High Bioavailabilty of α-Tocopherol Loaded into Poly (DL-Lactic-co-Glycolic Acid) Microspheres in Apolipoprotein B Knockout Mice

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Purpose. To assess the potential clinical value of α-tocopherol-loaded poly (DL-lactic-co-glycolic acid) (PLGA) microspheres, we examined the disposition kinetics of α-tocopherol after administration of the microspheres to apolipoprotein B (apo B) knockout mice as a model of abetalipoproteinemia.

Methods. PLGA microspheres containing α-tocopherol were prepared by a solvent-evaporation method. The concentration of α-tocopherol was measured by gas chromatography-mass spectrometry.

Results. The mean value of particle size of α-tocopherol-loaded PLGA microspheres was 108 μm. The loading and the trapping efficiency of α-tocopherol in PLGA microspheres were 20.8% and 86.6%, respectively. When α-tocopherol solution (25 mg/kg) was subcutaneously administered to *apob* (+/+) and *apob* (+/−) mice, the plasma concentrations of α-tocopherol reached a peak at 6 h and decreased to the endogenous level within 4 days in both types of mice. However, the area under the plasma concentration-time curve (AUC) of *apob* (+/−) mice was significantly smaller than that in the case of *apob* (+/+) mice. When α-tocopherol-loaded PLGA microspheres (100 mg/kg) were subcutaneously administered, the plasma concentrations of α-tocopherol increased slowly and remained about 2-fold higher than the endogenous level at 5 to 10 days after administration in both types of mice, and there was no significant difference between the AUC values.

Conclusions. The PLGA microsphere preparation of α-tocopherol is expected to be a very useful drug delivery system in vitamin E supplementation therapy for abetalipoproteinemia.

KEY WORDS: apolipoprotein B, knockout mice, α-tocopherol, microsphere, disposition kinetics

INTRODUCTION

Abetalipoproteinemia (ABL) is characterized by extremely low levels of plasma cholesterol and triglycerides in patients (1, 2). This causes defective absorption of lipophilic vitamins, especially vitamin E (3), and the symptoms include ataxia, chronic paralysis, and hypesthesia. In the absence of a primary therapy, patients are given high-dose vitamin E supplementation as secondary therapy (4, 5).

We have already clarified the disposition kinetics of α-tocopherol in apolipoprotein B (apo B) knockout mice as an ABL model (6). In that work, we found that after an oral administration of α-tocopherol, the intestinal absorption of α-tocopherol is very low in apoB knockout mice, so that the area under the plasma concentration-time curve (AUC) for *apob* (−/−) mice was significantly less (about one-fifth) than that of *apob* (+/+) wild-type mice. We concluded that it is necessary to establish a parenteral dosing route to improve the quality of life for ABL patients.

Recently, various carriers for drug delivery systems have been developed. For vitamin E, there are systems using liposomes (7, 8) or lipoproteins (9), but these are not yet ready for clinical application. On the other hand, several preparations using poly (DL-lactic-co-glycolic acid) (PLGA) microspheres have been used clinically, and they have made a great contribution to the improvement of drug therapy (10). It is well known that PLGA microspheres have several advantages, such as prolonged maintenance of an effective drug level and increase of the bioavailability of short-lived drugs through controlled release (11). Further, PLGA microspheres are very safe, because they are excreted from the body after degradation ultimately to carbon dioxide and water by hydrolysis.

In this study, we prepared α-tocopherol-loaded PLGA microspheres and examined their disposition kinetics in apoB knockout mice.

MATERIALS AND METHODS

Materials

(±) α-Tocopherol, 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMC) and propylene glycol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Poly (DL-lactic-co-glycolic acid) (PLGA, lactic/glycolic 50/50, molecular weight 40,000-90,000) and N,O-bis-TMS-trifluoroacetamide (MTBSTFA) were from Tokyo Kasei Organic Chemicals Co. (Tokyo, Japan). HCO-60 was supplied by Nippon Chemicals Co. (Tokyo, Japan). Oligonucleotide primers were custom-synethesized by Amersham Pharmacia Biotech (UK).

