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Blood Clearance and Tissue Distribution of Various Formulations of α-Tocopherol Injection after Intravenous Administration

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The *in vivo* elimination and tissue distribution (2 h after administration) of various types of formulations of α-tocopherol as a model drug were examined in rats. An organic cosolvents formulation of α-tocopherol (α-tocopherol/EtOH/polyethylene glycol 400) was rapidly removed from the blood circulation, the ratio of the distributed α-tocopherol was 20% in the lungs, about 70% in the liver and about 10% in the spleen. The main reason for this was considered to be the appearance of droplets more than 10 μm in diameter in the blood. Elimination of the micelle formulation using Brij58 from the blood was the most rapid, followed by Tween80 and HCO60. This order is thought to be due to the difference in the polyoxyethylene group among these surfactants.

In liposome formulations, liposomes having a diameter of about 80 nm were the most stable in the blood circulation, and α-tocopherol was distributed in the reticuloendothelial system (RES) in the smallest ratio. The uptake of liposomes by the spleen and lungs depended on the liposome size. The uptake by the liver was in the decreasing order of 28 nm ≫ 42 nm, 151 nm, 195 nm, 3656 nm > 106 nm > 75 nm.

The 100 nm lipid emulsion was eliminated from the blood more rapidly than the 100 nm liposome formulation.

Keywords tocopherol; liposome; tissue distribution; micelle; organic cosolvent; elimination

Introduction

Recently many studies on the drug delivery system (DDS) have been investigated for the effective targeting of drugs and the prolongation of the drug concentration in the blood. On the other hand, several techniques for preparing the injection of poorly water-soluble or water stable drugs have been proposed. In these techniques, organic cosolvents,[1] emulsions, liposomes, micelles[2-7] and cyclodextrins[8] are included. Especially, the DDS using liposomes has been investigated by many researchers.

Intravenously injected liposomes are rapidly taken up by the reticuloendothelial system (RES) in the liver and spleen.[9] This is a big problem for researchers of liposomes. Uptake by RES is affected by the liposome size,[10-14] the surface charge,[15,16] the dose of liposomes[17,18] and the lipid composition.[19-21] Previous studies have demonstrated that larger liposomes are rapidly removed from the circulation by uptake into Kupffer cells of the liver and fixed macrophages of the spleen.[10-14] Small liposomes less than 100 nm in diameter can pass through the fenestrated endothelium.[22] Many researchers have reported that small liposomes less than 100 nm in diameter are stable in the circulation for a long time.[13,14] Allen *et al.* reported that RES uptake of liposomes decreased with decreasing liposomal size down to 80 nm, but 50 nm liposomes showed an increased liver uptake.[23] This was obtained only in mice and the details of that phenomenon have never been explained.

On the other hand, many studies about the pharmacokinetics of the emulsion or the micelle have been reported.[6,22,24] Yamaguchi *et al.* investigated the pharmacokinetics of the emulsion and the micelle of CoQ_{10}.[6] The studies of the pharmacokinetics on different dosage forms have been investigated by using the isotope. In this report, we present the differences in the blood clearance and the tissue distribution of α-tocopherol among liposomes, emulsions, micelles and organic cosolvent formulation in rats. We chose α-tocopherol as a model drug for the water-insoluble drug. Moreover α-tocopherol is useful for preventing the lipids of the liposomes from oxidation.[25] We especially investigated the effect of liposome size on blood clearance and tissue distribution after the intravenous administration of liposomes.

Materials and Methods

Materials *dl*-α-Tocopherol, methanol, ethanol, 2-propanol, diethyl ether, chloroform, acetic acid and ammonium acetate were purchased from Kanto Chemical Co., Ltd. Polyethylene glycol 400 (PEG400) and Brij58 were purchased from Wako Pure Chemical Co., Ltd. Tween80 was purchased from Kao Co., Ltd. HCO60 and soybean phosphatidylcholine (soybean PC; Epikuron200) were purchased from Nikko Chemicals. Soybean oil was purchased from Nikko Pharmacetuicals Co., Ltd.

