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The characterization and biodistribution of cefoxitin-loaded liposomes

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Abstract

To conquer the clinical restriction of relative short half-life and poor tissue retaining activities, liposomes containing cefoxitin were prepared using three methods in this study. The physicochemical properties including cefoxitin encapsulation percentage, vesicle size, stability, as well as the in vivo biodistribution were studied. The highest entrapment percentage was observed by using reverse phase evaporation method, and the molar ratio of cefoxitin to phospholipids was 1:3, DMPC to cholesterol was 2:1, respectively. From the result of stability, the freeze-drying powder and then stored in the frozen condition of cefoxitin-loaded liposome was an ideal storage state. Accordingly, the formulation by reverse-phase evaporation method was selected to investigate the biodistribution of cefoxitin-loaded liposome and compared to free cefoxitin in rats. It was observed that the cefoxitin levels and the duration retained in the liver, spleen, and pancreas of liposome-injected animals were higher and longer than that of free cefoxitin-injected animals. The drug concentrations of bile after post-injection of liposomal cefoxitin at 0.5, 1 and 2 h were all approximately 2.7 times higher than that of free cefoxitin injection group.
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Keywords: Cefoxitin; Liposome; Stability; Biodistribution

1. Introduction

Cefoxitin, a semi-synthetic cephalosporin, can rapidly generate therapeutic levels within the blood and peritoneal fluid after intravenous administration. A prerequisite to antibiotic therapy is that the levels of antimicrobial agents at the site of infection must be sufficient to kill the bacteria or to arrest the growth and replication until host defense mechanisms are reinforced (Young, 1990). However, the half-life of cefoxitn is very short, as well, its poor intracellular diffusion and retention that the sustained therapeutic drug levels with organs or tissues may not be attained (Tally, 1981; Lau et al., 1986; Sweet et al., 1988; Sanchez et al., 1983; Lefrock et al., 1983). Therefore, the treatment of infections caused by obligate or facultative intracellular microorganisms seem to be difficult. To conquer this problem, by associating cefoxitin with liposome was considered to develop endocytosable drug carriers.

Liposomes are microscopic vesicles consisting of membrane like phospholipid bilayers surrounding an aqueous medium. The lipid vesicles are formed spontaneously when phospholipids are hydrated

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in aqueous medium. Because of their entrapping ability and high degree of biocompatibility, liposomes are suitable for many routes of administration (Siler-Marinkovic et al., 1997; Fielding, 1991). For the purpose of cefoxitin-loaded liposome treatment as an appropriated pharmaceutically formulation, three common methods were applied to prepare liposome using different compositions of phospholipids in this study (Fang et al., 1997; Sharma and Sharma, 1997; Taylor et al., 1990; Lin et al., 1996). The stability of liposomes in the blood seemed to be an important factor which determined the carrier potential of liposome (Kiwada et al., 1988). That is, drug would immediately release into the blood and excrete from the body after administration resulting in the invalidity of liposomes as drug carriers if they were unstable in the plasma. Therefore, the stability of cefoxitin liposome formulation in different storage states and in plasma was evaluated. Notwithstanding some articles had reported that the therapeutic efficacy of liposome entrapped cefoxitin was improved compared with that of free cefoxitin (Price et al., 1989). However, the biodistribution of liposome entrapped cefoxitin after intravenous administration was still not clear. Accordingly, from the results obtained in this study, the optimal formulations of cefoxitin formulated in liposome with appropriate drug encapsulation percentage and homogenous particle size distribution were selected to investigate the biodistribution after intravenous administration utilizing rats as an animal model of free cefoxitin and liposome entrapped cefoxitin, in the attempt to establish the in vivo animal model for liposome entrapped cefoxitin in order to offer the basic knowledge for further development and evaluation of liposome encapsulated cefoxitin formulations.

2. Materials and methods

2.1. Materials

Cefoxitin, dimyristoyl-L-α-phosphatidylcholine (D-MPC), dipalmitoyl-L-α-phosphatidyl-choline (DPPC), L-α-lecithin (from fresh egg yolk, approximately 99%, EPC), cholesterol (CH) were obtained from Sigma Chemical Co. (USA). All other chemicals and solvents were of analytical grade.