Preparation of microspheres

PLGA microspheres were prepared by a solvent-evaporation method according to Wakiyama *et al.* (12) and Urata *et al.* (13). Briefly, about 10 mg of α-tocopherol and 50 mg of PLGA were dissolved in 2 mL of methylene chloride and the solution was cooled to 4°C. Then the solution was dispersed in 0.1% polyvinyl alcohol solution at a rate of 7,000 rpm by means of a magnetic stirrer. The stirring was continued for 2 h at room temperature, and then methylene chloride was evaporated off. The microspheres were collected by filtration using a 0.2 μm filter (Nihon Millipore Ltd., Yonezawa, Japan), washed with distilled water, and dried under reduced pressure at room temperature for 2 days. The size and shape of the microspheres were observed under an optical microscope (×100, Nikon TMD300)

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ABBREVIATIONS: ABL, abetalipoproteinemia; ApoB, apoprotein B; AUC, the area under the blood concentration-time curve; GC-MS, gas chromatography-mass spectrometry; MTBSTFA, N,O-bis-TMS-trifluoro- acetamide; PMC, 2,2,5,7,8-pentamethyl-6-hydroxychroman; PLGA; Poly (DL-lactic-co-glycolic acid.

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Determination of Drug Content in Microspheres

Weighed amounts of microspheres (about 1 mg) were dissolved in 2 mL of methylene chloride in a screw-capped test tube. Then 5 mL of hexane was added, and the mixture was shaken for 15 min to extract α-tocopherol. After centrifugation at 3,000 rpm for 15 min, the supernatant was taken and dried, and α-tocopherol was determined by gas chromatography-mass spectrometry (GC-MS, Model GC-17 system Class 5000, Shimadzu, Kyoto, Japan).

Drug Release in Vitro

Weighed amounts of microspheres (about 3 mg) were taken into a screw-capped test tube with 3 mL of phosphate-buffered saline (PBS, pH 7.4) containing 0.1% Tween 80. The test tube was immersed in a shaker bath maintained at 37°C and shaken horizontally. At designated time intervals, after centrifugation at 2,000 rpm for 5 min, 100 μL of the supernatant was sampled and α-tocopherol was determined by GC-MS.

Animals and Propagation

Male and female *apob* (+/–) mice (C57BL/6J-Apo b^{tm1Unc}) as apoB knockout mice were purchased from the Jaxson Laboratory (JAX MICE®, ME, USA). By mating male *apob* (+/–) mice with female *apob* (+/–) mice, we obtained *apob* (–/–), *apob* (+/–) and *apob* (+/+) mice. Genotyping of littermates was performed according to the polymerase chain reaction (PCR) method using primers for *apob* (–/–); 5′-CAC CTC CTG TCC AAG CCG CCT ATC A-3′and 5′-CAG ATA TAC ATT GGC TTC ATT GGC A-3′ (400 bp), and for *apob* (+/+); 5′-CAG ATA TAC ATT GGC TTC ATT GGC A-3′ and 5′-GCA GTA CAA ATT AGA GGG AAC ATC A-3′ (430 bp), according to the instructions of the Jaxon Laboratory.

Animal Experiments

All animal experiments complied with the guidelines of the Institutional Animal Care and Use Committee of Kanazawa University. Experiments were performed on 8-week-old *apob* (+/+) and *apob* (+/–)mice. α-Tocopherol was dissolved in 50% ethanol, 10% HCO-60 and 10% propylene glycol, and the α-tocopherol solution was subcutaneously (s.c.) injected into the back in a volume of 40 μl. α-Tocopherol-loaded PLGA microspheres were suspended in a solution of 0.5% carmellose sodium and 0.1% Tween 80 and were s.c. injected into the back in a volume of 400 μl. Blood samples were collected from the intraorbital venous plexus using a heparinized capillary tube under light ether anesthesia, at designated time intervals. The plasma was separated by centrifugation, and stored at –30°C until assay. The tissues were quickly excised, rinsed well with ice-cold saline, blotted dry and weighed. The samples were homogenized in ice-cold saline (10%, w/v). The samples were kept at –30°C until assay.