Preparation of α-Tocopherol Micelles Formulation The formulation of micelles was chosen after preliminary tests. For the Tween80 preparation, 20 mg, of α-tocopherol and 500 mg of Tween80 were dissolved in 6 ml of 2-propanol. For the Brij58 preparation, 20 mg of α-tocopherol and 200 mg of Brij58 were dissolved in 6 ml of 2-propanol. For the HCO60 preparation, 20 mg of α-tocopherol and 750 mg of HCO60 were dissolved in 40 mg of methanol. These solvents were dried under vacuum in glass test tubes. After the addition of 5 ml of distilled water, α-tocopherol/surfactant micelles were formed by vortex mixing.

Preparation of Liposomes Multi lamellar vesicles (MLVs; 3656 nm) were prepared by the method of Bangham *et al.*[26] essentially. Forty milligrams of α-tocopherol and 1 g of soybean PC were dissolved in 5 ml of 2-propanol, and the solvent was dried in vacuum in a glass test tube. After the addition of 9 ml of distilled water, lipid films were hydrated by repeated vortex mixing.

Small unilamellar vesicles (SUVs; 28 nm) were prepared from MLVs. The MLVs suspension was sonicated for a total of 6 min (2 min sonication with 1 min cooling period × 2 times) at 0 °C using a sonicator (Branson model W185, U.S.A.) with a titanium probe at 70 W under the argon gas flow as described by Huang *et al.*[27]

Large unilamellar vesicles by extrusion techniques (LUVETs; 42—195 nm) were prepared from MLVs. The MLVs suspension was passed ten times through a polycarbonate membrane filter (Nucleopore, pore size 0.05—0.3 μm, Nomura Micro Science Co., Ltd.) to size the liposomes by the method of Hope *et al.*[28]

Preparation of Emulsion Forty milligrams of α-tocopherol, 500 mg of soy PC and 500 mg of soybean oil were dissolved in 5 ml of 2-propanol in a glass test tube, and the solvent was dried in vacuum. After the addition

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of 9 ml of distilled water, the lipids were hydrated with vortex mixing and then the suspension was sonicated (2 min × 3, at 0 °C, under the argon gas flow) as SUV preparation.

Animal Experiments Male Wistar rats (200—300 g) were fed commercial food pellets (F-2, Funabashi Farm, Japan) containing 50 mg vitamin E per kg, 20.8% protein, 4.5% fat, 3.4% fiber, 10.7% amino acids and 58.6% carbohydrates. Rats anesthetized with urethane (1 g/kg, i.p.) were cannulated in the jugular artery and the femoral vein. Various formulations containing α-tocopherol were injected through the femoral vein cannula. The dose of α-tocopherol was 4 mg/kg body weight. At selected times, blood samples (0.3 ml) were collected through the jugular artery. Rats were killed at 2 h after administration of various formulations, and their liver, lungs spleen and kidneys were collected.

Analysis of α-Tocopherol in Plasma Blood samples were heparinized, and after centrifugation (3000 rpm–15 min) blood plasma was obtained. One ml of methanol was added to a 0.15 ml portion of blood sample which was extracted with 5 ml of diethyl ether. Insoluble proteins were removed from the solution by centrifugation (3000 rpm–5 min). The solvent was dried in vacuum in a glass test tube, and α-tocopherol was reconstituted with 0.25 ml of HPLC mobile phase described below. α-Tocopherol was determined by HPLC analysis. Recovery of α-tocopherol from plasma was about 100% by recovery tests. Endogenous α-tocopherol in rats was measured before each experiment.

Analysis of α-Tocopherol in Organs Collected organs were homogenized with 2-propanol, and diethyl ether was added to the homogenate. After 10 min of shaking, the solvent layers were collected and dried in vacuum in a glass test tube. α-Tocopherol was reconstituted with 1 ml of HPLC mobile phase. α-Tocopherol was determined by HPLC analysis. Recovery of α-tocopherol was about 80% in all organs by recovery tests.