2.2. Preparation of liposome

Liposomes were prepared by the following three methods. Method A was the thin film method (Yu and Liao, 1996). The required amount of phospholipid was added directly into an organic solvent solution composed of 50% chloroform and 50% ether. After they dissolved completely, then the organic solvent was slowly removed at reduced pressure, 40 °C water bath on a rotary evaporator to make thin film of the phospholipid mixture formed in the inner wall of the flask. Aqueous phase (cefoxitin in McIlvaine buffer, pH 5.0) was added to the dried phospholipid film and shaken on a vortex mixer for 2 min to produce multi-lamellar vesicles (MLVs). Then the liposomal dispersions were maintained at the temperature exceeding the phospholipid Tc (EPC: 37 °C, DMPC: 38 °C, DPPC: 56 °C) for one hour to anneal the liposome structure, and were shaken intermittently during that hour. The small MLVs were produced from MLVs by sonication for 1 h at 45 °C in an ultrasonic bath (Branson 3200, 50/60 MHz, USA). Method B was the reverse phase evaporation techniques (Taylor et al., 1990). The sufficient amount of phospholipids were weighed into a round-bottom flask and dissolved in six fold greater volume of cefoxitin containing McIlvaine buffer (pH 5.0) of organic solvent (chloroform/ether: 1/1). Then the cefoxitin in McIlvaine buffer solution was directly added to phospholipids dispersed solution, and then, the mixture was sonicated for seven minutes at 45 °C in an ultrasonic bath. The organic solvent was then removed at 45 °C water bath on the rotary evaporator. Then the liposomal emulsion was maintained under the control temperature to make the liposome structure more stable. Method C was solution injection method (Lin et al., 1996). A ration of cefoxitin was added directly to an alcoholic solution of phospholipids and the solution was then rapidly injected into a fivefold greater volume of magnetically stirred pH 5.0 McIlvaine buffer. The liposomal dispersions were frozen in a dry ice-acetone bath and were dried by means of a freeze-dryer (Labconco, USA) under 10 μmHg vacuum at −50 °C for 24 h. The lyophilized liposome powder was then reconstituted with same volume of the McIlvaine buffer added in the preparation process described above.

2.3. Cefoxitin encapsulation percentage determination

The cefoxitin-containing liposomes were separated from untrapped drug by filtering the liposome dispersion under vacuum (5 mmHg) through a 0.025 μm filter (Millipore, USA), the liposomes were then washed with buffer to complete the removal of the free drug. The amount of entrapped cefoxitin was determined by lyse of the liposome with absolute alcohol to give a clear solution and the cefoxitin concentration was estimated by HPLC. The percentage of drug encapsulated was calculated by the ratio of cefoxitin in liposome vesicles to the cefoxitin of the total amount in the aqueous suspension.

2.4. Vesicle size analysis

Light scattering measurements were performed with a Coulter submicron particle-size analyzer (Model N4MD, Coulter, USA). The liposome preparation in sample vial was diluted with 10 ml twice-distilled water to detect the vesicle distribution at 20 °C.

2.5. The stability of liposome in different storage states

To estimate the influence of storage condition to the drug-encapsulated percentage, the freeze-drying powder of liposome and the liposome solution were placed in −20 °C freezer and were sampled at appropriate time intervals. All samples were frozen at −20 °C until HPLC assay.

2.6. In vivo organ distribution study

The male Wistar rats (110–180 g) were obtained from Kaohsiung Medical University (Kaohsiung, Taiwan ROC). The rats were fasted for 24 h before intravenous administration of cefoxitin. Each rat was given an IV bolus of either cefoxitin sodium or cefoxitin-entrapped liposomal emulsions into the tail vein at the dose of 30 mg/kg. After intravenous administration of drug, the 2 ml blood sample was collected by heart puncture from different rats at 1st hour, 3rd hour and 5th hour, respectively. After collecting the blood sample of each rat, the rat was sacrificed and we obtained its liver, spleen, pancreas, and kidney specimens. The tissues were elutriated by using 0.9% normal saline, weighed and a ration of tissue specimens was placed in a homogenizing tube with an appropriate volume of absolute alcohol and of internal standard alcohol solution, and homogenization was done in ice-bath for 20 min. The tissue homogenate was centrifuged at 5 °C, 1200 rpm for 15 min. The supernatant was then injected directly into the HPLC for analysis.