Assay for α-Tocopherol

Concentrations of α-tocopherol in plasma and tissues were determined by GC-MS. The assay for α-tocopherol was carried out according to Nakanishi *et al.* (14). Aliquots of 100 μl of plasma or tissue homogenates were each mixed with 100 μL of 1% ascorbic acid in water, 100 μL of 0.1 μg/mL PMC in ethanol, as an internal standard, and 1 mL of *n*-hexane. The mixture was shaken for 20 s and centrifuged for 5 min at × 3000*g*. The supernatant organic phase was transferred to another glass tube and preconcentrated under a stream of nitrogen gas at 37°C in a heating block. Then, 150 μL of acetone and 50 μl of MTBSTFA were added to the residue, and the mixture was shaken vigorously. The sample was transferred to an automated-sampler microvial, and incubated for 12 h at room temperature. An aliquot (1 μl) of sample was injected into the GC-MS system.

Analyses were carried out in the selected-ion monitoring mode, monitoring ions at *m/Z* 502 and *m/Z* 292 for α-tocopherol and PMC, respectively. Chromatographic separation of α-tocopherol was achieved with a 5% phenylmethylpolysiloxane-crosslinked capillary column (DB-5; 30 m × 0.315 mm I.D.; J&W Scientific Inc., USA) in a gas chromatograph equipped with a splitless injector. The oven temperature was set at 60°C for 1 min and then programmed up to 280°C at 20°C/min. The final temperature was maintained for 12 min.

Data Analysis

The pharmacokinetic parameters were estimated according to model-independent moment analysis as described by Yamaoka *et al.* (15). The data were analyzed using Student's *t* test to compare the unpaired mean values of two sets of data. The number of determinations is noted in each table and figure. A value of $p < 0.05$ or 0.01 was taken to indicate a significant difference between sets of data.

RESULTS

Characteristics of PLGA Microspheres Containing α-Tocopherol

Figure 1 shows the distribution histogram of particle size of the prepared α-tocopherol-loaded PLGA microspheres. The microspheres were clear, round particles. The number of microspheres in the size range of 60–140 μm was about 82% of the total, and the corresponding values for seven preparations were 75–85%. The mean value of particle size for the seven preparations was 108.1 ± 11.3 μm (mean ± SD). The loading of α-tocopherol in PLGA microspheres (w/w) was 20.8 ± 3.5%. The trapping efficiency (percentage of α-tocopherol trapped in the PLGA preparation) was 86.6 ± 11.9%.

Figure 2 shows the release profile of α-tocopherol from the PLGA microspheres in 0.1% Tween 80/PBS solution. After the initial stage, α-tocopherol was gradually released over 2 weeks, at which point, the cumulative release amounted to 26.8 ± 7.0% (n = 5) of the trapped drug in the microspheres.

Plasma Concentration-Time Course of α-Tocopherol After Subcutaneous Administration

Figure 3 shows the plasma concentration-time courses of α-tocopherol after an s.c. administration of α-tocopherol solution (25 mg/kg) in *apob* (+/+) and *apob* (+/–) mice. The plasma concentration represents the increased values after subtracting the endogenous concentration from the observed concentration at each time. The plasma concentrations in the

