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Liposomal Sustained-Release Delivery Systems for Intravenous Injection. II. Design of Liposome Carriers and Blood Disposition of Lipophilic Mitomycin C Prodrug-Bearing Liposomes

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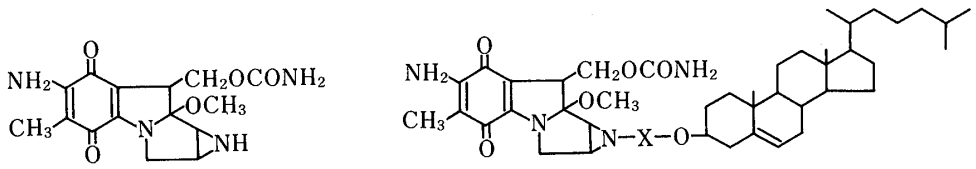
Various types of unilamellar liposomes possessing a narrow size distribution were prepared by controlled dialysis and their blood clearance was studied in mice and rats to assess their suitability as drug carriers for intravenously injectable liposomal sustained-release delivery systems. Also, the utility of these liposomal carrier systems combined with lipophilic prodrugs of mitomycin C (MMC) was evaluated. The fate of the liposomes was monitored using *N*-4-nitrobenzo-2-oxa-1,3-diazole-dipalmitoylphosphatidylethanolamine (NBD-PE), a liposomal membrane marker. The effect of vesicle size on the blood clearance was investigated for neutral liposomes. Small-sized (S-) liposomes (90 ± 15 nm) were cleared slowly compared with medium-sized (M-) liposomes (181 ± 31 nm) and large-sized liposomes (281 ± 38 nm). Surface charge was also an important determinant of the disposition of small-sized liposomes. S-Liposomes were retained in the circulation longer than positively (S^+-) and negatively (S^--) charged liposomes. The integrity of S-liposomes in the circulation was examined by simultaneous determination of NBD-PE and carboxyfluorescein (CF) entrapped in the liposomal membrane and liposomal aqueous phase, respectively. CF administered in the liposome-encapsulated form was cleared slowly in a fashion similar to NBD-PE, while free CF, administered as an aqueous solution, was rapidly removed from the circulation. These results reveal that S-liposomes show the best pharmacokinetic properties as a carrier vehicle for intravenously injectable sustained-release delivery systems. S-Liposomes loaded with the lipophilic MMC prodrug, *N*-(cholesteryloxycarbonyl)glycyl MMC or cholesteryloxyacetyl MMC, successfully gave sustained blood levels of the parent drug following intravenous injection. Thus, the potential utility of MMC prodrug-bearing S-liposomes as an intravenously injectable MMC sustained-release dosage form was demonstrated.

Keywords——liposome; controlled dialysis; narrow size distribution; surface charge; vesicle size; blood clearance; sustained release; intravenous injection; mitomycin C; lipophilic prodrug

In cancer chemotherapy, it is necessary to control the pharmacokinetics of cytotoxic agents for effective treatment, and a great deal of effort has been made to improve the therapeutic efficacy of such drugs by encapsulation within liposomes.[1] However, there still remain some problems in the systemic application of drug-bearing liposomes. The first is the biological disruption of intravenously-injected liposomes by the attack of high density lipoprotein[2] and other components.[3] The second problem is their rapid clearance by uptake through the reticuloendothelial system (RES) in such organs as the liver and spleen.[4] A predominant RES uptake might be convenient if the RES is a target site,[5] but it is generally a drawback in the use of liposomes as carriers for controlled release of a drug or for delivery of a drug to other target organs. For the latter, it would become necessary to overcome both problems for prolongation of the presence of intact liposomes in the circulation.