2.7. Measurement of the cefoxitin concentration within the bile

The male Wistar rat (110–180 g) was anesthized with pentobarbital sodium (Nembutal®, 50 mg/kg) and underwent laparotomy. The bile drainage was performed and the peritoneal cavity was lavaged with appropriate volume of normal saline and sutured with the surgical suture silk. The rat was given an IV bolus of either cefoxitin sodium or cefoxitin-entrapped liposomal emulsion into the tail vein at the dose of 30 mg/kg. At 0.5, 1, 2, 3, 4, 5, and 5.5 h post-injection, the bile samples were collected from bile drainage tube. All samples were frozen at −20 °C until assay. Each data represents the average determination of six rats.

2.8. Analytical condition of cefoxitin by HPLC

The cefoxitin content of each sample was determined by high-performance liquid chromatography (HPLC) using timolol as internal standard. The HPLC system used was the Water Associates (Milford, MA, USA) LC Module 1. A Nova-Pak C_{18} reverse-phase column (150 mm × 3.9 mm i.d.) was used, with a μBondapak C_{18} guard-column in series. The mobile phase was an actonitrile-buffer solution containing 88% 0.05 M phosphate buffer and 12% acetonitrile. The pH value was adjusted to 3.1 with 30% phosphoric acid. The flow rate was 1.0 ml/min with UV detector at wavelength 234 nm.

3. Results

For the preparation of the liposomes, the types of phospholipid and the methods selected were the main factors influencing the efficiency of drug encapsulation. To obtain the higher drug encapsulation

Table 1
Characterization of cefoxitin liposome by encapsulation, vesicle size after preparation by three methods

Phospholipid[a]	Method[b] A		Method B		Method C	
	Encapsulation (%)	vesicle size (nm)	Encapsulation (%)	vesicle size (nm)	Encapsulation (%)	vesicle size (nm)
EPC	31.22 ± 9.40[c]	711 ± 530	34.44 ± 6.68	784 ± 570	61.43 ± 2.84	1080 ± 420
DMPC	50.39 ± 2.47	600 ± 530	34.69 ± 1.24	1030 ± 1100	64.48 ± 2.90	984 ± 83
DPPC	42.67 ± 11.31	1320 ± 1100	27.97 ± 1.18	1350 ± 1500	55.83 ± 5.21	3000 ± 810
EPC/CH	38.43 ± 4.06	1597 ± 1420	35.90 ± 1.53	953 ± 820	63.92 ± 1.95	1320 ± 350
DMPC/CH	50.65 ± 0.66	904 ± 630	40.41 ± 2.15	1050 ± 450	65.81 ± 2.67	1370 ± 140
DPPC/CH	41.98 ± 8.78	1529 ± 144	28.11 ± 7.51	1845 ± 1450	61.50 ± 2.20	2820 ± 2100

[a] EPC: L-α-phosphotidylcholine obtained from fresh egg yolk, DMPC: dimyristoyl-L-α-phosphotidylcholine, DPPC: dipalmitoyl L-α-phosphotidylcholine, CH: cholesterol.
[b] Method A: thin film method, Method B: solvent injection method, Method C: reverse-phase evaporation techniques.
[c] Each data are mean ± S.D., $n = 3$.

efficiency, these three methods were used in this present study to evaluate the drug encapsulation percentage and the mean particle size. The results are shown in Table 1. Cefoxitin- loaded liposome was prepared by reverse-phase evaporation techniques using natural phospholipid of EPC and the encapsulation was approximately two folds higher than those by thin film method and solvent injection method. The same results were also observed using synthetic phospholipids, DMPC and DPPC as materials. The drug-encapsulation percentage of liposomal formulations prepared by 1:1, 2:1 and 3:1 of DMPC and cholesterol were 68.80 ± 1.92, 72.99 ± 3.43, and 73.14 ± 2.22.The increase of drug encapsulation efficiency and mean particle size were obtained by reverse-phase evaporation techniques when the phospholipids mixture was composed of 33% cholesterol and 67% phospholipids. To evaluate the optimal molar ratio of cefoxitin and phospholipid in liposome, the 1:1, 1:3, 1:5, and 1:8 drug-lipids molar ratio were selected to prepare the liposome by reverse-phase evaporation techniques. The drug encapsulation percentages prepared in 1:3, 1:5 and 1:8 of molar ratio were 79.36 ± 1.36, 81.14 ± 0.62, and 80.87 ± 2.56, respectively. They were all significantly higher than that of 1:1 of molar ratio (74.69 ± 0.89, $P < 0.05$). However, the difference among the encapsulation efficiency of the 1:3, 1:5 and 1:8 molar ratio groups were not significant.

