Modification of Liposomes by Addition of HCO60. I. Targeting of Liposomes to Liver by Addition of HCO60 to Liposomes

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The influence of HCO60 on the blood clearance and tissue distribution of soybean phosphatidylcholine (PC) liposomes, encapsulating α -tocopherol as a marker, was studied in rats. The liposomes were prepared by the hydration method from a lipid film containing different amounts of HCO60, and by extrusion through a 0.1 μ m polycarbonate membrane filter. The blood clearance and liver uptake of α -tocopherol after i.v. administration increased with increasing the amount of HCO60 the liposome contained. With 80 wt% HCO60 liposomes, the accumulation of α -tocopherol in the liver was approximately three-fold that of the 100% PC liposomes. The uptake by lungs, spleen and kidneys did not change with the addition of HCO60. The findings obtained in a gel-filtration study suggested that α -tocopherol is not removed from the liposomes, with or without HCO60, by rat plasma proteins and the HCO60 micelle may form a complex with rat plasma proteins. Our findings suggest that liposomes containing large amounts of HCO60 (more than 60 wt%) will be useful for delivering drug to the liver.

Keywords liposome; nonionic surfactant; liver; targeting; α -tocopherol

Liposomes have been considered as a carrier for delivering drugs to the target organ and many studies have been reported on the targeting of drugs to the liver using liposomes. There are many asialoglycoprotein receptors in the liver parenchymal cell that can recognize the galactose residue and several methods for modifying the liposomal surface with glycolipids or glycoproteins that have a galactose residue have been reported.^{1–4)} Recently, Tsuji *et al.*⁵⁾ reported the targeting of liposomes to the liver using liposomes modified with a glycyrrhizin derivative. It has also been reported that targeting of liposomes or low density lipoproteins (LDL) to the liver has been achieved using cholesterol derivatives of galactose.⁶⁾ Bijsterbosch *et al.* reported that more of the apoprotein of LDL accumulated in Kupffer cells than in parenchymal cells in the liver when apoprotein was conjugated reductively with lactose.⁷⁾

In addition, many interesting findings have been obtained from *in vitro* studies. Funato *et al.*⁸⁾ investigated the interaction of liposomes composed of hydrogenated egg phosphatidylcholine with complements inducing phagocytosis. Kronberg *et al.*⁹⁾ reported the incorporation of Tween 80 into liposomal membrane, the liposomes being taken up by the macrophage-like cell (J774) *in vitro*. Muller *et al.*^{10,11)} studied the blood clearance and tissue distribution of microcapsules with nonionic surfactants after their intravenous administration in rats. In their report, the uptake of microcapsules by the liver was decreased by the addition of nonionic surfactants, while the uptake of microcapsules by spleen and lung increased. It has been reported that coating latex particles with poloxamers prolonged the blood levels of the latex particles after i.v. administration.^{12–14)} These findings suggest that hydrophilic polyoxyethylene residues prevent the particles from opsonization, and this prolongs their life. Recently, the use of polyoxyethylene derivatives has been reported for preventing liposomes being taken up by the reticuloendothelial system (RES).^{15–20)} The report by Kronberg *et al.* seems to be anomalous with respect to these prolongation effects in the blood by polyoxyethylene residues. They discussed the stabilization of liposomes by the addition of Tween 80,

but they did not describe any interaction between liposomes and blood components.⁹⁾

We previously reported that HCO60 micelles containing α -tocopherol were selectively taken up by the liver after i.v. administration.²¹⁾ For the investigation described here, we studied the influence of HCO60 (nonionic surfactant) on blood clearance and tissue distribution in rats after the i.v. administration of liposomes encapsulating α -tocopherol as a model drug. Liposomes consisting of more than 60 wt% HCO60 significantly accumulated in the liver.

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. HCO60 and soybean phosphatidylcholine (soybean PC; Epikuron200) were purchased from Nikko Chemicals. Ammonium molybdate was purchased from Wako Pure Chemical Industries Ltd.

Preparation of α -Tocopherol Micelle Formulation For the HCO60 micelle preparation, 20 mg α -tocopherol and 500 mg HCO60 were dissolved in 40 ml methanol. These solvents were dried under vacuum in glass test-tubes. After addition of 5 ml distilled water, α -tocopherol/surfactant micelles were formed by vortex mixing.

