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COMMUNICATION

Preparation and Pharmacodynamic Evaluation of Liposomes of Indomethacin

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ABSTRACT

The side effects of indomethacin, such as ulceration of the kidney and central nervous system (CNS) toxicity, limit its use as a drug for rheumatoid arthritis. Encapsulation of this drug in liposomes may reduce the toxic effects. The aim of this study was to determine the factors influencing encapsulation of indomethacin in liposomes and to determine anti-inflammatory potential of liposomal indomethacin. A series of liposomal formulations of indomethacin were prepared using various phospholipids. The effects of method of preparation, lipid composition, charge, and cholesterol (CH) on encapsulation of indomethacin in liposomes were investigated. A significant variation in encapsulation of the drug in liposomes was observed when prepared by different methods. With all the methods of preparation tried, the favorable lipid composition for high encapsulation of this drug was egg phosphatidyl choline:CH:stearlyamine (PC:CH:SA) at a 1:0.5:0.1 molar ratio. Inclusion of cholesterol did not affect the encapsulation efficiency of the drug in liposomes. The drug release profile from the liposomes was biphasic, and the highest percentage drug release was observed with large unilamellar vesicles (LUVs) (100 nm). Inclusion of stearylamine (PC:CH:SA 1:0.5:0.1) and phosphatidyl glycerol (PG) (PC:CH:PG 1:0.5:0.2) in the liposomes reduced the release of the drug in comparison to the neutral liposomes (PC:CH 1:1). The slow release of the drug from stearylamine-containing liposomes may be explained by the electrostatic interaction between the acid moiety of the drug and the amine moiety of the lipid. It is assumed that the

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possible hydrogen bonding between —OH groups of phosphatidyl glycerol and the —COOH group of the drug might be the reason for the slow release of the drug from PC:CH:PG (1:0.5:0.2) containing liposomes. Pharmacodynamic evaluation of the liposomes was performed by carrageenan-induced rat paw edema (acute) and adjuvant arthritis (chronic) models. The anti-inflammatory activity was increased from the first to fifth hour PC:CH:PG (1:0.5:0.2) and PC:CH:SA (1:0.5:0.1) liposomes showed the highest percentage inhibition of edema. In both these models, anti-inflammatory activity of liposomal indomethacin was significantly higher than that of free indomethacin ($p < .01$). The ulcer index of the free drug was about three times more than the encapsulated drug when administered at the same dose intraperitoneally to arthritic rats consecutively for 21 days.
Key Words: *Anti-inflammatory; Encapsulation efficiency; Liposomes; Ulcer index.*

INTRODUCTION

Indomethacin is the most useful drug for rheumatoid arthritis and nonrheumatoid inflammatory diseases. Its usefulness is limited, however, by the high incidence of side effects, including gastrointestinal complications, headache, and other central nervous system (CNS) disturbances, bone marrow suppression, rashes, and asthmatic attacks. Various attempts have been made to reduce these side effects (1).

Colloidal drug delivery systems such as liposomes have been investigated as potential drug carriers for site-specific delivery of antitumor agents, antibiotics, enzymes, and the like as they are preferably taken up by reticulo endothelial system (RES)–rich areas such as tumors, infected areas, and inflamed tissues. It has been established that vasopermeability increases during the process of inflammation (2). Many inflammatory mediators were reported to increase the permeability by opening the gaps between adjacent endothelial cells at the level of postcapillary venules. Anatomically, these gaps appear sufficiently large enough for extravasation of colloidal particulate systems in the size range 0.2 μm. These earlier findings prompted us to develop a liposomal system of indomethacin to reduce the side effects of this drug on chronic administration.

The diversity in the design of liposomes, such as composition, structure, and size, makes it possible to develop this drug delivery system, which is more efficacious than the use of free drug. The objective of the present study was to determine the factors influencing encapsulation of indomethacin in liposomes and to optimize these factors to achieve a suitable liposomal system. The liposomal system was evaluated further for anti-inflammatory potential using acute and chronic inflammatory models.