To estimate the influence of storage condition to the drug-encapsulated percentage, the freeze-drying powder of liposome and the liposome solution were placed in −20 °C freezer and sampled at 0, 12, 24, 48, 96, and 144 h, respectively. Fig. 1 shows the retention percentage-time profile of cefoxitin with both freeze-drying powder type and liposome solution stored in −20 °C condition. After thawing of frozen liposome, the cefoxitin retention percentage was decreased from original 83.43 to 72.75% during 48 h of liposome solution freezing. Moreover, the retention of cefoxitin (%) was lessened to 50.60% during 144 h. In order to achieve the high drug retention affinity with specific tissue or organ, the stability of liposome in plasma was important to maintain the structure integrally of liposome until it was transported to the specific sites. Fig. 2 shows the drug-retention percentage after the various time intervals of liposome solution in plasma and normal saline. There was not statistically significant difference in both groups.

Accordingly, the optimum liposome-loaded cefoxitin formulation by reverse-phase evaporation method was used to investigate the biodistribution of liposomal cefoxitin in rats and evaluate its relative retaining activities to the certain organs such as liver, spleen, pancreas and kidney. After the single intravenous bolus of liposomal cefoxitin or free cefoxitin, the amount of drug in different organs was studied. Table 2 shows the concentration of cefoxitin after the administration of free cefoxitin or liposomal cefoxitin in various organs at the scheduled time.

Fig. 3 shows that the drug concentration of bile after post-injection of liposomal cefoxitin at 0.5, 1 and 2 h. There were approximately 2.7 times higher than that of free cefoxitin injection group. After 3 h of drug administration, the drug levels of bile were still about 1.5 times compared with the free cefoxitin injection.

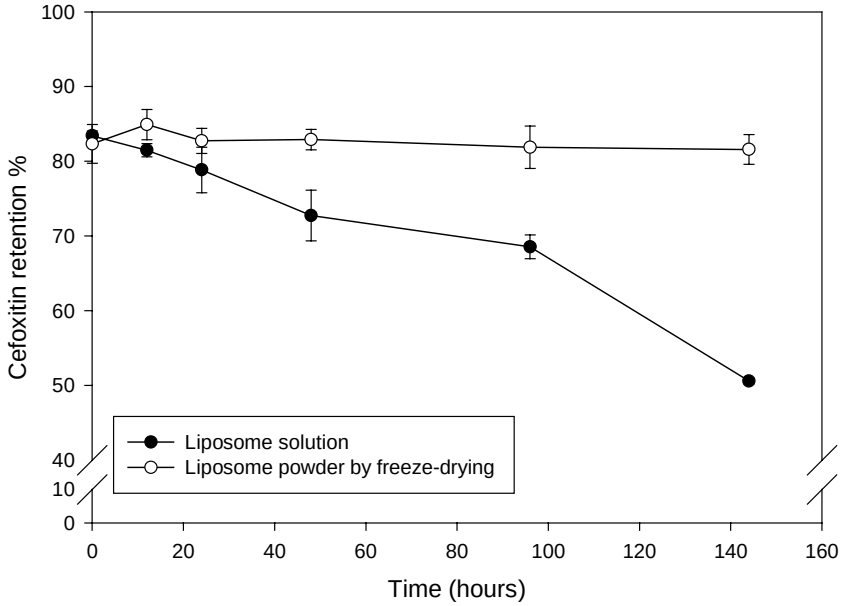

Fig. 1. The cefoxitin retention percentage after the various time intervals of cefoxitin liposome solution and liposome powder by freeze-drying method.