Preparation of Liposomes Multi-lamellar vesicles (MLV) were prepared by the method of Bangham *et al.*²²⁾ Forty milligrams α -tocopherol, soybean PC and HCO60 (total amount of PC and HCO60 was 1 g) were dissolved in 5 ml 2-propanol, and the solvent was dried in vacuum in a glass test-tube. After addition of 9 ml distilled water, lipid films were hydrated by repeated vortex mixing. The MLV suspension was passed ten times through a polycarbonate membrane filter (Nuclepore, pore size 0.1 μ m, Nomura Micro Science Ltd.) to size the liposomes by the method of Hope *et al.*²³⁾

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

Measurement of Zeta Potential Zeta potentials of liposomes and micelles were determined in 0.05 M pH 7 phosphate buffer solution by electrophoretic measurements using a dynamic laser light-scattering detection electrophoretic apparatus (ELS-800, Otsuka Electronics Ltd.).

Electron Microscopy Negative-stain electron microscopy was performed according to the method of Magin and Weinstein.²⁴⁾ Carbon-coated copper grids (150-B mesh) were purchased from Ohken Shoji Ltd. A drop of liposome was placed on the grid for 1 min and then removed. A drop of 2% ammonium molybdate was placed on the grid and removed after 8 min. The grid was air dried and then examined by electron microscopy (Hitachi H-7000, 75 kV of voltage).

Animal Experiments Animal experiments were performed as described in a previous report.²¹⁾ 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. A series of 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 2 h after administration of the different formulations and their liver, lungs, spleen and kidneys were collected.

Analysis of α -Tocopherol Blood samples were heparinized and, after centrifugation (3000 rpm–15 min), plasma was obtained. One ml methanol was added to 0.15 ml plasma 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 under vacuum in a glass test-tube, and α -tocopherol was reconstituted with 0.25 ml HPLC mobile phase as described below. α -Tocopherol was determined by HPLC analysis. The recovery of α -tocopherol from plasma was about 100% as determined by recovery tests. Endogenous α -tocopherol was measured before each rat experiment.

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

For the α -tocopherol determination by HPLC, a Shimadzu HPLC system was used consisting of a LC-9A Liquid Chromatograph, an RF-530 fluorescence HPLC monitor, an SCL-6B system controller and a model C-R6A chromatopac. Separation was carried out on a 4.6 $\times$ 250 mm LiChrosorb[®] RP-8, 10 μ m, column and the guard column was a 4 $\times$ 25 mm YMC ODS-A, 5 μ m. The mobile phase was a mixture of methanol and 0.2 M pH 4.0 acetate buffer (92:8). Detection of α -tocopherol was carried out at excitation and emission wavelengths of 286 and 330 nm respectively.

Binding Studies Removal of lipids from rat plasma was performed with ether as described by Scanu *et al.*,²⁵⁾ and then powdered plasma proteins were obtained by drying with N₂ gas and under vacuum. The powdered proteins were reconstituted with distilled water. Insoluble particulate matter were removed by centrifugation (3000 rpm–10 min at 5 °C) and then the supernatant was filtered through polysulfone membrane (0.2 μ m, Filterite). The plasma proteins were transferred to polypropylene tubes and stored at –80 °C. The protein concentration in solution was 40 mg/ml, determined using the Bio-Rad Protein assay dye reagent (Bio Rad).

For binding studies, frozen protein solution was thawed at room temperature. Then 0.5 ml liposome solution was added to 0.5 ml plasma protein solution and the mixture incubated at 37 °C for 60 min. After incubation, 0.1 ml of the mixture was immediately subjected to molecular sieve chromatography on a Sepharose CL-6B (Pharmacia) column (1.5 $\times$ 20 cm), equilibrated with Dulbecco phosphate buffered saline without Mg²⁺ and Ca²⁺, at 25 °C. The eluate was collected using a fraction collector (3 ml/fraction).

One ml of each fraction was subjected to HPLC analysis (100 μ l injection loop) to measure α -tocopherol.

1.5 ml of each fraction was transferred to a glass test-tube and then 3 ml ethanol and 1 ml ether were added. Organic solvent/buffer solutions were dried in vacuum to remove water. After drying, 3 ml ethanol and 1 ml ether were added to each test-tube and sonication carried out using a bath-type sonicator. Then the suspensions were centrifuged (3000 rpm–10 min) to separate protein precipitates from the lipid solutions and α -tocopherol and the supernatants collected. This separation procedure was performed twice. Protein precipitates were dissolved in 0.1 ml 0.02 N HCl after drying with N₂ gas and under vacuum, and then 5 ml of a 5-fold aqueous dilution of Bio-Rad Protein assay dye reagent (Bio Rad) was added to the protein solution for the determination of the protein concentration. For addition, the supernatants containing the lipids were dried *in vacuo* and reconstituted with 1 ml of methanol. Lipid concentrations were determined by enzyme assay (Determiner PL, Kyowa Medex 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 shown as the means $\pm$ standard deviation (σ_n).