MATERIALS

Egg phosphatidyl choline (PG), cholesterol (CH), stearylamine (SA), phosphatidyl glycerol (PG), and phosphatidyl ethanolamine (PE) were obtained from Fluka (Buchs, Switzerland). Carrageenan, Freund's complete adjuvant and dialysis tubing were procured from Sigma (St. Louis, MO). Membrane filters were purchased from Nucleopore Incorporated (Pleasanton, CA). Indomethacin was a gift sample from Natco Pharma Limited, Hyderabad, India. All other reagents were analytical grade.

METHODS

Preparation of Liposomes

Liposomes were prepared by various methods. A brief discussion of the procedures follows.

Thin-Film Hydration Method

Multilamellar vesicles (MLVs) were prepared by the conventional method described by Shaw et al. (3). The specified quantity of lipid(s) and the drug were dissolved in chloroform in a round-bottom flask. The solvent was evaporated under reduced pressure to obtain a thin film. The flask was stored overnight under vacuum to remove traces of the solvent. The lipid film was hydrated with phosphate-buffered saline (PBS), pH 7.4.

Ether Infusion Method

For the ether infusion method (4), the lipid mixture and the drug were dissolved in diethyl ether at a concentration of 2 mg/ml and kept on ice after complete drying.

A sage syringe (pump) coupled to a crimped 19-gauge needle with polypropylene tubing was employed for the infusion process. The other end of the tube was dipped in PBS, pH 7.4, maintained at 45°C with constant stirring. An icebag was placed on top of the syringe so that a high temperature difference between the lipid solution and the buffer was ensured for the flash evaporation of the solvent. The lipid solution was injected into the buffer at a flow of 0.25 ml/min to prepare large unilamellar vesicles (LUVs).

Proliposome Method

For the proliposome method (5), the lipid mixture was dried thoroughly and dissolved in warm ethanol (80 mg) along with the drug, and 25mM Tris-HCl, pH 7.4 (200 mg) was added to yield a 100:80:20 w/w/w lipid:ethanol:water mixture. This mixture was heated to 60°C for a few minutes and then allowed to cool to room temperature to facilitate the formation of a proliposome mixture. This proliposome mixture was finally converted to a liposome suspension by dropwise addition of the buffer with continuous stirring.

High-Speed Dispersion Method

For the high-speed dispersion method (6), the lipid (100 mg) along with the drug and Poloxamer 188 (10% by weight of total lipid) was dissolved in a sufficient quantity of ethanol and warmed at 50°C for a few minutes to evaporate ethanol until the product looked like a gel. To this, 300 mg of glycerin was added and stirred well to get a homogeneous mixture. At the same time, PBS at pH 7.4 was heated to 50°C and was added to the above lipid mixture to get a viscous liposomal suspension.

Determination of Encapsulation Efficiency

A volume of 100 ml of stock liposomal dispersion was taken in a standard flask, and 50 ml of 10% w/v Triton X-100 was added, vortexed thoroughly, and allowed to incubate at room temperature for 4 hr for complete release of the drug from the liposomes. The volume of the solution was made up to 100 ml, and the absorbance was noted at 320 nm using a Shimadzu double-beam spectrophotometer.

Determination of Lipid Content

Determination of lipid content gives an idea about the efficiency of the method of preparation of liposomes. A volume of 100 ml of the liposomal dispersion was taken, and the total phosphorous was measured per the method described by Stewart (7). First, the lipid was extracted in methanol, and the methanol portion was separated, dried, and further dissolved in the required amount of chloroform. To this, 2 ml of ammonium ferrothiocynate solution and enough chloroform were added to make the final chloroform volume 2 ml. The biphasic system was then vigorously mixed on a rotomixer for 1 min. On separating, the lower chloroform phase was removed with a syringe, clarified if necessary with a pinch of anhydrous Na_2SO_4, and the optical density of chloroform was read at 488 nm using chloroform as the blank.

In Vitro Release of Indomethacin from Liposomes

A volume of 1 ml of liposome preparation was taken in a dialysis tube, and both the ends were tied. The dialysis bag was suspended in 40 ml of PBS, pH 7.4, maintained at 25°C ± 2°C, and the solution was stirred at 200 rpm. Periodically, 1-ml samples were drawn, and absorbance was noted at 320 nm using a double-beam spectrophotometer. The volume of the buffer was maintained at 40 ml throughout the experiment.

Pharmacodynamic Evaluation

Indomethacin-encapsulated liposomes were evaluated for anti-inflammatory activity by two experimental models. They are briefly described next.

