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LIPOSOMES WITH ANTI-INFLAMMATORY STEROID PREDNISOLONE PALMITATE

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ABSTRACT

To increase the efficiency of therapeutic anti-inflammatory steroid, prednisolone palmitate was synthesized and encapsulated within a liposome which has a Tm value of 40°C. This steroidal liposome showed higher anti-inflammatory activity than prednisolone hemisuccinate, following intraveneous injection into rats, using a λ-carrageenin paw edema test.

INTRODUCTION

High-dose and prolonged corticosteroid therapy may be associated with a variety of complications and side effects [1]. To

avoid these toxic side effects, many different approaches were devised, such as chemical modifications of glucocorticoids, local applications and alternate day therapy. Anti-inflammatory steroids encapsulated within a liposome showed a great advantage over the free steroid [2-4]. Such phenomena may be attributed to the fact that liposomal steroids are more concentrated in the inflamed region and facilitated by migrating phagocytic cells. Another contributing factor is that interaction of free steroids with blood proteins may be avoided by use of a liposome. Once the steroid-containing liposomes reach the target site, their content may be delivered by endocytosis or fusion, thereby improving localization at the inflamed site and enhancing therapeutic effects at lower dosage [5,6]. This could diminish serious dose-related toxic side effects.

In this study, to deliver prednisolone into the inflamed site effectively, 1) liposome was prepared, of which the size was smaller than 1 μm in order to minimize its propensity for deposition in the liver and spleen of which the reticuloendothelial system is highly developed; 2) high incorporation of prednisolone palmitate and retention under normal physiologicals condition was achieved by using various liposomes containing different lipid constituents and prednisolone palmitate; and 3) a liposome having a Tm value near 40°C was chosen for an effective release of its content at the inflamed site. Anti-inflammatory activity was evaluated in a λ-carrageenin induced rat paw edema test.

MATERIALS AND METHOD

Materials

Egg yolk phosphatidic acid (PA), egg yolk phosphatidylcholine (PC), sphingomyeline (SM), cholesterol (CHL), dipalmitoylphosphatidylcholine (DPPC), diheptadecanoylphosphatidylcholine (DHPC), distearoyl-phosphatidylcholine (DSPC), palmitoyl chloride, λ-carrageenin and prednisolone hemisuccinate were obtained from Sigma Chemical Co. (St. Louis, MO). Prednisolone and prednisolone acetate were purchased from the Upjohn Co. (Kalamazoo, MI). All other chemicals were reagent grade or analytical grade.

Preparation of Prednisolone Palmitate

Prednisolone palmitate was prepared by conventional synthetic procedure with palmitoyl chloride.

Prednisolone (240 mg, 0.66 mmol) was dissolved in pyridine (5ml) at 21°C. Palmitoyl chloride (0.4 ml, 1.32 mmol) was added and the mixture was stirred at 21°C for 20 hours. The solvent was removed in vacuo at 40°C. Methylene chloride (20 ml) and 0.9 M sodium chloride solution (20 ml, .3 aliquots) were added and the two phases were shaken vigorously. After removal of aqueous phase three times, the organic phase was dried with magnesium sulfate and evaporated to dryness. The dried residue was dissolved in chloroform (1.5 ml) and applied to a silica gel column (1.2 x 25 cm) using chloroform/acetonitrile (5:2, v/v) as a mobile phase. After combining the fractions-containing product, checked by TLC, evaporation gave prednisolone palmitate as a pure white solid. The purity and identification were checked by the HPLC assay

system described below, and the chemical structure was confirmed by IR spectroscopy.