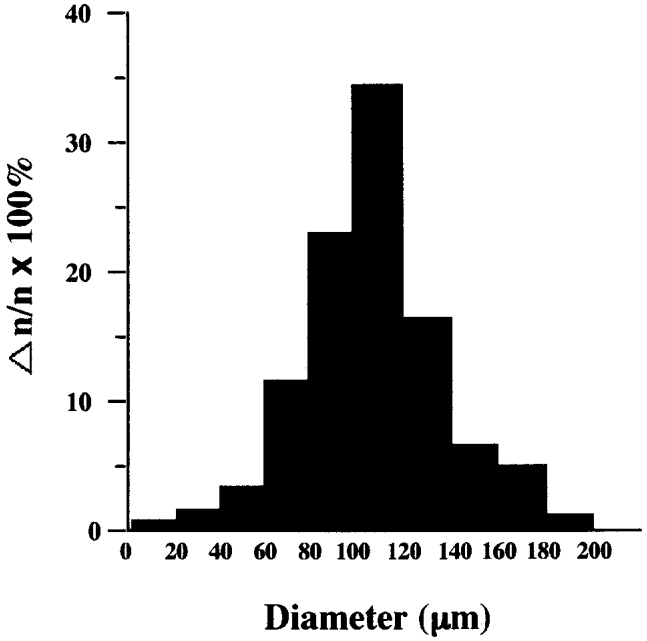

Fig. 1. Distribution histogram of particle size of α-tocopherol-loaded PLGA microspheres. Each area of column represents the particle size distribution on a number basis. n is the number of particles in a size increment of 20 μm: n is the total number of particles.

two genotypes increased gradually until 6 h after administration, but the plasma concentrations of *apob* (+/–) mice were significantly lower than those of *apob* (+/+) mice. The plasma concentrations in the two genotypes decreased to the endogenous level at 4 days after the administration.

To calculate the pharmacokinetic parameters, data were fitted to one-compartment model using the MULTI program (15). The apparent volumes of distribution (Vd/F) for *apob* (+/+) and *apob* (+/–) mice were 149 ± 22 and 152 ± 27 mL (mean ± SD), respectively, and not a significant difference. F is the fraction of the administration dose that is absorbed

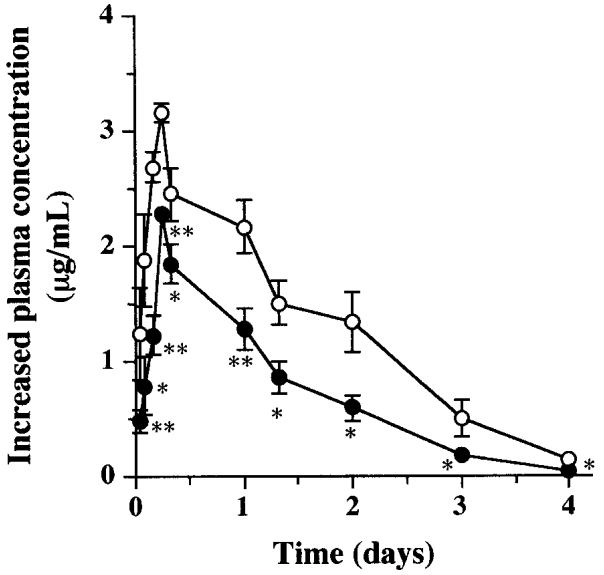

Fig. 3. Time course of the increase in plasma concentration of α-tocopherol after a s.c. administration of α-tocopherol solution (25 mg/kg) in *apob* (+/+)and *apob* (+/–) mice. The endogenous plasma concentration of α-tocopherol was 2.62 ± 0.27 μg/mL in *apob* (+/+) mice and 1.61 ± 0.34 μg/mL in *apob* (+/–) mice. Each point with a bar represents the mean ± SE of five mice. *·**Significantly different from *apob* (+/+) mice at $p < 0.05$ and 0.01, respectively.
Key: ○, *apob* (+/+) mice; ●, *apob* (+/–) mice

following an s.c. administration. The apparent first-order absorption rate constant (k_a) for *apob* (+/–) mice (2.77 ± 0.68 day^{-1}) was significantly smaller than that for *apob* (+/+) mice (7.38 ± 2.16 day^{-1}), $p < 0.01$. The apparent first-order elimination rate constant (k_e) for *apob* (+/–) mice (1.48 ± 0.16 day^{-1}) was significantly larger than that for *apob* (+/+) mice (0.861 ± 0.074 day^{-1}), $p < 0.01$.