Carrageenan-Induced Rat Paw Edema Model

The carrageenan paw edema was induced as described by Winter et al. (8). In the first experiment, male Wistar rats weighing 150 ± 10 gm were divided into six groups containing five rats per group. To the first group, free indomethacin was given at a dose of 3 mg/kg (i.p.), and the second group was given empty liposomes (3 mg of lipid/kg). Various liposome formulations (PC:CH 1:1, PC:CH:PE 1:0.5:0.16, PClCH:PG 1:0.5:0.2, and PC:CH:SA 1:0.5:0.1) were given to each of the remaining rats at a dose equivalent to 1.5 mg of drug/kilogram i.p. After 1 hr, 0.1 ml of 1% (w/v) carrageenan solution was injected intraplantarly in the right hind paw. The edema volume was measured periodically up to 5 hr using a plethysmometer (Ugo Basile, Comerio [VA], Italy), and percentage inhibition of edema was calculated using the following equation:

$$\text{Percentage Inhibition} = [(V_{\text{control}} - V_{\text{treated}})/(V_{\text{control}})] \times 100$$

where V_{control} is the mean edema volume of rats in the control group, and V_{treated} is the edema volume of each rat in the test group.

In another experiment, male Wistar rats each weighing 150 ± 10 gm were divided into five groups containing five rats per group. Each group of rats was given one formulation as follows. Empty liposomes were given to the first group (3 mg of lipid/kg). The second group was given free indomethacin (3 mg/kg). The last two groups were given liposomes of 50 nm and 100 nm size (1.5 mg of drug/kg). The rest of the procedure was the same as that of the above experiment.

Adjuvant Arthritis

Male Wistar rats weighing 200 ± 20 gm were given a single intradermal injection of 0.25 ml of Freund's adjuvant into the subplantar area of the right hind paw. Rats were divided into three groups containing five rats in each group, and the test preparations were administered at the same dose equivalent to 3 mg/kg of indomethacin intraperitoneally once a day for 15 consecutive days, beginning 1 day before the injection of the adjuvant. At regular intervals, paw volume of both the paws was measured by plethysmometer.

Statistical analysis of the above data was performed by analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Table 1 shows the effect of lipid composition and charge on encapsulation efficiency of indomethacin in MLVs. Based on encapsulation efficiency, the thin-film hydration method was found to be the better method for encapsulation of indomethacin in phosphatidyl choline liposomes. It was observed that inclusion of positively charged lipid (stearylamine) increased the encapsulation efficiency of the drug by more than twofold compared with neutral phosphatidyl choline liposomes (PC:CH 1:1). It may be assumed that, as indomethacin is an acetic drug, its encapsulation was better when a basic lipid such as stearylamine was used because of the electrostatic interaction of the drug with the bilayers. Improvement in encapsulation efficiency by the addition of a negatively charged phospholipid such as phosphatidyl glycerol (from 30% to 54%) may be because of hydrogen bonding between the —COOH group of the drug and the —OH groups of the phospholipid. Addition of phosphatidyl ethanolamine increased encapsulation of indomethacin, but to a lesser extent compared to the addition of the charged lipids. Determination of lipid content of a liposomal formulation reflects the efficiency of method of the prepara-

Table 1

Effect of Lipid Composition on Encapsulation of Indomethacin in Liposomes Based on Egg Phosphatidyl Choline Using Various Methods of Preparation

Lipid Composition	Encapsulation Efficiency % (mol/mol)			
	Thin-Film Hydration Method	Proliposome Method	Ether Infusion Method	High-Speed Dispersion Method
Phosphatidyl choline (PC)	30.10 ± 4.53 (3.33)	25.64 ± 6.42 (3.47)	25.62 ± 6.16 (3.47)	31.66 ± 8.49 (2.85)
Phosphatidyl choline:cholesterol (1:1)	31.57 ± 3.09 (3.67)	29.19 ± 7.68 (4.10)	30.37 ± 9.54 (3.03)	31.35 ± 4.17 (2.56)
Phosphatidyl choline:cholesterol: stearylamine (1:0.5:0.1)	70.16 ± 11.16 (2.96)	65.52 ± 7.03 (4.22)	57.63 ± 13.24 (3.17)	41.82 ± 6.27 (2.74)
Phosphatidyl choline:cholesterol: phosphatidyl ethnolamine (1:0.5:0.16)	58.90 ± 12.61 (3.01)	40.64 ± 5.14 (3.79)	40.59 ± 5.14 (3.50)	30.15 ± 8.23 (2.65)
Phosphatidyl choline:cholesterol: phosphatidyl glycerol (1:0.5:0.2)	54.27 ± 6.48 (3.75)	31.33 ± 3.60 (4.06)	36.64 ± 7.56 (3.11)	31.11 ± 4.98 (3.11)