HPLC Analysis α-Tocopherol was determined by the HPLC analysis method. The Shimadzu HPLC system consisted of a model LC-9A Liquid Chromatograph, a model RF-530 fluorescence HPLC monitor, a model SCL-6B system controller and a model C-R6A chromatopac. The column was a 4.6 × 250 mm LiChrosorb® RP-8, 10 μm and the guard column was a 4 × 25 mm YMC ODS-A, 5 μm. The mobile phase was the mixture of methanol and 0.2 M pH 4.0 acetate buffer solution (92 : 8). Detection of α-tocopherol was carried out by using excitation and emission wave lengths of 286 nm and 330 nm, respectively.

Measurement of Particle Size The mean particle size was determined by using a dynamic laser light scattering instrument (a model DLS-700, Ohtuka Electronics Co., Ltd.).

Data Analysis The area under the plasma α-tocopherol concentration–time curve (*AUC*) was estimated by the trapezoidal rule for each rat. The data are presented as means ± standard deviation (σ_n).

Results

Measurement of the Endogenous α-Tocopherol in Various Tissues of Rats The endogenous α-tocopherol in various tissues of rats was measured first. The value of α-tocopherol in every tissue had good accordance with the previous report[29] and good uniformity among rats (CV values of endogenous α-tocopherol in the liver, the spleen, lungs and kidneys were 7, 5, 6 and 11%, respectively). The amount of α-tocopherol in each tissue did not change within 2 h after intravenous administration of the placebo formulations. The data of the tissue distribution of α-tocopherol at 2 h after intravenous administration were calculated by the equation as follows:

α-tocopherol in the tissue = measured α-tocopherol − endogenous α-tocopherol

Formulation Studies of Micelles Containing α-Tocopherol The size of the micelles containing α-tocopherol formed with Brij58, Tween80 or HCO60 was determined by using a dynamic laser light scattering. These sizes were minimum when these surfactants were used for solubilizing 4 mg/ml of α-tocopherol as follows as Brij58: 40 mg/ml, Tween80 : 100 mg/ml or HCO60 : 150 mg/ml. The micelle formulations in 1 ml of water were decided under the following conditions.

A. α-tocopherol 4 mg, Brij58 40 mg

B. α-tocopherol 4 mg, Tween80 100 mg

C. α-tocopherol 4 mg, HCO-60 150 mg

Plasma Concentration and Tissue Distribution of Water-Miscible Multi-cosolvents or Micelle Formulations α-Tocopherol of PEG/EtOH (0.7 ml/0.3 ml) formulation rapidly disappeared from the blood (Fig. 1, Table I). The blood clearance of the Brij58 formulation was fastest in micelle formulations in our studies. The blood clearance of Tween80 formulation was slower than that of the Brij58 formulation and the HCO60 formulation was slowest in micelle formulations. Figure 2 shows the tissue distribution at 2 h after intravenous administration. All of the α-tocopherol in the PEG400/EtOH formulation was taken up by RES. Especially, 72% of the dose was taken up by the liver and 20% of the dose was taken up by the lungs. In the case of Brij58, 80% of the dose was taken up by the liver. On the other hand, only 53% of the α-tocopherol in the HCO60 micelles was taken up by the liver. The uptake of α-tocopherol in the Tween80 micelles by the liver was intermediate between Brij58 and HCO60.

Plasma Concentration and Tissue Distribution of Liposome Formulations The size of the liposome is an important factor affecting the uptake by RES. Different size liposomes containing α-tocopherol were prepared, and then plasma concentrations and tissue distributions of α-tocopherol after

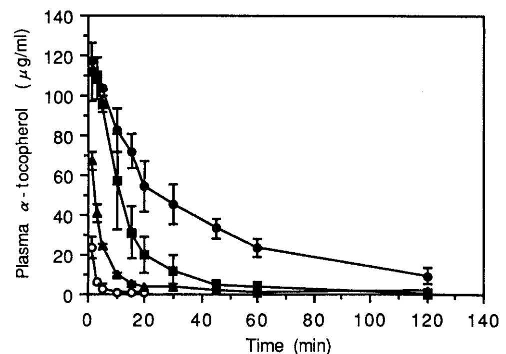

Fig. 1. Plasma Concentration of α-Tocopherol after i.v. Administration of Micelles or Water-Miscible Multi-cosolvents Formulation to Rats

Data are presented as the mean ± S.D. of three rats. —○—, PEG400 EtOH; —■—, Tween 80; —●—, HCO 60; —▲—, Brij58.

