STUDIES ON LIPOSOME-ENCAPSULATED CARBOQUONE. II.¹⁾ EFFECT OF LIPOSOMAL ENCAPSULATION OF CARBOQUONE ON DISTRIBUTION AND CLEARANCE IN ANIMALS

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The effects of encapsulation of carboquone (CQ) in the liposomes were investigated on the plasma clearance and tissue distribution of CQ in rabbit and rat. CQ-Liposome, the liposomes in which CQ was encapsulated, was prepared by the method described in a previous report.¹⁾ By intravenous administration at the same dose, CQ-liposome showed approximately three times higher plasma concentration than free CQ in rabbit and rat. However, prolonged release from liposomes or stabilization of CQ in plasma was not observed by encapsulation. The distribution to lung and reticuloendothelial-rich organs such as liver and spleen was enhanced, but on the contrary the distribution to the peripheral tissues such as muscle and skin was decreased. In rats pretreated with drug free liposomes, high plasma CQ concentration was obtained after intravenous administration of CQ-liposome.

From these results, it is considered that the large parts of CQ-liposome is distributed into reticuloendothelial system (RES) and RES is saturable with liposomes. And it is also presumed that the high plasma concentration obtained after intravenous administration of CQ-liposome, would be caused by altering the tissue distribution of CQ by encapsulation.

Keywords—drug delivery system; liposome; carboquone; tissue distribution; clearance; rat; rabbit

Cancer chemotherapy with cytotoxic drugs is interrupted by their indiscriminate action on both tumor and normal cells. Any approach by which cytotoxicity could be directed selectively to tumor cells would be of great value. Many attempts have been made to deliver the antitumor drugs into target sites within the tumor by means of drug delivery systems.^{2–4)} Most of approaches are the uses of drug carriers such as immunoglobulins,⁵⁾ DNA,⁶⁾ dextran,⁷⁾ albumin microsphere,⁸⁾ oil emulsion⁹⁾ and liposomes.¹⁰⁾ One of these carriers, liposomes have been used as a membrane model for a long times. However, in recent years, they have also been used for the delivery of enzymes,^{11,12)} steroids^{13,14)} and some antitumor agents.^{15–18)} These studies were carried out by using various kinds of liposomes, and we have planned to study the relationship between the pharmacokinetics and antitumor effects of

CQ by using liposome encapsulated preparation. The method of preparation and physical characteristics of liposomes were shown in the previous report.¹⁾

The aims of present studies were to investigate the fate of CQ encapsulated in liposomes after intravenous administration in an attempt to examine the distribution characteristics.

MATERIALS AND METHODS

Materials—CQ was synthesized in Sankyo Co., Ltd. Esquinon for injection[®], a commercial preparation of Sankyo Co., Ltd., was used for animal experiments as free CQ. Distearoyl phosphatidylcholine was purchased from Sigma Chemical Co., Ltd., (London, England). 2,5-Diethyleneimino-3,6-dimethylbenzoquinone (MEB), synthesized in Sankyo Co., Ltd., was used for an internal standard on high performance

liquid chromatography (HPLC). All other reagents used were of analytical or reagent grade.

Preparation of CQ-Liposome — CQ-Liposome was prepared as described previously,¹⁾ by modifying the method of Szoka and Papahadjopoulos.¹⁹⁾ Briefly, 15.6 μmol of CQ and 330 μmol of distearoyl phosphatidylcholine were dissolved in a mixture of 35 ml of chloroform and 35 ml of isopropyl ether. After adding 10 ml of 1/10 phosphate buffered saline (pH 7.2),¹⁹⁾ the mixture was sonicated for 5 min at room temperature, and the chloroform and isopropyl ether were

evaporated with a rotary evaporator. This crude liposomal suspension was subjected to gel chromatography on a Sephadex G-50 column. The fractions containing liposomes were pooled and centrifuged at $27000 \times g$ for 20 min. Precipitated liposomes were collected and resuspended in 1/10 phosphate buffered saline. Concentration of CQ in liposome suspension was estimated by HPLC as described in a previous report¹⁾ and the amount of phospholipid was estimated by weighing the liposomes after drying. CQ concentration in this suspension was 465 $\mu\text{g}/40 \text{ mg}$ of dis-