Each experiment was repeated in triplicate. Initial total lipid content in each experiment was kept at 4 mg/ml. Average lipid content of each preparation is expressed as mg/ml in parentheses.

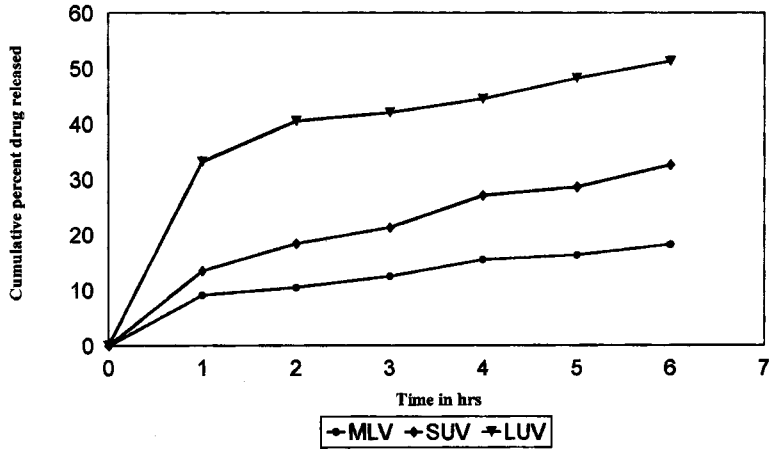

Figure 1. In vitro release profiles of indomethacin from various liposomes prepared with PC:CH (1:1) molar composition. All experiments were repeated in triplicate.

tion of liposomes. It was observed from the lipid content data from the above table that the proliposomal method was an efficient method for the preparation of MLVs.

The MLVs were sonicated using a Branson ultrasonifier at 100% duty cycle for 7 min and were further extruded through 0.05-μm polycarbonate membrane to get small unilamellar vesicles (SUVs). This also removes titanium particles if any are present in the dispersion.

Table 1 also shows the effect of lipid composition and charge on encapsulation of indomethacin in LUVs prepared by the ether infusion method. This method resulted in a heterogeneous size population of liposomes as observed by transmission electron microscopy.

Based on encapsulation efficiency, LUVs were observed not to be better candidates for encapsulation of indomethacin compared to MLVs with the lipid compositions tried. As indomethacin is a lipophilic drug, it will be incorporated into the lipid layers. Large unilamellar vesicles, in spite of their larger size, will have a volume in the lipid compartment that will be lower than in the aqueous compartment. Therefore, encapsulation efficiency was not improved. Incorporation of cholesterol

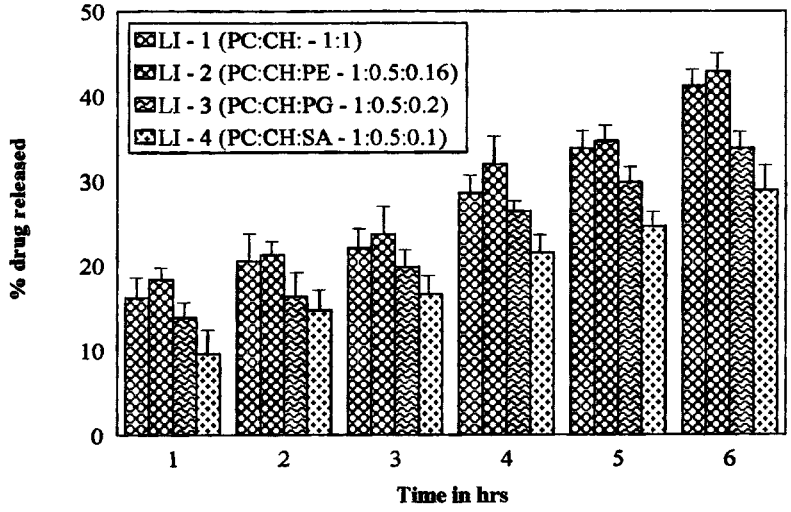

Figure 2. Effect of lipid composition on in vitro drug release profile of indomethacin from liposomes prepared with various lipid compositions. All experiments were repeated in triplicate.