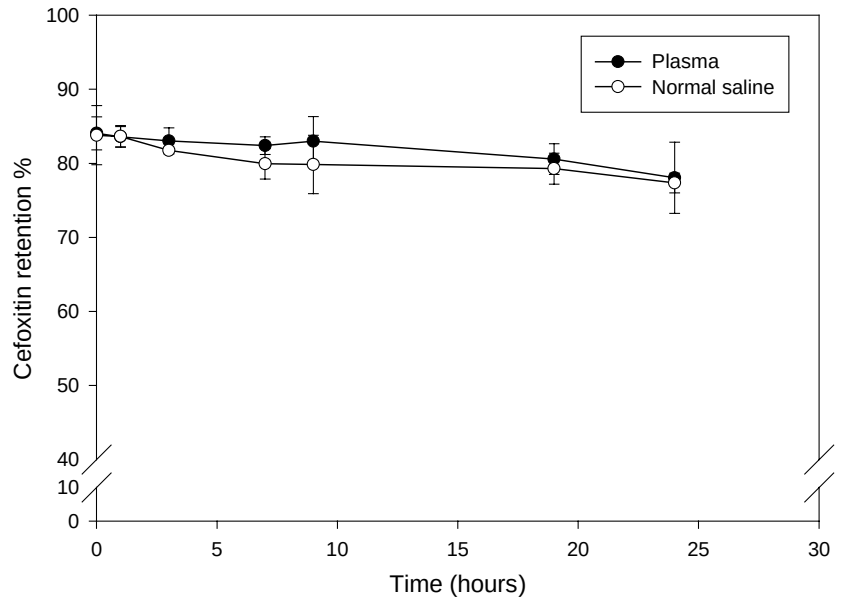

Fig. 2. The cefoxitin retention percentage after the various time intervals of liposomal cefoxitin solution in plasma and normal saline.

Table 2
The cefoxitin levels of plasma, liver, spleen, pancreas and kidney after free cefoxitin and liposomal cefoxitin administration in rats

Time (h)	Cefoxitin concentration				
	Plasma (μg/ml)	Liver (μg/g)	Spleen (μg/g)	Pancreas (μg/g)	Kidney (μg/g)
Free form					
1	60.17 ± 22.04	53.81 ± 35.01	76.84 ± 29.87	7.38 ± 4.56	212.28 ± 112.63
3	19.66 ± 8.50	7.12 ± 5.88	7.71 ± 5.69	7.14 ± 6.04	6.82 ± 6.83
5	4.94 ± 5.37	6.20 ± 3.23	5.64 ± 2.57	4.65 ± 1.99	7.67 ± 6.27
Liposome form					
1	46.98 ± 29.35	80.30 ± 55.62	57.57 ± 24.89	267.87 ± 209.97[a]	126.81 ± 99.67
3	4.13 ± 1.70[b]	40.20 ± 26.60[a]	52.02 ± 25.36[a]	508.74 ± 241.60[a]	23.04 ± 21.22[a]
5	3.27 ± 1.72	43.48 ± 13.41[a]	82.66 ± 85.41[a]	398.10 ± 308.97[a]	19.65 ± 16.65

Each data are mean ± S.D., $n = 6$.
[b] The concentration of liposome form was significant higher than that of free form ($P < 0.05$).
[a] The concentration of liposome form was significant lower than that of free form ($P < 0.05$).

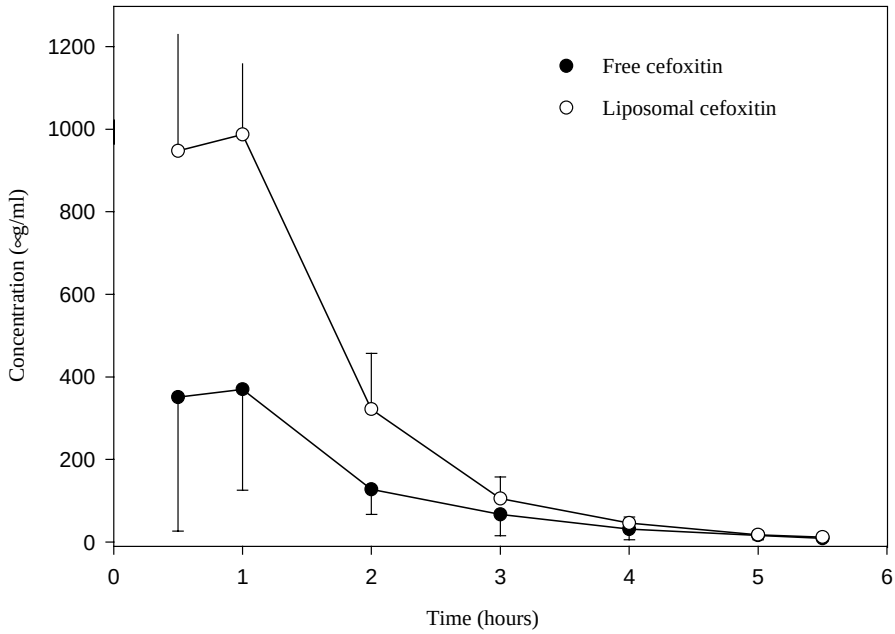

Fig. 3. The comparative drug concentration-time profile in the bile after post-injection of liposomal cefoxitin and free cefoxitin at 0.5, 1 and 2 h.