FIG. 1. Plasma Concentrations of CQ after Intravenous Administration of Free CQ and CQ-Liposome at Various Doses in Rat

---●---: 4 mg/kg, ---○---: 2 mg/kg, —●—: 1 mg/kg, —○—: 0.5 mg/kg, ---▲---: 0.25 mg/kg, ---△---: 0.125 mg/kg. Results are expressed as the mean $\pm$ S.E. of three or four rats.

tearoyl phosphatidylcholine per 1.0 ml.

Animal Experiments — Plasma Concentration in Rat: Male Wister-Imamichi rats weighing 430–480 g were used. CQ-Liposome was administered intravenously as a sterilized saline suspension and free CQ was administered intravenously as a sterilized solution. The dose of CQ-liposome was expressed as an amount corresponding to parent CQ. Free CQ and CQ-liposome were administered into femoral vein. After administration, about 0.6 ml of blood was obtained from tail blood vessel at various time intervals for 2–4 h.

Plasma Concentration in Rabbit: Male rabbits weighing 3.0–3.3 kg were used. After intravenous administration of drugs at the dose of 0.33 mg/ml/body, venous blood was obtained from the

opposite ear of drug administration at various time intervals for 4 h.

Organ Distribution in Rat: Male Wister-Imamichi rats weighing 170–180 g were used. Free CQ and CQ-liposome were administered into femoral vein at the dose of 1 mg/kg. At various time intervals after administration, rats were killed with depletion of blood and various organs were taken out. Organs were homogenized rapidly with Polytron[®] homogenizer (Kinematica GMBH.) under cooled condition with 4 times the weight of distilled water, and a certain volume of homogenate was used for the determination of CQ concentration.

Effect on Liposomal Encapsulation on Stabilization of CQ in Organ Homogenates of Rat — Fresh blood and plasma were used. Organ homo-

FIG. 2. Relationship between the Dose and AUC
---○---: (a) free CQ, —●—: (b) CQ-liposome, ---△---: (c) CQ-liposome which contains approximately the same amount of liposomes at each doses (about 90 mg of total lipid/kg). Results are expressed as the mean $\pm$ S.E. of three rats.

FIG. 3. Plasma Concentrations of CQ after Intravenous Administration of CQ-Liposome in Rat Pretreated with Drug Free Liposomes

—●—: pretreated with 30 mg of liposomes by dry weight per kg of body weight, ---○---: pretreated with 60 mg of liposomes by dry weight per kg of body weight. Results are expressed as the mean $\pm$ S.E. of three rats.

genates were prepared by homogenizing fresh organs with twice the weight of distilled water. CQ and CQ-liposome were adjusted to contain 1 μg of CQ in 1.0 ml of sample solutions. The sample solutions such as blood, plasma and 1/10 phosphate buffered saline (pH 7.2) were incubated at 37°C. The definite volume of incubated solution was withdrawn for the determination of CQ concentration.

Analytical Method of CQ — The method was described previously.²⁰⁾ Sample solution (0.1–1.0 ml) was added to the mixture of 3.0 ml of distilled water, 1.0 ml of MEB solution and 10 ml of chloroform, and the mixture was mechanically shaken for 10 min. After centrifugation at $900 \times g$ for 5 min, organic layer was withdrawn and evaporated following the addition of 2 drops of ethylene glycol. Adequate volume (100–200 μl) of MeOH was added to the residue, and 10 μl of this solution was injected on HPLC system (combination of Altex Pump 110A and Waters Detector 440). After separation with μ -bondapak C_{18} column, CQ was detected with UV absorption at 340 nm.

Pharmacokinetics — Plasma CQ concentration curve after intravenous administration were

analyzed using the program of Hanano *et al.* by two compartment open model given the form of $C_1 = Ae^{-\alpha t} + Be^{-\beta t}$ where C_1 is the plasma concentration. Pharmacokinetic parameters were calculated from the values of A , B , α and β . The first-order rate constant, k_{el} , represents all elimination processes and k_{12} and k_{21} represent the first order transfer constant of distribution between compartment 1 and 2. Compartment 1 is called the central compartment with a distribution volume, V_1 , and compartment 2 is called the peripheral compartment with a distribution volume, V_2 .