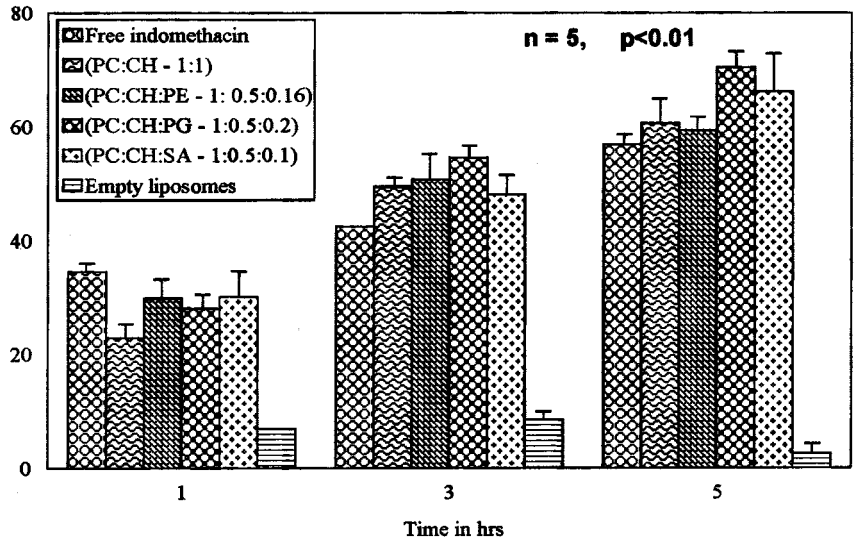

Figure 3. Effect of lipid concentration on anti-inflammatory activity of indomethacin encapsulated in liposomes (50 nm). The dose of free indomethacin was 3 mg/kg, and of liposomal indomethacin, it was 1.5 mg/kg. Free indomethacin versus liposomes: $p < .01$. LUVs versus SUVs: $p > .05$.

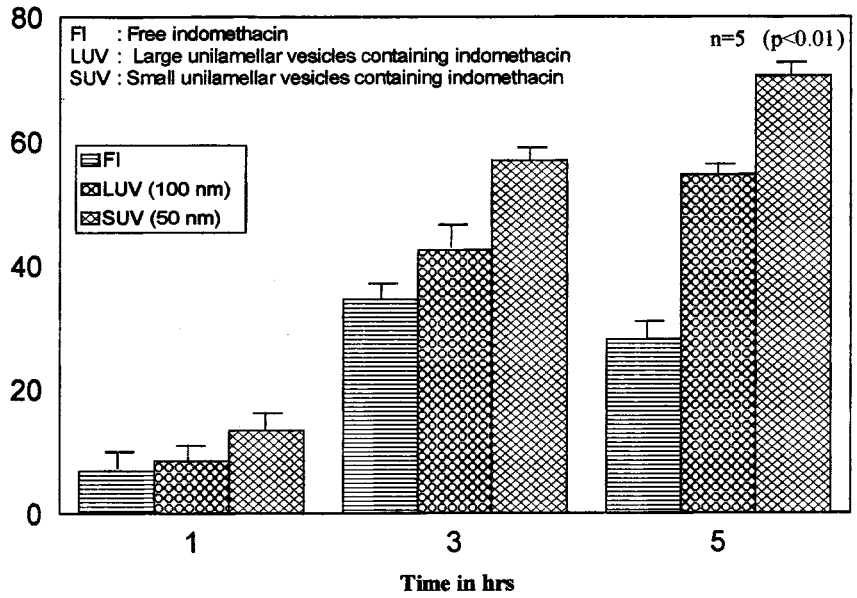

Figure 4. Effect of liposome size on anti-inflammatory activity of indomethacin encapsulated in liposomes prepared with PC:CH (1:1) molar composition. The dose of free indomethacin was 3 mg/kg, and of liposomal indomethacin, it was 1.5 mg/kg. Free indomethacin versus liposomes: $p < .01$. LUVs versus SUVs: $p > .05$.