Table I. *AUC* after i.v. Administration of Various Formulations of α-Tocopherol

Formulation		AUC_{0-2h} (μg·min/ml)
Micelles	Brij58	516 ± 34
	Tween80	1580 ± 333
	HCO60	3943 ± 636
Cosolvents	PEG400/EtOH	61 ± 21
Liposome	28 nm	6342 ± 446
	42 nm	9334 ± 182
	75 nm	12941 ± 1288
	106 nm	9840 ± 704
	151 nm	9544 ± 400
	195 nm	7926 ± 1782
	3656 nm	1479 ± 110
Emulsion		3372 ± 416

Male Wistar rats were i.v. injected 4 mg/kg α-tocopherol. Data are presented as the mean ± S.D. of three rats.

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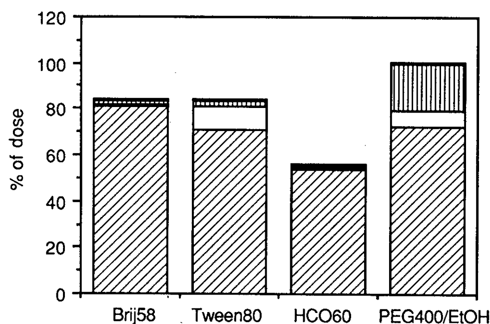

Fig. 2. Tissue Distribution of α-Tocopherol at 2 h after i.v. Administration of Micelle or Water-Cosolvents Formulation to Rats

Data are presented as the mean of three rats. ▨, liver; □, spleen; ■, kidney; ▥, lung.

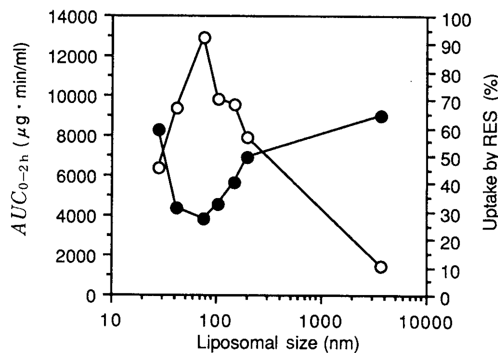

Fig. 5. Influence of Liposomal Size on AUC and Uptake by RES (Liver, Lung, Spleen)

—○—, AUC; —●—, RES.

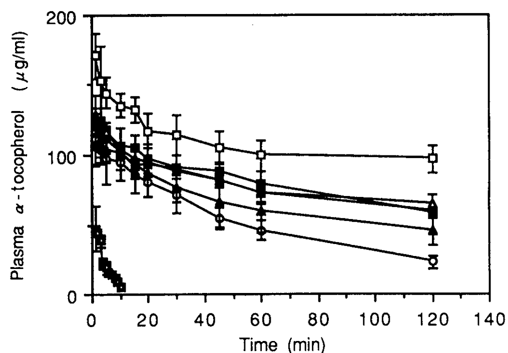

Fig. 3. Plasma Concentration of α-Tocopherol after i.v. Administration of Liposome Formulation

Data are presented as the mean ± S.D. of three rats. —○—, 28 nm; —●—42 nm; —□—, 75 nm; —■—, 106 nm; —△—, 151 nm; —▲—, 195 nm; —◘—, 3656 nm.

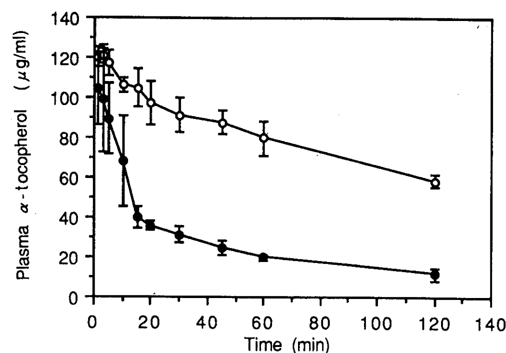

Fig. 6. Plasma Concentration of α-Tocopherol after i.v. Administration of Liposome or Emulsion Formulation

Data are presented as the mean ± S.D. of three rats. —○—, liposome (106 nm); —●—, emulsion (101 nm).