4. Discussion

4.1. Optimization of cefoxitin-encapsulated liposome formulations

Numerous studies have discussed the method for preparing the liposome (Fang et al., 1997; Sharma and Sharma, 1997; Taylor et al., 1990; Lin et al., 1996) including thin film method, solvent injection method and reverse phase evaporation techniques. In general, the choice phospholipids are often limited to the family of the phosphatidylcholines because of toxicological considerations and availability of pure compounds. Compounds with long and saturated alkyl chains provide bilayers with low permeabilities for small compounds. Moreover, phospholipids containing unsaturated fatty acids are known to undergo oxidative reactions. Accordingly, phospholipids of phos-

phatidylcholine with saturated fatty acids of C_{14} (DMPC), C_{16} (DPPC) and L-α-lecithin obtained from fresh egg yolk were selected for characterization. Generally, phospholipids have a characteristic phase transition temperature (T_c), thus depending on the surrounding temperature, they exhibit different physical states. The gel-like state means that the lipids are a well-ordered arrangement below the T_c, and exist in a disordered, fluid-like or liquid-crystalline state above the T_c. In the liquid-crystalline state, lipid bilayer was less stable and more permeable to solute, especially hydrophilic solute, than the solid gel-like state (Siler-Marinkovic et al., 1997; Fielding, 1991). On the other hand, the encapsulation efficiencies of solutes were correlated with the fluidity of lipid bilayer. The maximum bilayer permeability occurs at the phase transition temperature T_c (Sharma and Sharma, 1997), since the gel state and liquid crystalline state coexist when the temperature was close to T_c, leading to the leakage of encapsulated drug. After the incorporation of cholesterol into lipid bilayer, the rotational freedom of hydrocarbon chains of phospholipids decreased. Besides, cholesterol could entirely eliminate phase transition and decreased at temperatures above T_c (Sharma and Sharma, 1997), these mechanisms made the lipid bilayer more stable and less permeable to the encapsulated drug. Moreover, the vesicle size became larger and the apparent capture volume increased relatively after the incorporation of cholesterol into the lipid bilyers.

According to the results obtained by the three liposome preparing methods, DMPC showed a higher encapsulation percentage than DPPC and EPC did. Regarding the efficiency of drug encapsulated as shown in Table 1, correlating to the different preparation methods, the mean encapsulation percentage of cefoxitin prepared by reverse-phase techniques was elevated significantly and the particle size distribution was more homogeneous as compared to that of thin film or solvent injection method. However, it should be noted the fine drug encapsulation efficiency of liposome was obtained using 1:5 molar ratio of cefoxitin and phospholipids. The encapsulation percentage was not elevated significantly accompanied with the increment of phospholipid amount. This phenomenon is probably due to the enlargement of the vesicle size after adding the larger amount of phospholipid and the aggregation or fusion of vesicle happened. Therefore, the break of vesicle membrane and drug leakage was observed. Hence, the 1:3 molar ratio of drug to phospholipid was selected to perform the following investigations. Along with the influence of drug/phospholipid molar ratio, the amount of cholesterol added might affect the stability of liposome. From the previous report (Betageri, 1993; Dean and Hider, 1993), cholesterol increased the stability of lipid bilayers and then augmented the drug-entrapped efficiency. The difference of drug encapsulation percentage between the molar ratio of 1:1 and 2:1 was similar, and the lowest cefoxitin encapsulation percentage was observed at the 3:1 molar ratio group among three liposome formulations. Subsequently, the 1:3 molar ratios of drug to phospholipid and the 2:1 molar ratios of phospholipids to cholesterol was prepared by reverse-phase technique for the following study based on the optimal drug-entrapped efficiency and stability for cefoxitin liposomes.