Table 2

Effect of Free and Encapsulated Indomethacin on Edema Volume, Induced Using Freund's Adjuvant

		Average Edema Volume (ml), Days After Inoculation with Adjuvant										
Treatment	Dose	1	2	3	5	7	9	11	13	15	18	21
Empty liposomes	4 mg/kg, i.p.	1.10 ± 0.22	1.67 ± 0.13	1.13 ± 0.15	1.54 ± 0.32	1.64 ± 0.17	1.61 ± 0.19	1.65 ± 0.18	1.54 ± 0.21	1.57 ± 0.26	1.71 ± 0.18	1.79 ± 0.16
Free indomethacin	3 mg/kg, i.p.	1.34 ± 0.20	1.32 ± 0.25	0.89 ± 0.12	1.22 ± 0.20	1.28 ± 0.13	1.31 ± 0.43	1.27 ± 0.11	1.19 ± 0.45	1.17 ± 0.31	1.11 ± 0.28	1.16 ± 0.17
Liposomal indomethacin (PC:CH: PG 1:0.5:0.2)	3 mg/kg, i.p.	0.96 ± 0.10	1.26 ± 0.15	0.77 ± 0.06	1.31 ± 0.17	1.14 ± 0.36	1.09 ± 0.73	1.10 ± 0.26	0.91 ± 0.11	0.90 ± 0.28	0.84 ± 0.33	0.86 ± 0.29

Test preparations were given intraperitoneally equivalent to a dose of 3 mg/kg of indomethacin ($N = 5$; $p < .01$).

did not affect the encapsulation efficiency in both MLVs and LUVs, but as cholesterol was found to impart the rigidity to bilayers and thereby increase the stability, it was used in all the preparations.

There is a limitation of size for the use of colloidal drug carriers for effective localization at the inflammatory sites. It has been reported that particles in the size range <200 nm will be deposited efficiently at the inflammatory sites. Although for hydrophobic drugs MLVs are suitable drug carriers, because of their size limitation, MLVs were further sonicated, extruded through 0.05-μm polycarbonate membrane, and used for in vivo studies.

In vitro release studies are often performed to predict how a delivery system might work in ideal situations, which might give some indication of its performance. Hence, a comparative drug release study was performed among MLVs, SUVs, and LUVs prepared with the same lipid composition (PC:CH 1:1). About 19% of the encapsulated drug was released from MLVs at 6 hr, whereas SUVs released around 32%, and about 50% of the encapsulated drug was released from LUVs. From Fig. 1, it is evident that the percentage drug release from MLVs was much lower compared to ULVs. This may account for the number of aqueous barriers to be crossed to be released outside the vesicle. The percentage release of the encapsulated drug was much higher with LUVs than SUVs.

Apart from lamellarity, the composition of bilayers also influences the rate of drug release from the liposomes. Hence, a series of liposomes with varied lipid compositions was prepared by the proliposome method. These liposomes were sonicated and further extruded through a 0.1-μm pore size polycarbonate membrane, and drug release studies were performed as mentioned above. As shown in Fig. 2, the rate of drug release was slow from negative and positively charged liposomes (PC:CH:PG 1:0.5:0.2 and PC:CH:SA 1:0.5:0.1 liposomes, respectively) than the neutral liposomes. The slow release of the drug may be attributed to the electrostatic interaction between the drug and the lipid bilayers.

The effect of lipid composition on anti-inflammatory activity was studied by administering the above series of liposomes to rats with carrageenan-induced inflammation at a dose equivalent to half the dose of free indomethacin. Empty liposomes were also administered to one group of rats to find if the lipid vesicles have any anti-inflammatory effect. It was observed that empty liposomes (3 mg of lipid/kg) could not inhibit the edema formation significantly. From Fig. 3, it is evident that 1 hr after administration of the formulations, the percentage inhibition of edema with all the liposomal formulations (1.5 mg of drug/kg) was almost same as that of the free drug (3 mg/kg), indicating that the anti-inflammatory activity of the liposomes was twofold higher than the free drug. The