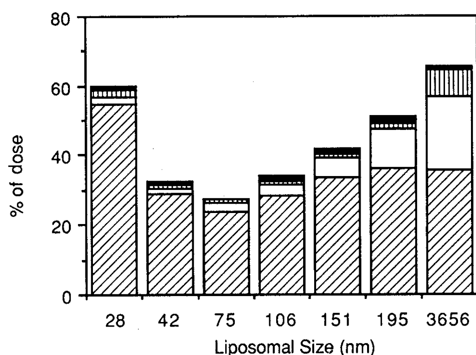

Fig. 4. Tissue Distribution of α-Tocopherol at 2 h after i.v. Administration of Liposome Formulation to Rats

Data are presented as the mean of three rats ▨, liver; □, spleen; ■, kidney; ▥, lung.

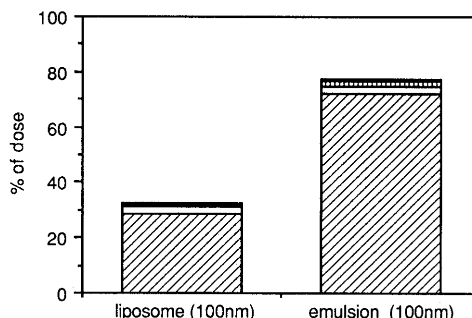

Fig. 7. Tissue Distribution of α-Tocopherol at 2 h after i.v. Administration of Liposome or Emulsion Formulation to Rats

Data are presented as the mean of three rats. ▨, liver; □, spleen; ■, kidney; ▥, lung.

intravenous administration were determined by HPLC. Figure 3 and Table I show the time course of plasma concentration and AUC, respectively. The blood clearance of 75 nm liposomes was slowest, and the 2 h AUC of 75 nm liposomes was greatest in all liposomes. Figure 4 shows the tissue distribution of α-tocopherol. The amount of α-tocopherol in RES (total of lungs, spleen and liver) at 2 h after intravenous administration was minimum when the liposome size was 75 nm. The uptake by the spleen was increased depending on the liposome size from 42 to 3656 nm. The uptake by the lungs was maximum when the liposome size was 3656 nm. On the other hand, the uptake by the liver was maximum in our studies when the liposome size was 28 nm. Figure 5 summarized the influence of liposome size on AUC and the uptake by RES. It is clear from the data in Fig. 5 that there is a significant correlation between AUC and the uptake by RES.

Plasma Concentration and Tissue Distribution of α-Tocopherol of the Emulsion Formulation The emulsion with an average diameter of 100 nm was rapidly decreased in the blood compared to the same size liposomes (Fig. 6). AUC of emulsions was about one third of that of the

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Table II. Particle Size of Micelles and Liposomes

Formulation		Mean ± S.D. (nm)
Micelles	Brij58	9.2 ± 1.0
	Tween80	9.6 ± 1.1
	HCO60	10.0 ± 2.2
Liposomes	28 nm	28.4 ± 7.9
	42 nm	42.2 ± 4.0
	75 nm	74.5 ± 4.0
	106 nm	105.8 ± 10.5
	151 nm	151.4 ± 3.8
	195 nm	194.5 ± 11.7
	3656 nm	3655.6 ± 171.3
Emulsion		100.5 ± 21.8

Data are presented as the mean ± S.D. of three experiments.

liposomes (Table I). Figure 7 shows the distributions of α-tocopherol of emulsions or liposomes. At 2 h after administration of emulsions, 72% of the dose was accumulated in the liver and 76.3% of the dose was taken up by RES (total of lungs, liver and spleen) in the case of emulsions. On the other hand, only 32.7% of the dose was taken up by RES in the case of liposomes.