RESULTS

Time-Courses of CQ in Plasma after Intravenous Administration of Free CQ or CQ-Liposome in Rat

Fig. 1 shows the time-courses of plasma CQ concentration after intravenous administration of free CQ or CQ-liposome in rat at various doses. Plasma CQ concentrations of CQ-liposome were higher than those of free CQ after administration of several doses. Comparing the time-courses at the dose of 1 mg/kg, plasma CQ concentrations at 5 min after intravenous administration of free CQ and CQ-liposome were 81 ± 4 ng/0.1 ml (Mean

TABLE I. Pharmacokinetic Parameters of Free CQ and CQ-Liposome after Intravenous Administration in Rat and Rabbit

Parameter		Rat ^{a)}		Rabbit ^{b)}	
		Free CQ	CQ-Liposome	Free CQ	CQ-Liposome
n		3	4	3	4
A	ng/0.1 ml	131 ± 35	439 ± 62	19 ± 6	43 ± 4
B	ng/0.1 ml	70 ± 7	199 ± 12	6 ± 1	14 ± 3
α	h^{-1}	18.0 ± 3.4	15.3 ± 2.2	19.5 ± 0.8	15.4 ± 2.1
β	h^{-1}	1.9 ± 0.2	2.0 ± 0.1	3.1 ± 0.1	3.2 ± 0.3
k_{el}	h^{-1}	4.6 ± 0.9	5.1 ± 0.7	8.5 ± 1.0	8.0 ± 0.7
k_{12}	h^{-1}	7.8 ± 1.9	6.2 ± 1.3	6.8 ± 0.6	4.5 ± 1.4
k_{21}	h^{-1}	7.5 ± 0.7	6.0 ± 0.2	7.3 ± 0.9	6.1 ± 0.8
V_1	l/kg	0.53 ± 0.09	0.16 ± 0.01	0.46 ± 0.10	0.19 ± 0.00
V_2	l/kg	0.51 ± 0.04	0.15 ± 0.02	0.42 ± 0.06	0.15 ± 0.05
$[\text{AUC}]_0^\infty$	ng.h/0.1 ml	44 ± 3	129 ± 8	3 ± 1	8 ± 1

a) Dose: 1 mg/kg. b) Dose: 0.33 mg/body. Results are expressed as the mean $\pm$ S.E.

$\pm$ S.E., $n=3$) and 287 ± 15 ng/0.1 ml (Mean $\pm$ S.E., $n=4$), respectively. And both of those plasma concentrations decreased rapidly below the sensitivity limit (0.4 ng/0.1 ml) at 2 h. From these results, it was shown that plasma CQ concentration was approximately three times higher

than that of free CQ. But the differences became smaller with the lower doses.

Fig. 2 shows the relationship between the values of area under the concentration-time curve (AUC) and the doses. Dose-response of AUC of free CQ (a) was shown to be linear up to 4 mg/kg

FIG. 4. Distribution of Free CQ and CQ-Liposome after Intravenous Administration in Rat
Dose: 1 mg/kg. ---○---: free CQ, —●—: CQ-liposome. Results are expressed as the mean $\pm$ S.E. of three rats.

dose, but that of CQ-liposome (b) deviated from linearity. In this experiment, the particle number of liposomes at high doses were more than those at low doses, because the same preparation of CQ-liposome was used at every doses. In order to equalize the injected amount of liposome particle per se at different doses, liposomes which contained 48 μg , 101 μg , 186 μg and 358 μg of CQ in 32 mg of phospholipid were prepared. Each liposomal preparations were suspended in 1.0 ml of normal saline solution and used for the study at the dose of 0.125 mg/kg, 0.25 mg/kg, 0.5 mg/kg and 1 mg/kg, respectively. The results obtained with these liposomal preparations were shown in line (c) in Fig. 2. Dose-response curves of AUC now became linear at this experiment. From these results, it can be considered that more than certain number of liposome particles play an important role in plasma level of liposomal preparation, probably through the saturation of favored organs with a constant amount of liposomes. To ascertain the distribution kinetics, CQ-liposome was administered intravenously 5 min after intravenous administration of drug free liposomes (Fig. 3). As shown in Fig. 3, in rats pretreated with 30 mg and 60 mg of drug free liposomes per kg body weight, plasma CQ concentrations were approxi-