Through numerous studies on the biological disposition of liposomes, the size,[6] surface charge[7] and lipid composition[8] have been proved to be the important parameters affecting the fate of liposomes. Furthermore, it has been demonstrated that the use of well size-defined,



mitomycin C (MMC)

I : X=-$COCH_2NHCO$-
II : X=-$COCH_2$-

Fig. 1. Structures of Mitomycin C (MMC) and Its Lipophilic Prodrugs

homogeneous liposomes is essential to obtain reproducible results in disposition studies.[6a] Although a number of studies have been carried out in attempts to utilize liposomes as drug carriers for biological and therapeutical applications, only a little is known about the precise pharmacokinetic characteristics of well size-defined, unilamellar liposomes possessing a narrow size distribution.[6a,e]

In the present study, various types of homogeneous unilamellar liposomes of different sizes and/or surface charges were prepared by the controlled dialysis method[9] and their blood clearance was investigated to select optimal carrier systems for sustained-release intravenous (i.v.) injection. In addition, for practical use of these carrier systems, lipophilic prodrugs of mitomycin C (MMC),[10] *N*-(cholesteryloxycarbonyl)glycyl MMC (I) and cholesteryloxyacetyl MMC (II) (Fig. 1), were incorporated into the optimal liposome carriers and the utility of these preparations as an intravenously injectable MMC sustained-release dosage form was evaluated.

Experimental

Materials——Egg phosphatidylcholine (PC), egg sphingomyelin (SM) and bovine brain phosphatidylserine (PS) were purchased from Sigma Chemical Co. Stearylamine (SA) was from Tokyo Chemical Industries. Detergents were obtained from the following sources: sodium cholate, Ishizu Pharmaceutical Co.; sodium deoxycholate, Nakarai Chemicals; and 1-*o*-(*n*-octyl)-D-glucopyranoside, Sigma Chemical Co. *N*-4-Nitrobenzo-2-oxa-1,3-diazole-dipalmitoyl-phosphatidylethanolamine (NBD-PE) was prepared by the method of Monti *et al.*[11] Carboxyfluorescein (CF) was purchased from Eastman Kodak Co. and used without further purification. MMC was obtained from Wako Pure Chemical Industries. Prodrugs I and II were synthesized according to the method previously described.[10] All other chemicals were reagent grade or purer.

Preparation of Liposomes——Unilamellar liposomes were prepared largely according to the controlled dialysis method described by Zumbuehl and Weder.[9b] Liposomal lipid composition is summarized in Table I and all liposome preparations contained a trace amount of NBD-PE as a liposomal membrane marker. Lipids (150 μmol) were dissolved in chloroform–methanol (2 : 1, v/v) and the organic solvents were evaporated off under vacuum. The dried lipid film was suspended in 6 ml of pH 7.4 phosphate-buffered saline (PBS). Sodium cholate (375 μmol), sodium deoxycholate (375 μmol) or 1-*o*-(*n*-octyl)-D-glucopyranoside (1500 μmol) was added to the lipid suspension and the mixture was agitated by hand. The formed mixed micellar solution was dialyzed against PBS at 37 °C for 16 h using a Lipoprep dialyzer (Diachema). After completion of dialysis, the liposome suspension was purged with nitrogen gas and stored at 4 °C ready for use.

For encapsulation of CF within small-sized (S-) liposomes, the dried lipids were suspended in PBS containing 0.25 mM CF and sodium cholate was added. The resulting mixed micellar solution was dialyzed against PBS containing 0.25 mM CF as described above. Non-encapsulated CF was separated from liposomes by chromatography on a Sepharose CL-4B column (Pharmacia Fine Chemicals, 26 × 200 mm).

Prodrug-bearing S-liposomes composed of PC, SM and the prodrug (I or II) in a molar ratio of 7 : 3 : 1.33 were prepared in a similar manner. After completion of dialysis, the formed liposome suspension was sequentially extruded through 0.2 and 0.1 μm pore-sized polycarbonate membranes (Nuclepore) and subsequently sterilized by filtration through a Millex GV (0.22 μm, Millipore). The content of entrapped prodrugs was determined by high performance liquid chromatography (HPLC) as described previously.[10] The samples were diluted with sterile PBS to obtain the desired drug concentration (2.77 mM) and kept under nitrogen at 4 °C until use.

Determination of Vesicle Size——Liposome size was measured by the use of a dynamic laser light scattering instrument (Coulter, model N4).

Animal Experiments——For evaluating the blood clearance of different types of liposomes, female ICR mice weighing 22—25 g were injected with 0.2 ml of the liposome preparations containing 2 μmol of lipids into the tail vein. At various times after injection, blood was obtained by cardiac puncture. The blood samples (0.5 ml) were immediately frozen in liquid nitrogen and lyophilized.