Particle Size of Micelles, Liposomes or Emulsions Table II shows the particle size of micelles, liposomes or emulsions containing α-tocopherol measured by a dynamic laser light scattering method.

Discussion

There are many proteins in the serum. Among them, the lipoproteins play an important role as carriers of cholesterols and triglycerides in the serum. Receptors for ApoB, ApoE or ApoA-I included in chylomicrons, low density lipoproteins (LDL) or high density lipoproteins (HDL) exist in the liver.[30] Interactions between the lipophilic drug and serum proteins are considered to be an important factor in the disposition and the metabolism of the lipophilic drug after intravenous administration.

Yamaguchi *et al.* have reported that micelles of CoQ_{10} with HCO60 did not decrease from the blood for 2 h after intravenous administration to rats.[6] On the other hand, emulsions of CoQ_{10} with soybean oil and egg PC decreased rapidly from the blood. By contrast, the micelles of α-tocopherol with HCO60 decreased smoothly from the blood during the 2 h after intravenous administration in our study. These differences were considered to be attributed to the difference in the amount of surfactant used for the micelle formulation. Yamaguchi *et al.* used 7 mg of HCO60 per 1 mg of CoQ_{10} in 1 ml of solution. On the other hand, we used 150 mg of HCO60 per 4 mg of α-tocopherol in 1 ml. We used a 20 times larger amount of HCO60 than that used by Yamaguchi *et al.* We speculate that serum proteins were taken up by micelles, and then mixed micelles were formed because these micelles had an excess amount of surfactants. These mixed micelles might be rapidly taken up by the liver through the receptors for serum proteins. This manner was dependent on the kind of surfactant. The structure of the surfactant may affect the clearance of micelles. The number of polyoxyethylene (POE) groups was 20, 20 and 60 for Tween80, Brij58 and HCO60, respectively. Brij58 has a straight POE chain, but Tween80 and HCO60 have a branched one. Recently Mori *et al.* reported that a long POE chain was more effective to prolong the circulation time in the blood of egg PC liposomes than a short length POE.[31] HCO60 had a very similar effect. Tween80 micelles were more slowly removed from the blood than Brij58 micelles. The branched POE chain may be effective to coat the surface of the micelles containing α-tocopherol. Helenius and Simons reported that Triton X-100 took up apoproteins from low density lipoprotein, and the amount of the apoproteins taken up was 2 g per 1 g of TritonX-100.[32] Brij58 has the straight POE chain similar to TritonX-100 micelles. Brij58 micelles may take up apolipoproteins from LDL similar to TritonX-100 micelles. The micelles that contain apolipoproteins may be recognized by the LDL receptors that exist in the liver and then internalized by endocytosis.

Of the α-tocopherol in organic cosolvents formulation, 20% was distributed in the lungs. In general, the particles with a diameter of more than 7 μm are easily taken up by the lungs.[33] In our studies, oil droplets of α-tocopherol with a diameter of more than 10 μm are formed by the dilution of organic cosolvent formulation with the blood after intravenous administration. We have observed the formation of oil droplets more than 10 μm of α-tocopherol by the dilution with the phosphate buffered saline containing 5% of bovine serum albumin by using the particle analyzer (data were not shown).

Small liposomes are cleared from the circulation at a slower rate than large liposomes.[10,11,13] Allen *et al.* reported the role of the liposome size on the circulation of liposomes in mice.[23] In their report, the blood/RES ratio increased with decreasing liposome size down to about 80 nm, but the blood/RES ratio of about 50 nm liposomes was lower than that of 80 nm liposomes. They speculated that 50 nm liposomes could penetrate the liver sinusoids and gain access to the hepatocytes. In our studies, the *AUC* of 80 nm liposomes was the maximum and the distribution of the 80 nm liposomes to the liver, the spleen and lungs was lowest. The disposition of liposomes in the liver was increased when the liposome size was reduced from 75 to 28 nm. More 28 nm liposomes were distributed to the liver than 195 nm liposomes in spite of their similar *AUC*. These findings suggest that the uptake of liposomes by the liver cannot be explained by the physical trapment such as the filtration by RES alone. On the other hand, the increase in the uptake of liposomes by the spleen depended on the liposome size. This suggests that the uptake of liposomes by the spleen is caused by a physical interaction like the filtration mechanism. In the case of the lungs, significantly, more 3656 nm liposomes than smaller liposomes were distributed to the lungs, which is in agreement with the previous report that demonstrated that the critical size of the liposomes trapped by the lungs is about 3 μm.[34]