Figure 4 shows the increased plasma concentration-time courses of α-tocopherol after an s.c. administration of α-to-

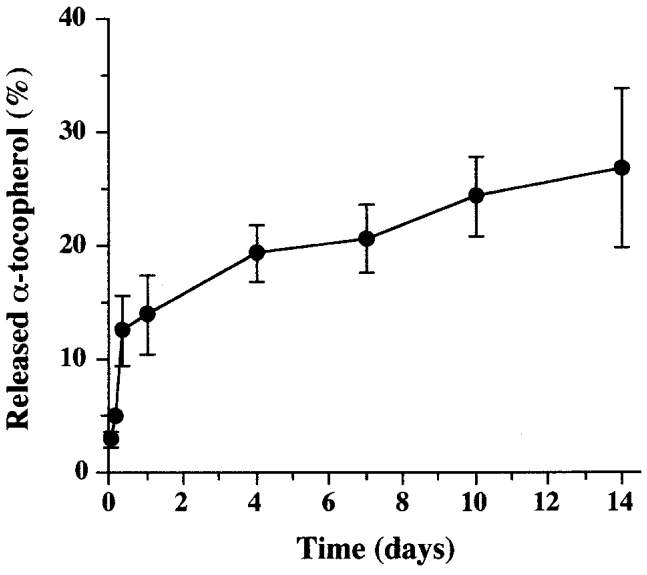

Fig. 2. Release profile of α-tocopherol from PLGA microspheres in PBS at 37°C. Each point with bar represents the mean ± SE of five experiments.

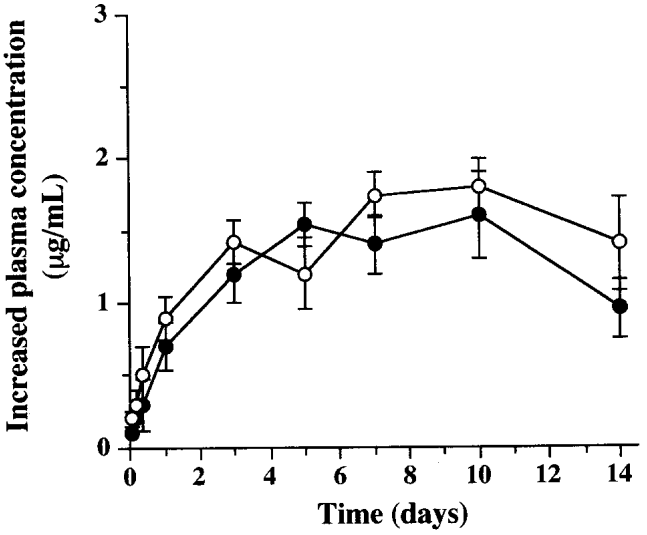

Fig. 4. Time course of the increase in plasma concentration of α-tocopherol after a s.c. administration of α-tocopherol-loaded PLGA microspheres (100 mg/kg) in *apob* (+/+) and *apob* (+/–) mice. Each point with bar represents the mean ± SE of five mice. Key: ○, *apob* (+/+) mice; ●, *apob* (+/–) mice

copherol-loaded PLGA microspheres (100 mg/kg of α-tocopherol) in *apob* (+/+) and *apob* (+/−) mice. The plasma concentrations in the two genotypes increased gradually, then remained about 2-fold higher than the endogenous level for 5 to 10 days, and thereafter decreased slowly. There was no significant difference between the courses of plasma concentration in these two genotypes.

The values of the area under the plasma concentration-time curve (AUC) of α-tocopherol after the s.c. administration of α-tocopherol solution or PLGA microspheres containing α-tocopherol in *apob* (+/+) and *apob* (+/−) mice are listed in Table I. The AUC values after intravenous or oral administration of α-tocopherol solution in mice of three genotypes that we reported previously (6) are also listed for comparison. The value of $AUC_{0-4\ day}$ after s.c. administration of α-tocopherol solution in *apob* (+/+) mice was significantly higher than that in *apob* (+/−) mice. However, the value of $AUC_{0-14\ day}$ after s.c. administration of PLGA microspheres containing α-tocopherol in *apob* (+/+) mice was not significantly different from that in *apob* (+/−) mice.