Results

Physical Properties of the PC Liposome, PC/HCO60 Liposomes or HCO60 Micelles Figure 1 shows the electron micrograms of liposomes using the negative-staining method. Partial lipid lamella were found in the liposome containing 90% HCO60. Table I shows the particle size and zeta potential of the liposome or micelle. The mean size of the liposomes containing 0–80% HCO60 was approximately 100 nm. The size of the liposomes containing 90% HCO60 was 64 nm. The zeta potential of the liposomes containing 0–90% HCO60 was approximately constant

TABLE I. Physical Properties of HCO60/Soybean PC Liposome or HCO60 Micelle Containing α -Tocopherol

HCO60 (%)	Size (nm)	Zeta potential (mV)
0	94.0 $\pm$ 13.7	2.02 $\pm$ 1.80
10	105.5 $\pm$ 5.3	0.46 $\pm$ 2.83
50	98.6 $\pm$ 3.9	—
60	92.7 $\pm$ 4.5	4.98 $\pm$ 1.03
70	92.4 $\pm$ 7.3	2.27 $\pm$ 2.84
80	95.0 $\pm$ 11.8	—
90	64.1 $\pm$ 14.4	3.24 $\pm$ 0.60
100	43.8 $\pm$ 4.8	4.92 $\pm$ 0.55

Data presented are means $\pm$ S.D. from more than three experiments.

Fig. 1. Electron Microscope Images of Liposomes Containing Various Amounts of HCO60 by Negative Staining

Magnification, $\times$ 100000; size of bar, 100 nm. A, 100% PC; B, 60% HCO60; C, 90% HCO60.

(0.5–5 mV). The mean size and zeta potential of the HCO60 micelle were 43.8 nm and 4.9 mV, respectively.

Plasma Concentration and Tissue Distribution Liposomes

Fig. 2. Plasma Concentration of α -Tocopherol after i.v. Administration of Liposomes Containing Different Amounts of HCO60 or HCO60 Micelles to Rats

Data presented are means from more than three rats. ●, HCO60 0%; ○, HCO60 10%; ◆, HCO60 50%; ◇, HCO60 60%; ■, HCO60 70%; □, HCO60 80%; ▲, HCO60 90%; △, HCO60 100%.

Fig. 3. Tissue Distribution of α -Tocopherol 2h after i.v. Administration of Liposomes Containing Different Amounts of HCO60 or HCO60 Micelles to Rats

Data presented are means from more than three rats. ●, liver; ○, spleen; ▲, lung; △, kidney.

Fig. 4. Relationship between AUC and Liver Uptake

Data presented are means $\pm$ S.D. from more than three rats. ●, AUC; ○, liver uptake.

some composed of PC, with or without HCO60, and HCO60 micelles containing α -tocopherol were prepared and then the plasma concentrations and tissue distribution of α -tocopherol after i.v. administration were determined by HPLC. Figure 2 shows the time-course of the plasma concentration. The blood clearance of liposomes containing more than 60% HCO60 was greater than that of 100% PC liposomes. Figure 3 shows the tissue distribution of α -tocopherol. The accumulation of α -tocopherol in the liver after intravenous administration increased with increasing content of HCO60, above 60%. The amount of α -tocopherol accumulating in the liver with liposomes containing 80% HCO60 was approximately three folds greater than that seen with 100% PC liposomes. The HCO60 content did not affect the uptake of liposomes by the spleen, lungs and kidneys. There was no difference in accumulation in the liver or blood clearance when 80% HCO60, 90% HCO60 liposomes, and HCO60 micelles were compared. Figure 4 summarizes the relationship between AUC and the liver

Fig. 5. Gel Filtration Profile of 100% PC Liposomes, 60% HCO60 Liposomes, or HCO60 Micelles

A, 100% PC liposomes; B, 60% HCO60 liposomes; C, HCO60 micelles. ●, α -tocopherol; ○, PC.

Fig. 6. Gel Filtration Profile of 100% PC Liposomes, 60% HCO60 Liposomes, or HCO60 Micelles after Incubation with Rat Plasma Protein at 37°C

A, 100% PC liposomes; B, 60% HCO60 liposomes; C, HCO60 micelles. ●, α -tocopherol; ○, PC; ▲, proteins; △, rat plasma proteins only.