The clearance from the blood and the distribution of liposomes have been considered related to the opsonization by plasma proteins.[35] There are many reports that plasma proteins bind to the surface of liposomes or emulsions and play an important role in their metabolism.[36-39] The opsonized liposome was considered to have bound to the receptor (ApoB : E receptor, *etc.*) in the liver and the liposomes taken up by the liver.[40] Tajima *et al.* reported that ApoA-I bound to the surface of a small emulsion (27 nm diameter) more easily than a large one (230 nm diameter).[41]

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Liu *et al.* reported that a larger amount of plasma proteins bound to a small DOPE/OA liposome than to a large one.[42] We consider that the amphiphilic proteins bind to the small lipid particle easily because the small lipid particles have a low surface pressure. The 28 nm liposomes were bound with ApoE or other plasma proteins, and the receptor mediated uptake of liposomes by the liver occurred. The lipid transfer from liposomes to HDL seems to have occurred to degrade the liposomes in the blood. Further experiments are needed to explain these phenomena.

PC/soybean oil emulsions 100 nm in size were rapidly removed from the circulation and were distributed to the liver compared to the same size liposome. Mims *et al.*[43] and Funahashi *et al.*[44] reported an interaction of ApoE and emulsion. Yamaguchi *et al.* reported that emulsions containing CoQ_{10} decreased rapidly from the blood in rats when the amount of soybean oil was increased.[6] Amphiphilic plasma proteins may be able to bind onto the surface of emulsions more easily than liposomes because emulsions containing soybean oil and PC have a more hydrophobic surface than liposomes.

We have discussed the blood clearance and the tissue distribution of various formulations for α-tocopherol on the basis that the interaction between plasma proteins and various formulations is an important factor. Of course, the difference of formulation may affect the distribution of α-tocopherol in blood components such as erythrocytes or platelets. Stabilities of various formulations of α-tocopherol in the blood circulation may be different and are thought to be important for the blood clearance and the tissue distribution after intravenous administration. Further experiments are needed to obtain a full detail of these phenomena.

Finally we summarized *AUC* and RES uptake of various formulations of α-tocopherol after intravenous administration. The *AUC* ratio based on organic cosolvents formulation after intravenous administration of various formulations of α-tocopherol were calculated as follows; 75 nm liposome: 212 > 106 nm liposome: 161, 151 nm liposome: 156, 42 nm liposome: 153 > 195 nm liposome: 130 > 28 nm liposome: 104 ≫ HCO60 micelle: 65, 100 nm emulsion: 55 > Tween80 micelle: 26, 3656 nm liposome: 24 ≫ Brij 58 micelle: 8.5 ≫ organic cosolvents: 1.

The uptake by RES at 2 h after intravenous administration was in the decreasing order of organic cosolvents: 100% > Tween80 micelle, Brij58 micelle: 83% > 100 nm emulsion: 76% > 3656 nm liposome: 64%, 28 nm liposome: 59%, HCO60 micelle: 56% > 195 nm liposome: 49% > 151 nm liposome: 41% > 106 nm liposome: 33%, 42 nm liposome: 31% > 75 nm liposome: 27%.

In our experiments, 75 nm liposome is the best formulation for prolonged circulation of α-tocopherol in the blood. However, organic cosolvents formulation is not efficient. Organic cosolvents formulation, micelle formulation, 3656 nm liposome and 28 nm liposome are suitable for the target delivery of α-tocopherol to RES. Nevertheless, organic cosolvents formulation of a water insoluble drug such as α-tocopherol must be used carefully because particles of the drug may be produced in the blood.

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