Male Sprague–Dawley rats weighing 220—250 g were used in *in vivo* stability studies of S-liposomes. CF in either free or liposome-encapsulated form was injected into ether-anesthetized rats *via* the femoral vein at a volume of 1 ml/rat. At various time periods post injection, blood samples (0.25 ml) were taken from the jugular vein and freeze-dried.

For evaluating the blood disposition of liposome-entrapped MMC prodrugs, male Sprague–Dawley rats weighing 240—300 g were used. The animals were anesthetized with ether and injected with 1 ml of prodrug-bearing liposome preparations at a dose of 2.77 μmol prodrug/rat into the femoral vein. At various times after injection, blood samples (0.6 ml) were withdrawn *via* the jugular vein. For simultaneous determination of the prodrugs and NBD-PE, an aliquot (0.15 ml) of the blood was immediately frozen in liquid nitrogen to avoid postcollection degradation of the prodrugs, and lyophilized. A residual aliquot of the blood was centrifuged to obtain plasma for assay of MMC. To avoid postcollection regeneration of MMC, an aliquot (0.2 ml) of the plasma samples was immediately frozen in liquid nitrogen and stored in a freezer until assay.

Extraction Procedure——NBD-PE and the prodrugs were extracted with ethanol from the freeze-dried blood samples as described previously.[10]

For simultaneous analysis of NBD-PE and CF, the lyophilized blood samples were homogenized in 3 ml of 0.05 M phosphate buffer (pH 7.0). To an aliquot (0.15 ml) of the homogenate, an equal volume of 10% Triton X-100 was added, and the mixture was agitated. The supernatant after centrifugation was taken for assay of CF. A residual aliquot of the homogenate samples was re-lyophilized for analysis of NBD-PE.

Extraction of MMC from the plasma was carried out as follows. The plasma samples (0.2 ml) were deproteinized by addition of cold acetonitrile (2 ml) at 0 °C soon after thawing in 1 ml of cold distilled water containing phenacetin (0.5 μg/ml) as an internal standard. After centrifugation, the supernatant was applied to a Sep-pak C_{18} cartridge (Waters) to remove the prodrugs. The eluent was evaporated and the residue was dissolved in 0.25 ml of distilled water for analysis of MMC.

HPLC Assay——NBD-PE, MMC and its prodrugs were determined by HPLC as described previously.[10] Phenacetin, an internal standard for analysis of MMC, was detected at a wavelength of 254 nm (Hitachi model 638).

CF was determined by means of a HPLC system equipped with a Waters model 510 pump, a Waters WISP 710B automatic sample injector and a Hitachi 650-10S fluorometer. CF was detected at excitation and emission wavelengths of 495 and 525 nm, respectively. The stationary phase used was a Develosil ODS-5 packed column (4.6 × 100 mm, Nomura Chemical Co.) and 15% (v/v) methanol in 0.05 M phosphate buffer (pH 7.0) was used as the mobile phase with a flow rate of 1.0 ml/min.

Results

Characterization of Liposomes

The size and lipid composition of liposomes used in the blood clearance study are summarized in Table I. On the basis of the vesicle size, liposomes were classified into three types: small-sized (S-), medium-sized (M-) and large-sized (L-) liposomes. All types of liposome preparations showed a fairly narrow size distribution. No significant differences in

Table I. Size and Lipid Composition of Liposomes Used in the Blood Clearance Experiments

Liposomes	Lipid composition	Diameter[a] (nm)
S-Liposomes	PC/SM (7:3)[b]	90±15
S^--Liposomes	PC/SM/PS (7:3:0.5)	88±17
S^+-Liposomes	PC/SM/SA (7:3:0.5)	87±16
M-Liposomes	PC/SM (7:3)	180±31
L-Liposomes	PC/SM (7:3)	281±38
S-Liposomes (CF)[c]	PC/SM (7:3)	102± 6

a) Liposome diameters were determined by dynamic laser light scattering and are expressed as the mean ± S.D. *b*) The values in parentheses represent the molar ratios of lipids. *c*) S-Liposomes encapsulating CF.