Tissue Distribution of α-Tocopherol After s.c. Administration

Figure 5 shows the tissue concentrations of α-tocopherol after s.c. administration of different α-tocopherol preparations in *apob* (+/+) and *apob* (+/−) mice. At 24 h after administration of α-tocopherol solution (25 mg/kg), the α-tocopherol concentration in the liver was higher than in other tissues, and moreover, was significantly higher in *apob* (+/−) mice than in *apob* (+/+) mice. After administration of α-tocopherol-loaded PLGA microspheres (100 mg/kg), α-tocopherol was approximately equally distributed into all tissues in both types of mice, and there was no significant difference among the concentrations in these tissues.

DISCUSSION

The particle size of microspheres is known to influence the disposition kinetics of drugs after s.c. administration (16,17). Microspheres of 0.5–5.0 μm in diameter remain at the injection site for a short time, then are distributed rapidly into the systemic circulation. However, microspheres of over 10 μm in diameter remain at the injection site for a long time, and the drug is released slowly. In this study, we prepared α-tocopherol-loaded PLGA microspheres with a mean diameter of 108 μm. These microspheres slowly released α-tocopherol to the extent of 27% of their loading over 2 weeks. Wakiyama *et al.* (18) reported that the *in vivo* release is faster than the corresponding *in vitro* release, because of a higher degradation rate of PLGA *in vivo*. Therefore, large microspheres are expected to be useful for a sustained-release preparation of α-tocopherol.

It is well known that intestinal absorption and secretion of α-tocopherol into blood from the liver are mediated by apoB-containing lipoproteins such as chylomicrons and very-low density lipoproteins (6,11,12). Therefore, when α-tocopherol solution is orally administered, the AUC value is significantly lower in wild-type mice than in ApoB knockout mice (6). After intravenous administration of α-tocopherol solution, although the liver concentration of α-tocopherol was very high in ApoB knockout mice, not only the AUC value, but also the distribution volume at steady state was significantly lower in the knockout mice than in wild-type mice, as previously reported (6). After s.c. administration of α-tocopherol solution, the kinetic characteristics of ApoB knockout mice were lower for the absorption and faster for the elimination than that of wild-type mice, and the liver concentration of α-tocopherol was very high similar to intravenous administration, as confirmed in this study. It seems that superfluous α-tocopherol in blood is trapped in the liver, fails to re-enter the blood circulation, and is therefore not distributed to other tissues, because of ApoB deficiency.

Next, α-tocopherol-loaded PLGA microspheres were s.c. administered to wild-type mice and heterozygotes of ApoB knockout mice. The plasma concentrations were maintained at apparently higher than endogenous levels during 14 days, and there was no difference between the plasma concentrations and the AUC values in mice of the two genotypes. α-Tocopherol was, furthermore, equally distributed into various tissues. These results indicate that α-tocopherol is slowly released into the systemic circulation from PLGA microspheres which remain at the injection site, without being trapped in the liver. Consequently, the s.c. administration of PLGA microspheres containing α-tocopherol resulted in high bioavailability of α-tocopherol, despite the ApoB deficiency.

Moreover, in this study, the concentrations of α-tocopherol in plasma and tissues after the administration of the PLGA microspheres increased to about 3 μg/g wet tissue. The trapping efficiency of PLGA microspheres containing α-tocopherol was very high (about 90%), as expected, because α-tocopherol is a highly lipophilic drug. Therefore, because it should be easy to increase the loading of α-tocopherol in

Table I. Area Under the Plasma Concentration-Time Curve (AUC, μg day/mL) of α-Tocopherol after Various Dosages in Mice

Dosage	*apob* (+/+)	*apob* (+/−)	*apob* (−/−)
Solution			
$AUC_{0-8\ h}$[a] (i.v., 25 mg/kg, n = 4)	5.42 ± 0.46	4.29 ± 0.42*	3.89 ± 0.55**
$AUC_{0-32\ h}$[a] (p.o., 100 mg/kg, n = 4)	4.02 ± 0.65	—	0.738 ± 0.346**
$AUC_{0-4\ day}$ (s.c., 25 mg/kg, n = 5)	5.08 ± 0.81	2.76 ± 0.38**	—
PLGA microspheres			
$AUC_{0-14\ day}$ (s.c., 100 mg/kg, n = 5)	20.1 ± 2.6	17.3 ± 2.8	—

Mice were intravenously, orally or subcutaneously administered α-tocopherol solution (25 or 100 mg/kg) or α-tocopherol-loaded PLGA microspheres (100 mg/kg).