mately two times higher than those in untreated rats (Fig. 1) at 5 min after administration of CQ-liposomes.

In rabbit, plasma CQ concentrations were obtained after intravenous administration of free CQ or CQ-liposome at the dose of 0.33 mg of CQ equivalent per body. In the case of free CQ, plasma CQ concentration at 5 min was 73 ± 13 ng/ml (Mean $\pm$ S.E., $n=3$) and it rapidly decreased to the concentration of less than the sensitivity limit at 1.5 h. But in case of CQ-liposome, plasma CQ concentration at 5 min was 246 ± 22 ng/ml (Mean $\pm$ S.E., $n=4$), which was 3 times higher than that of free CQ. It rapidly decreased to 3 ± 3 ng/ml at 1.5 h.

To obtain the clearance kinetics of CQ after intravenous administration of free CQ and CQ-liposome in rat and rabbit, mean rate constants ($\pm$ S.E.) were obtained from individualized fits with two compartment open model (Table I). Plasma CQ concentration in rat used for the pharmacokinetic analysis was the data at the dose of 1 mg/kg in Fig. 1. In rat, elimination rate constants (k_{el}) from free CQ and CQ-liposome showed almost the same value of 4.6 ± 0.9 h $^{-1}$ and 5.1 ± 0.7 h $^{-1}$, respectively. And the value of AUC of CQ-liposome was approximately three times

TABLE II. Tissue Distribution of Free CQ and CQ-Liposome at 5 Minutes after Intravenous Administration

Tissue	Concentration (ng/g or ml)		B/A
	Free CQ (A)	CQ-Liposome (B)	
Liver	63 $\pm$ 0	1144 $\pm$ 93	18.2
Spleen	271 $\pm$ 55	1311 $\pm$ 99	4.8
Lung	416 $\pm$ 40	3090 $\pm$ 403	7.4
Kidney	650 $\pm$ 129	1116 $\pm$ 124	1.7
Heart	348 $\pm$ 37	753 $\pm$ 51	2.2
Intestine	423 $\pm$ 20	336 $\pm$ 18	0.8
Stomach	230 $\pm$ 46	227 $\pm$ 0	1.0
Brain	26 $\pm$ 4	41 $\pm$ 0	1.6
Muscle	533 $\pm$ 14	262 $\pm$ 59	0.5
Skin	352 $\pm$ 38	195 $\pm$ 3	0.6
Serum	806 $\pm$ 77	2033 $\pm$ 198	2.5

Concentrations are expressed as the mean $\pm$ S.E. of three rats.

larger than that of free CQ. The distribution volume (V_1 and V_2) of CQ-liposome was one third that of free CQ, indicating higher plasma level of liposomal preparation. The same relationship of pharmacokinetic parameters were obtained by intravenous dose of each 0.33 mg/ml/body of the two CQ preparations in rabbit. Elimination rate constants of free CQ and CQ-liposome were $8.5 \pm 1.0 \text{ h}^{-1}$ and $8.0 \pm 0.7 \text{ h}^{-1}$ respectively, showing the same elimination rate. Almost the same tendencies as rat were obtained in comparing free CQ with CQ-liposome about the value of AUC and the distribution volume.