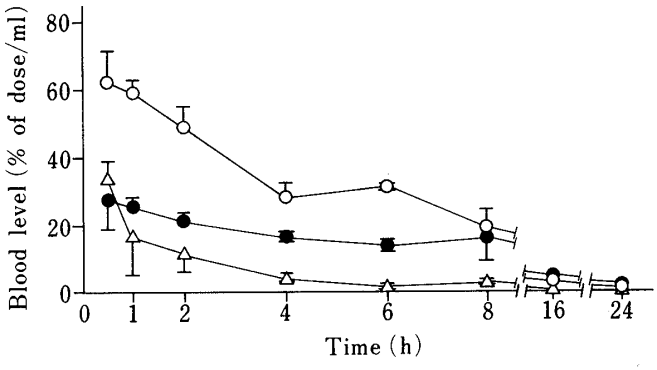


Fig. 2. Blood Levels of NBD-PE in Mice after i.v. Injection of S-, M- and L-Liposomes

○, S-liposomes; ●, M-liposomes; △, L-liposomes. Each result is the mean ± S.D. of four mice.

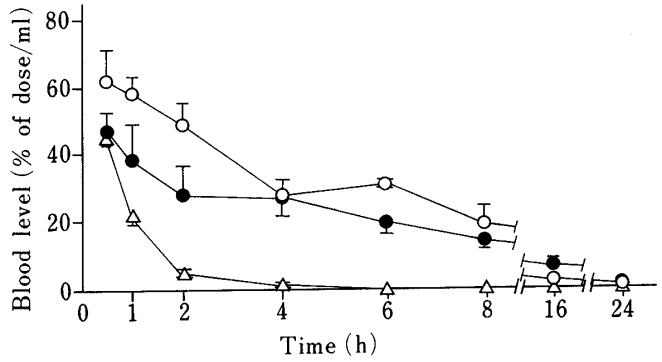


Fig. 3. Blood Levels of NBD-PE in Mice after i.v. Injection of S-, S^-- and S^+-Liposomes

○, S-liposomes; △, S^--liposomes; ●, S^+-liposomes. Each result is the mean ± S.D. of four mice.

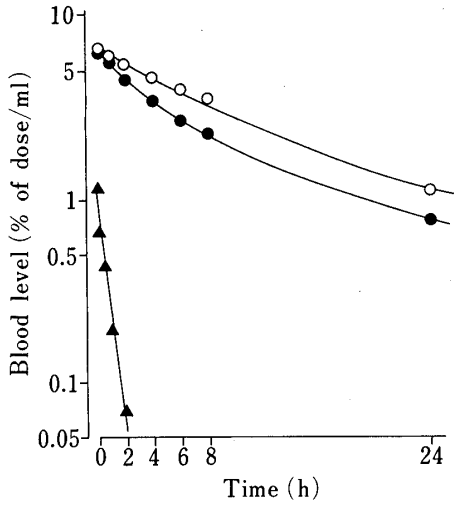


Fig. 4. Blood Levels of CF and NBD-PE in Rats after i.v. Injection of S-Liposomes Encapsulating CF

●, CF; ○, NBD-PE; ▲, CF (after i.v. injection in an aqueous solution). Each result is the mean of three rats.

size and size homogeneity were observed among small-sized liposomes possessing different surface charges.

Effect of Liposome Size on Blood Clearance

By monitoring NBD-PE, the blood clearance of liposomes following i.v. injection was studied in mice for three types of preparations of identical lipid composition but of different sizes. The results are shown in Fig. 2. The clearance of liposomes from the body circulation increased with increase of vesicle size. S-Liposomes exhibited a long lifetime and remained in the circulation at a high level for up to 8 h. On the other hand, L-liposomes were removed from the circulation within a few hours after i.v. injection.

Effect of Liposome Surface Charge on Blood Clearance

The clearance of small-sized liposomes differing in surface charge was investigated by determining NBD-PE, and the results are shown in Fig. 3. S-Liposomes were retained in the circulation longer than positively (S^+-) and negatively (S^--) charged liposomes. S^--Liposomes were most rapidly cleared and few remained in the circulation 6 h after injection.