TABLE II. Amount of Plasma Protein in Liposome or Micelle Fraction

HCO60 (%)	Protein (μ g/fraction No. 5, 6)
0	181
60	152
100	456

uptake of α -tocopherol. The AUC decreased as the uptake of α -tocopherol by the liver increased.

Incubation with Rat Plasma Protein Figure 5 shows the initial gel-filtration profile of PC liposome, 60% HCO60 liposome, and HCO60 micelle formulation before incubation with plasma proteins. Almost 100% of the α -tocopherol was detected in the liposome or micelle fraction.

Figure 6 shows the gel-filtration profile of the three formulations after incubation with rat plasma proteins at 37°C for 60 min. In the case of liposome formulations,

α -tocopherol existed in the liposome fraction without transfer to proteins. In the case of micelles, the fraction with the maximum amount of α -tocopherol shifted from the 5th to 7th. This suggests that the size distribution had broadened by formation of a complex between micelle and rat plasma protein.

Table II shows the amount of rat plasma proteins detected in the liposome or micelle fractions. There was no significant difference between liposome formulations, but a large amount of protein bound to the micelle.

Discussion

The size of the liposomes containing 0–80% HCO60 was maintained at a diameter of approximately 80–90 nm. The size of the liposome containing 90% HCO60 was about 64 nm. The 90% HCO60 liposome may be an emulsion. Nevertheless, partial lamellae were found in liposomes containing 90% HCO60 (Fig. 1). The zeta potential of HCO60-containing liposomes was not as large as that of liposomes bearing PEG1900-DSPE (7.5% PEG1900-DSPE: -8 mV²⁶). The plasma concentration of α -tocopherol in the liposomes decreased rapidly with increasing HCO60 content while liver uptake increased with the increasing in HCO60 content. The size of the liposomes used in this study was approximately 80–90 nm, except for the 90% HCO60 containing liposomes; this size of liposome is suitable for prolonged circulation as described previously.²¹ This size should be able to pass through the fenestration of the liver sinus since the average diameter of the holes there is approximately 100 nm.

The micelle used in this study should be able to pass through the fenestration in the liver, because its size is approximately 40 nm. The mechanism of micelle uptake by the liver may be related to opsonization by proteins in the blood. The study of the incubation of the three formulations with rat plasma proteins suggests that micelles form a complex with the plasma proteins as described below. The binding of protein to the micelle may play a role in the opsonization of micelles and receptors in the liver, parenchymal and non-parenchymal cells, recognize the proteins. After binding to the receptor, the micelle is taken up by the liver. This receptor-mediated uptake of liposomes by the liver has been considered to be enhanced by binding to apolipoproteins in the blood.^{27,28} Recently, C3, one of the complements, has been reported to be involved in the opsonization of liposomes²⁹ and fibronectin is thought to be an important factor in the opsonization of microparticles in the blood.³⁰

The gel-filtration technique has been reported to be a useful method for analysis of insulin-binding on the surface of liposomes.^{31,32} Large amounts of protein bound to 100% PC liposomes were not observed (Table II). Senior *et al.*¹⁹ reported that the binding of plasma proteins to liposomes was not strong, and the binding proteins could be removed from the liposome surface after gel-filtration; this explains our findings. There was no lipid transfer from the liposome. Therefore, rat plasma proteins did not contain any lipoproteins such as high density lipoprotein (HDL) or LDL because lipids had been removed from the rat plasma protein.

In the case of micelle formulation, the fraction with the maximum amount of α -tocopherol shifted from the 5th to

7th. This suggests that the size distribution of the micelles had broadened, or the electrostatic properties of the micelles had been changed by interaction of plasma proteins with the micelle. Proteins easily bind to non-ionic surfactants.³³⁾ This complex formation seems to be related to the liver uptake.

Coating of colloidal particles with non-ionic surfactants had been reported to suppress RES uptake of such particles. Muller *et al.*¹⁰⁾ demonstrated the prevention of serum protein-binding with nanoparticles using non-ionic surfactants, such as poloxamer 338, poloxamer 407, polysorbate 80, polysorbate 20, and POE (20) LE, using measurement of zeta potential. They also showed that all surfactants, except for POE (20) LE, suppressed the uptake of particles by the liver. On the other hand, Chonn *et al.*²⁹⁾ reported that the lipid composition of liposomes affected the amount and nature of the binding to the surface of the liposome. HCO60 may affect the binding properties of blood components to the surface of the liposome. We previously reported a high uptake of micelle formulations of α -tocopherol with nonionic surfactants.²¹⁾

We believe that the liposome-bearing HCO60 will be a useful carrier for liver-directed drugs such as α -tocopherol, insulin, and glutathione.

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