4.2. The stability of liposome in different storage state and plasma

The stability is always a limited factor for the application of liposome. After thawing of frozen liposome, the cefoxitin retention percentage was decreased. This result was interpreted by the interaction of acyl chain of phospholipids after freeze-thawing to the room temperature results in the transformation of the structure of large unilamellar vesicles aggregate to the large multilamellar vesicles. Therefore, the leakage of cefoxitin and the disorder of lipid bilayers seemed to be unavoidable. Lasic et al. had reported that when the temperature of liposome solution is close to the T_c of certain phospholipids, the lipid bilayer became more leakable. In other words, the stability of liposome and the efficiency of drug retaining represented as the tendency of declination. As the frozen liposome solution was thawing to room temperature (25–28 °C), the lipid bilayer possessed the coexistence status of liquid-crystalline state and gel-state because the T_c of DMPC was 23 °C, and this was approximate to room temperature. Therefore, the alignment of lipid bilayer was disordered and the substantial decrement of cefoxitin retention percentage was observed. On the contrary, the drug-encapsulation percentage of freeze-drying powder was not statistically significant at post-storage during 144 h compared to that of

fresh-prepared liposome. This result suggested that the freeze-drying liposome showed a more stable state than liposome solution did. As well, the freeze-drying powder and then stored in the frozen condition of cefoxitin-loaded liposome was an ideal storage state.

Otherwise, the comparison of liposome solution in plasma and normal saline was found that the components of plasma should not affect the stability of liposome and the structure of liposome was maintained for a sufficient period until the uptake by specific tissues or organs.

4.3. In vivo biodistribution

For most of intracellular infections, we knew it was difficult to eradicate the infections because most of commonly used antibiotics seem to have the low intracellular uptake ability or reduction of the antimicrobial activity at the acidic circumstance. In this study, the hepatic cefoxitin levels from liposome were higher than those of free form at 1 h post-injection, and were about six times higher at 3 and 5 h post-injection, compared with that of free cefoxitin injected groups. Similar results had been reported by Kresta et al. (1993) who showed the intraperitoneal injection of liposomal cefoxitin resulted in substantially elevated levels of hepatic drug at 3 and 5 h post-injection. Judging from the cefoxitin levels of spleen, after the injection of liposomal cefoxitin, the levels of cefoxitin at 1 h was lower than that of free cefoxitin. Nevertheless, after intravenous administration of liposomal cefoxitin, the levels of spleen at 3 and 5 h were substantially elevated about seven times and 16 times higher than that of free cefoxitin.

In regard to cefoxitin level of kidney, the level observed at 1 h was about 30 times higher than that of 3 and 5 h after the injection of free cefoxitin. However, the levels in the liposomal cefoxitin group were approximately one-half of that of free cefoxitin injection group at the same time. From the result, cefoxitin was rapidly excreted by kidney and retained in the body with a relatively short time interval. In contrast, after the same dose of liposomal cefoxitin injection, the accumulation of cefoxitin in kidney was slower and smaller than those of free cefoxitin group. The phenomenon showed that liposome-loading cefoxitin could be retained for a long time interval by slower clearance from kidney as compared with that of free cefoxitin.

These consequences represented in vivo biodistribution of cefoxitin were altered and retained in the reticuloendothelial system (RES) such as liver and spleen after using the liposome as a drug carrier for cefoxitin. The route of administration might be a factor that influenced the biodistribution of the liposomal injection. It had been reported that the intraperitoneal injection of liposome-loaded drug was mainly absorbed through the subdiaphramatic lymphatic and then accumulated in the RES (Hirano and Hunt, 1985). In comparison with this study, the route of intravenous administration was used and the liposome-loaded cefoxitin was distributed directly into the systemic circulation. Thus, the possibility of liposome that contacted the pancreas was increased and the phenomenon of higher pancreatic cefoxitin level was explained reasonably. According to the highest concentration of liposomal cefoxitin was found in the pancreas, the clinical treatment of acute pancreatitis may be considered to use the liposomal cefoxitin.

4.4. Cefoxitin concentration within the bile

Judging from the concentration of bile juice after the liposomal cefoxitin injection, the time of peak concentration in the bile was almost at 1 h after administration of drug either liposomal cefoxitin or free cefoxitin. This phenomenon suggested that the characteristic of liposomes were similar to that of some pathogens, so liposomes tended to be rapidly taken up after intravenous administration, principally by phagocytic elements of the RES such as hepatic kupffer cell and marcophage of spleen and others. Hence, after the administration of liposome-loaded cefoxitin, a higher drug concentration was found within the bile of the liver, than that of the free cefoxitin group. The drug recovery percentage in the bile was 16% after 5.5 h post-injection of liposomal cefoxitin and was significantly higher than that of free cefoxitin group which showed a value of 5.78%. However, utilization of liposome as a drug carrier could result in a higher drug amount in a specific tissue or organ. The information obtained in this study may be helpful in development of cefoxitin delivery system by liposomal dosage form.

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