Stability of S-Liposomes in the Systemic Circulation

To assess the integrity of S-liposomes in the systemic circulation, the blood levels of CF

and NBD-PE were simultaneously determined in rats intravenously injected with S-liposomes incorporating these markers. The results are shown in Fig. 4, together with the blood CF levels found after i.v. injection of CF aqueous solution. CF administered in liposome-encapsulated form was cleared slowly from the circulation in a fashion similar to NBD-PE, while free CF showed rapid clearance.

Characterization of MMC Prodrug-Bearing S-Liposomes

Table II summarizes the size of S-liposomes entrapping the lipophilic MMC prodrugs. Compared with plain S-liposomes (Table I), prodrug-bearing S-liposomes showed a slight increase in size, but still maintained size homogeneity.

Blood Disposition of MMC Prodrug-Bearing S-Liposomes

Figures 5 and 6 show the blood levels of the prodrugs, the MMC regenerated and NBD-PE in rats after i.v. injection of liposomes entrapping prodrugs I and II, respectively. The plasma clearance profile of MMC administered intravenously as an aqueous solution is included for comparison. Each concentration is expressed as a percent of dose/ml for comparison with liposome carriers. One percent of dose/ml is equal to 27.7 μM. MMC was

TABLE II. Size of S-Liposomes Entrapping Lipophilic MMC Prodrugs

Composition	Diameter[a] (nm)
PC/SM/prodrug I (7:3:1.33)[b]	119±23
PC/SM/prodrug II (7:3:1.33)	128±24

a) Liposome diameters were determined by dynamic laser light scattering and are expressed as the mean±S.D. *b*) The values in parentheses represent the molar ratios of lipids and prodrugs.

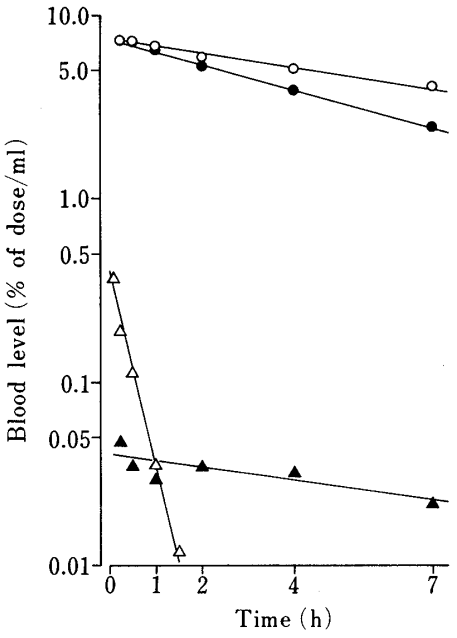


Fig. 5. Blood Levels of Prodrug I, Regenerated MMC and NBD-PE in Rats after i.v. Injection of S-Liposomes Entrapping Prodrug I

○, NBD-PE; ●, prodrug I; ▲, regenerated MMC; △, MMC (after i.v. injection in an aqueous solution). Each concentration of MMC and regenerated MMC is expressed as % of dose/ml plasma. Each result is the mean of three rats.

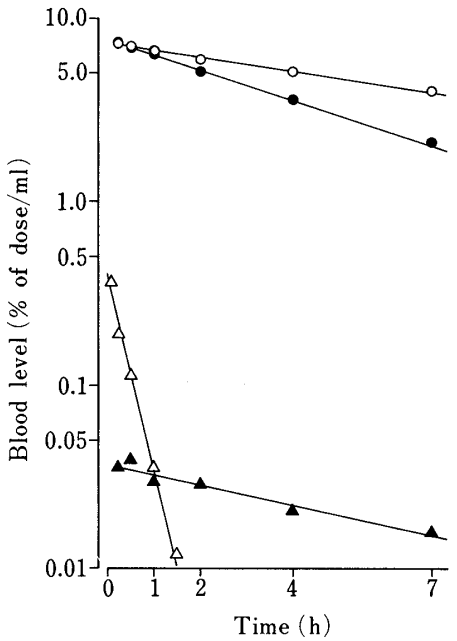


Fig. 6. Blood Levels of Prodrug II, Regenerated MMC and NBD-PE in Rats after i.v. Injection of S-Liposomes Entrapping Prodrug II