Each value represents the mean ± SD of 4–5 mice.

*,** Significantly different from the *apob* (+/+) mice at $P < 0.05$ and 0.01, respectively.

[a] Ref. 6

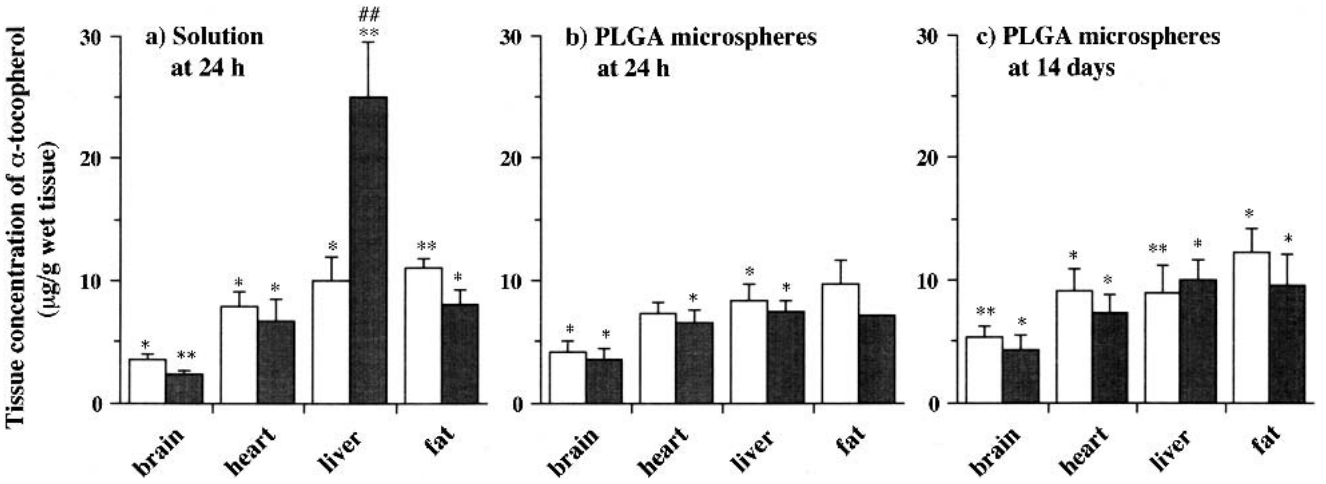

Fig. 5. Tissue concentrations of α-tocopherol after a s.c. administration of α-tocopherol solution and loaded PLGA microspheres (100 mg/kg) in *apob* (+/+) and *apob* (+/−) mice. The endogenous concentrations of α-tocopherol were 2.18 ± 0.36 and 1.48 ± 0.15 μg/g in the brain, 4.75 ± 0.61 and 3.91 ± 0.45 μg/g in the heart, 4.99 ± 0.66 and 4.64 ± 0.75 μg/g in the liver, and 6.81 ± 1.06 and 4.51 ± 0.84 μg/g in fat of *apob* (+/+) mice and *apob* (+/−) mice, respectively. Each column with bar represents the mean ± SE of five mice. ##Significantly different from *apob* (+/+) mice at $p < 0.01$. *,**Significantly different from each endogenous concentration at $p < 0.05$ and 0.01, respectively. Key: □, *apob* (+/+) mice; ■, *apob* (+/−) mice

PLGA microspheres, we suggest that it should be possible to control the α-tocopherol concentrations of plasma and tissues reached in ABL patients treated with such preparations. In conclusion, we demonstrated that PLGA microspheres have great potential value as a drug delivery system for vitamin E supplementation in ABL patients.

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