereoisomers of α -tocopheryl III. Biopotencies of all eight stereoisomers, individually or in mixtures, as determined by rat Resorption-Gestation Tests. *Int. J. Vit. Nutr. Res.*, **52**, 351–370.
- 2) Ingold, K. U., Burton, G. W., Foster, D. O., Hughes, L., Lindsay, D. A., and Webb, A. (1987): Biokinetics of and discrimination between dietary *RRR*- and *SRR*- α -tocopherols in the male rat. *Lipids*, **22**, 163–172.
- 3) Traber, M. G., Ingold, K. U., Burton, G. W., and Kayden, H. J. (1988): Absorption and transport of deuterium-substituted 2*R*,4'*R*,8'*R*- α -tocopherol in human lipoproteins. *Lipids*, **23**, 791–797.
- 4) Thomas, D. W., Parkurst, R. M., Negi, D. S., Lunan, K. D., Wen, A. C., Brandt, A. E., and Stephens, R. J. (1981): Improved assay for α -tocopherol in the picogram range, using gas chromatography-mass spectrometry. *J. Chromatogr.*, **225**, 433–439.
- 5) Yamaguchi, H., Itakura, Y., and Kunihiro, K. (1984): Analysis of the stereoisomers of α -tocopherol acetate by HPLC. *Iyakuhi Kenkyu*, **15**, 536–540.
- 6) Vecchi, M., Walther, W., Glinz, E., Netscher, T., Schmid, R., Lalonde, M., and Vetter, W. (1990): Chromatographische Trennung und quantitative Bestimmung aller acht Stereoisomeren von α -Tocopherol. *Helv. Chim. Acta*, **73**, 782–789.
- 7) Ueda, T., and Igarashi, O. (1987): New solvent system for extraction of tocopherols from biological specimens for HPLC determination of tocopherols from biological specimens for HPLC determination and the evaluation of 2,2,5,7,8-pentamethyl-6-chromanol as an internal standard. *J. Micronutr. Anal.*, **3**, 185–198.

- 8) Traber, M. G., Rudel, L. L., Burton, G. W., Hughes, L., Ingold, K. U., and Kayden, H. J. (1990): Nascent VLDL from liver perfusions of cynomolgus monkeys are preferentially enriched in *RRR*-compared with *SRR*- α -tocopherol: Studies using deuterated tocopherols. *J. Lipid Res.*, **31**, 687-694.
- 9) Traber, M. G., Burton, G. W., Ingold, K. U., and Kayden, H. J. (1990): *RRR*- and *SRR*- α -tocopherols are secreted without discrimination in human chylomicrons, but *RRR*- α -tocopherol is preferentially secreted in very low density lipoproteins. *J. Lipid Res.*, **31**, 675-685.