○, NBD-PE; ●, prodrug II; ▲, regenerated MMC; △, MMC (after i.v. injection in an aqueous solution). Each concentration of MMC and regenerated MMC is expressed as % of dose/ml plasma. Each result is the mean of three rats.

rapidly cleared from the circulation and little remained 2 h after injection of its aqueous solution. On the other hand, liposomes incorporating prodrug I showed sustained plasma levels of regenerated MMC ranging from 0.036% of dose/ml to 0.020% of dose/ml for the first 7 h after injection (Fig. 5). Prodrug I and NBD-PE were removed slowly from the circulation with half-lives of 4.2 and 7.9 h, respectively.

In the case of liposome-entrapped prodrug II, similar blood concentration–time profiles of the prodrug, regenerated MMC and NBD-PE were observed (Fig. 6).

Discussion

For successful application of drug-bearing liposomes to clinical therapy, it is important to design well-defined uniform preparations to obtain high reproducibility in pharmacokinetic behavior.[1e,6a] In the present study, we prepared liposomes by the controlled dialysis method devised by Milsmann *et al.*[9a] This method could be described as a kind of detergent removal method, and it provides unilamellar liposome preparations possessing a narrow size distribution. From the viewpoint of manufacturing, the method can be considered to be practical because of its good reproducibility and applicability to mass production.

As expected, liposomes used in this study showed quite a narrow size distribution (Table I). In the preparation method, the micellar lipid concentration, dialysis temperature and detergents used are the major size-determining factors. Of these, detergents proved to be the most important to control the size of liposomes. In the present study, sodium cholate, sodium deoxycholate and 1-*o*-(*n*-octyl)-D-glucopyranoside were chosen for the preparation of S-, M- and L-liposomes, respectively, because of adequate solubilization capacity and fast dialysis kinetics.

S-Liposomes were retained in the circulation over a considerable period of time, while M- and L-liposomes showed rapid clearance (Fig. 2). Slight differences in size ranging from 90 to 281 nm led to a drastic alteration in blood clearance of liposomes. Sato *et al.* have obtained similar results when investigating the blood clearance kinetics of negatively charged reverse-phase evaporation vesicles (REV) ranging from 150 to 430 nm.[6e] These findings reveal that it is of the utmost importance for the use of liposomes as drug carriers to control not only the mean diameter but also the size homogeneity.

As implied by several investigators,[7] surface charge is also an important factor in liposome disposition. As shown in Fig. 3, S-liposomes remained in the circulation longer than S^+- and S^--liposomes. This observation is in disagreement with the previous finding[6a] that no significant difference in the clearance was observed between positively charged and neutral small unilamellar vesicles having relatively narrow size distribution prepared by combined use of sonication and gel filtration. The rapid clearance of S^--liposomes might be due to the known tendency of vesicles to coalesce in the presence of protein and calcium ion.[12]

Stability is also an important parameter affecting the fate of liposomes.[8] In *in vivo* stability tests, liposome-encapsulated CF was cleared slowly from the circulation in a fashion similar to NBD-PE, while free CF showed rapid clearance (Fig. 4). These results indicate that S-liposomes are retained in the circulation, keeping the closed spherical structure. S-Liposomes composed of PC and SM in a molar ratio between 7/3 and 5/5 showed similar stability, while S-liposomes prepared from only PC were unstable in the presence of serum (unpublished data). SM is considered to participate in intermolecular hydrogen bonding[13] and to stabilize the membrane structure.[14] Addition of this component might inhibit phospholipid removal by high density lipoprotein. Cholesterol has been demonstrated to improve liposome stability.[8] However, cholesterol-containing liposomes of desired lipid composition (PC : cholesterol = 7 : 3) do not lend themselves to preparation by our methods.

On the basis of these results, S-liposomes proved to have the best pharmacokinetic

characteristics as a carrier vehicle for intravenously injectable sustained-release delivery systems.