Tissue Distribution in Rat

The distribution of CQ to various organs after injection of free CQ or CQ-liposome was shown in Fig. 4. The results showed that the favored sites for distribution of CQ-liposome seemed to be lung and tissues rich in reticuloendothelial cells such as liver and spleen. Also, the distribution of CQ to kidney and heart were relatively enhanced by encapsulation in liposomes. Table II shows CQ

concentration in various organs at 5 min after injection, as well as the concentration ratio of CQ-liposome against free CQ. In favored tissues such as liver, spleen and lung, CQ concentration ratios of CQ-liposome to free CQ were 18.2, 4.8 and 7.4, respectively. But in peripheral tissues such as muscle and skin, the ratios were 0.5 and 0.6, respectively. Total amount of CQ in whole body was suggested from these CQ concentrations by using the organ volume and the same amounts of CQ were shown in both cases of free CQ and CQ-liposome. So, it can be seen that the mass valances of CQ are obtained after intravenous administration of free CQ and CQ-liposome and the results of tissue distribution are reasonable.

In vitro Time-Courses of CQ in Blood and Organ Homogenates

Fig. 5 shows the comparative stabilities of free

FIG. 5. Stabilities of CQ contained in Free CQ and CQ-Liposome in Rat Plasma and Blood
CQ concentration in samples: $1.0 \mu\text{g/ml}$.
—●—: in phosphate buffered saline, ---○---: in plasma, —△—: in blood.

FIG. 6. Stabilities of CQ contained in Free CQ and CQ-Liposome in Rat Organ Homogenates at 37°C
CQ concentration in samples: $1.0 \mu\text{g/ml}$.
—▲—: spleen, ---○---: lung, —●—: kidney, ---△---: liver.

CQ and CQ-liposome in 1/10 phosphate buffered saline (pH 7.2), plasma and blood at 37°C. In both buffer solution and plasma, CQ was relatively stable, but in blood it was very unstable. The degradation rate of CQ in blood had the same value whether it was encapsulated or not. Fig. 6 shows the comparative stabilities in organ homogenates. In the homogenates of spleen, lung and kidney, the degradation rates of CQ were not so fast, but in that of liver it was very fast, and little difference was recognized between free CQ and CQ-liposome.

DISCUSSION

It has been reported that many drugs encapsulated into liposomes can dramatically alter the plasma clearance and tissue distribution of the drugs,^{21,22)} and also can protect the drugs against metabolic degradation.²³⁾ After intravenous injection of CQ-liposome, high plasma CQ concentration was obtained at earlier points (Fig. 1). Also, enhanced levels of drug distribution in the tissues rich in reticuloendothelial cells and reduced accumulation in the peripheral tissues were observed (Fig. 4, Table II). However, no alterations were observed in elimination rate from plasma (Table I) as well as stability in blood and tissue homogenates (Figs. 5, 6) even though CQ was encapsulated in liposomes. From these results, it is concluded that high level of CQ obtained at earlier points after administration of CQ-liposome was caused by the alteration of tissue distribution, but not by the protection of CQ from the environmental degradation.

Juliano *et al.* have reported that particle size or surface charge of particle can alter the pharmacokinetic behavior of liposome.^{22,24)} According to their results of clearance patterns of liposomes, CQ-liposome seemed to be a unilamellar liposome and charged positively on the surface. In our previous report,¹⁾ electron micrographs showed CQ-liposome to be a unilamellar liposome forming the large aggregates. The surface charge of CQ-liposome will be determined by electrophoresis in succeeding report.

As can be seen in Fig. 4 and Table II, the

preferred sites of uptake of CQ-liposome are clearly reticuloendothelial-cell rich organs corresponding to the previous reports.^{10,21)} However, the lung is also a very preferred organ for CQ-liposome distribution. It also became clear that these preferred organs for distribution are saturable with liposomes (Fig. 1—3). And it seemed that the organ saturation occurred with CQ-liposome at the dose of approximately 0.3 mg/kg as shown in Fig. 2. This amount of CQ-liposome corresponds to 28 mg by dry weight per kg body weight in rat. So, it is reasonable that the same high plasma levels were obtained in rats pretreated with 30 mg and 60 mg of drug free liposomes per kg body weight.

It is presumed that CQ-liposome is a valuable pharmaceutical carrier for the treatment of lung carcinoma because of its characteristics on tissue distribution. It is under planning to make an experiment on the effects of CQ-liposome on lung carcinoma in mouse.

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