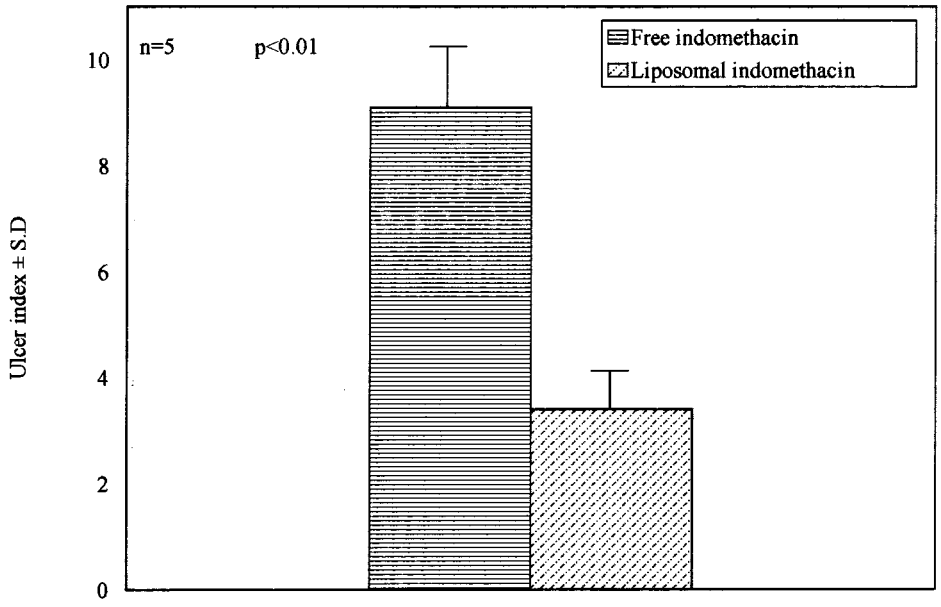

Figure 5. Ulcerogenicity of free and encapsulated indomethacin (PC:CH:PG 1:0.5:0.2) administered intraperitoneally consecutively for 21 days to adjuvant-induced arthritic rats. Dose was 3 mg/kg.

anti-inflammatory activity was observed to increase from the first hour to the fifth hour. After 5 hr, the highest percentage inhibition of edema was observed with PC:CH:PG (1:0.5:0.2) liposomes and PC:CH:SA (1:0.5:0.1) liposomes. Although stearylamine was used to find the effect of charge on anti-inflammatory potential of the liposomes, as it has been proved toxic, efforts have to be made to look for alternative cationic lipids. No significant difference in percentage inhibition in edema was found between PC:CH:PG (1:0.5:0.2) and PC:CH:SA (10.5:0.1) liposomes ($p > .05$).

It is evident from Fig. 4 that there is a significant difference in percentage inhibition of edema volume between the free and encapsulated indomethacin at a dose of 3 mg/kg. To understand the advantage in using the submicron size range of particles, liposomes of 50 nm and 100 nm were used for the animal experiments. At 3 hr, percentage inhibition of edema was about 55% with SUVs (50 nm) and about 50% with LUVs (100 nm), whereas with free indomethacin it was only 40% at the same dose. It may be assumed that liposomes were localized at the inflammatory site, therefore anti-inflammatory activity was more than the free drug.

As shown in Table 2, in the case of Freund's adjuvant arthritis model, peak swelling of the paw was observed on the second day with free indomethacin, whereas it was the fifth day with liposomal indomethacin, and neither the free drug nor the encapsulated indomethacin could completely prevent the inflammation. On the 21st day, a significant difference was observed in edema volume between the free drug and the encapsulated drug ($p < .05$), indicating better efficacy of liposomal indomethacin.

To understand the ulceration potential of liposomal indomethacin in comparison to free indomethacin, the ulcer index of the rats in both the groups was determined on the last day of the study by the method described by Robert et al. (9). It was found that the ulcer index with encapsulated indomethacin was one-third that of free indomethacin (Fig. 5).

It may be concluded that encapsulated indomethacin showed greater efficacy than the free drug in inhibiting the inflammation in both acute and chronic inflammatory models. It was also observed that the ulceration potential of the drug was reduced considerably when liposomal indomethacin was administered. However, as pharmacodynamic studies alone cannot give conclusive evidence of efficacy and safety of a formulation, biodisposition studies of various liposomal formulations with different lipid compositions in arthritic rats are being carried out in our laboratory.

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