In a previous study,[10] we reported that among the MMC derivatives tested, prodrugs I and II showed the best properties as regards liposome entrapment efficiency and lability. Furthermore, in contrast to previously reported lipophilic MMC prodrugs such as nonyloxycarbonyl MMC,[15] these compounds were firmly retained within the liposome carriers in the circulation. To assess the feasibility of employing S-liposomes loaded with compounds I or II as an intravenously injectable MMC sustained-release dosage form, a blood disposition study was performed. In the preparation by controlled dialysis, addition of the MMC prodrugs resulted in an increase of liposome size heterogeneity (unpublished data). By additional use of an extrusion technique,[16] however, relatively homogeneous liposome preparations were obtained (Table II).

As expected, i.v. injection of MMC prodrug-bearing liposomes resulted in well-maintained blood levels of the parent drug (Figs. 5 and 6). These results show the potential of S-liposomes loaded with I or II as a systemically applicable MMC sustained-release dosage form.

The clearance profiles of S-liposomes entrapping prodrugs I and II (Figs. 5 and 6) were similar to those of the empty liposomes (Fig. 2). This finding indicates that the blood disposition of a carrier vehicle is not altered by the entrapment of these compounds.

As previously reported,[10] almost all of the compounds I and II in the blood are firmly associated with liposome carriers, and intravascular hydrolysis of the prodrugs is considered to occur on the liposomal membranes. NBD-PE is reasonably stable in the blood (unpublished data). Therefore, the blood concentration ratios of the prodrugs to NBD-PE (P/N ratios) are considered to represent the extent to which the prodrugs remain intact in liposome carriers. Figure 7 shows the P/N ratios calculated from observed blood concentrations of the prodrugs and NBD-PE at various times after i.v. injection. The P/N ratios decreased gradually in monoexponential patterns having half-lives of 9.0 and 7.1 h for I and II, respectively. These results reveal that slow cleavage of the prodrugs to MMC occurs in the circulation and that their intravascular hydrolysis follows apparent pseudo-first-order kinetics. The *in vivo* conversion half-life of I (9.0 h) showed fairly good agreement with that (10.0 h) observed in rat serum *in vitro*.[10] On the other hand, II was found to be hydrolyzed more rapidly *in vivo* than expected from an *in vitro* hydrolysis experiment.[10] Erythrocytes and other blood components might play an important role in intravascular hydrolysis of II.

The observed plasma levels of regenerated MMC after i.v. injection of liposomes entrapping prodrugs (Figs. 5 and 6) were much higher than expected from the intravascular hydrolysis rates. This phenomenon might be partially explained by the retention of

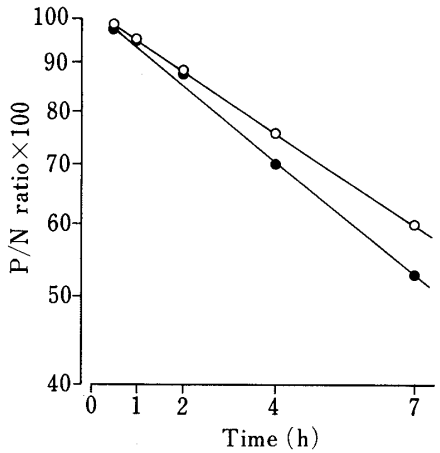


Fig. 7. P/N Ratio–Time Profiles after i.v. Injection of S-Liposomes Entrapping Prodrugs

○, liposomes containing prodrug I; ●, liposomes containing prodrug II.

regenerated MMC within the liposomal inner aqueous phase. As demonstrated by Hashida *et al.*,[17] MMC–dextran conjugate administered intravenously is accumulated in the RES, and acts as an intracellular sustained-release reservoir to maintain the plasma levels of MMC for a long period. Although, in the present case, it is probable that similar intracellular slow release may occur, further studies are necessary to clarify this possibility.

On the basis of the present results, S-liposomes showed the best pharmacokinetic properties for a carrier vehicle for sustained-release delivery systems to be administered by i.v. injection. Furthermore, it is apparent that S-liposomes entrapping MMC prodrugs have the potential to maintain blood MMC levels over a prolonged period of time. Further studies are under way to investigate the antitumor activity of MMC prodrug-bearing S-liposomes against solid tumors with the hope that prolonged blood levels of MMC might lead to an increase in therapeutic efficacy, as suggested by previous investigations.[18]

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