HPLC Analysis of Steroids

All anti-inflammatory steroids were analyzed by HPLC (Waters Associate) equipped with variable wavelength UV detector set at 254 nm. The Omniscribe recorder (Houston Instrument) was used for recording the chromatographic peak. Using the peak height of each steroid, the standard curves were constructed. Prednisolone and prednisolone acetate were quantitated according to the method of Ralph et al. [7] with μ-Bondapak analytical column using water/tetrahydrofuran/acetonitrile (77.5:12:10.5, v/v/v) as a mobile phase. The assay was linear from 50 to 250 ng at 0.02 AUFS. Prednisolone palmitate was analyzed with μ-Porasil column using cyclohexane/ethanol (76:24, v/v) at a flow rate of 1.5 ml/min. The assay was linear from 60 to 300 ng at 0.02 AUFS. Retention time was 3.0 min for prednisolone palmitate and 7.0 min for the internal standard theophylline.

Preparation of Liposomes

A basic procedure for preparation of liposomes was followed as described by Shaw et al. [8].

A combination of lipids (12 mg) and steroid (5 mg) was dissolved in chloroform (1.5 ml). The solvent was removed from the lipid mixture by rotary evaporation under nitrogen gas at a temperature 20°C higher than each constituent lipid's Tm value in order to make the lipids more compatible with the steroids. The dried lipid film was dispersed in 5mM sodium phosphate buffer, pH 7.35, containing 0.15 M NaCl (2.5 ml), and vigorously shaken at

the above corresponding temperature for 1 hr. After standing at 40°C for 30 min, the suspension was sonicated for 15 min using the microtip of a Branson sonifier (VWR Scientific Inc.). To anneal the prepared liposome, the suspension was allowed to stand alone at 40°C for 30 min. The resultant translucent suspension was centrifuged at 500 x g at 21°C for 10 min to remove any titanium particles shed by the probe during sonication. To separate unentrapped steroids from the liposome suspensions, they were washed either by centrifugation at 50,000 x g for 10 min or by passing through a Sephadex G-50 column (2.5 x 25 cm). After separation of unentrapped steroids, the pellets were resuspended in 2 ml of phosphate-buffered saline (PBS), pH 7.35.

Properties of the Prepared Liposomes

The size distribution of the prepared liposomes was observed by electron microscope according to the general method of negative staining [9].

The Tm value of each liposome was measured using liposomal pellets by differential scanning calorimeter (Perkin-Elmer) analysis.

To determine the amount of encapsulated steroids in each liposome, the liposomes were incubated at 73°C for 15 min to rupture the membrane. After that time, the steroids were extracted with solvent and analyzed by HPLC.

For measuring steroidal retention within the liposomes, each liposome suspension was diluted to 4 ml with PBS, and incubated at 37°C under nitrogen. After 24 hr, the liposome suspensions were

washed by centrifugation at 50,000 x g for 10 min (3 times) in order to remove leaked steroids and the amount of steroids remaining within the liposomes was determined by HPLC analysis.

Pharmacological Evaluation

The relative anti-inflammatory activity was measured by using a λ-carrageenin induced rat paw edema test [10]. Male Sprague-Dawley rats weighting 120-150 g (Southern Animal Farms, Prattville, AL) were maintained on a standard diet with water ad libitum under controlled lighting conditions for 1 week prior to use. Groups of 6-8 rats were injected intravenously via the tail vein with liposomal suspension or free prednisolone hemisuccinate in 40% DMSO solution immediately prior to injecting 0.1 ml of 1% λ-carrageenin solution in saline into the left hind paw. Paw volumes were determined with mercury displacement plethysomography by immersion of the paw to the anatomical hairline. Paw volumes were measured before and 4 hrs after injection of λ-carrageenin solution. The increase in paw volume over a 4 hr period was calculated and a dose-response curve was constructed. Significance among the groups was determined by using Duncan's multiple range test.

RESULTS

Properties of Liposomes

The size distribution of the liposomes ranged from 600 to 1,500 Å in diameter, but the mean size was 800 to 1,000 Å (Fig. 1).

As shown in Figure 2, using prednisolone or prednisolone acetate, the degree of incorporation was found to be less than 21%

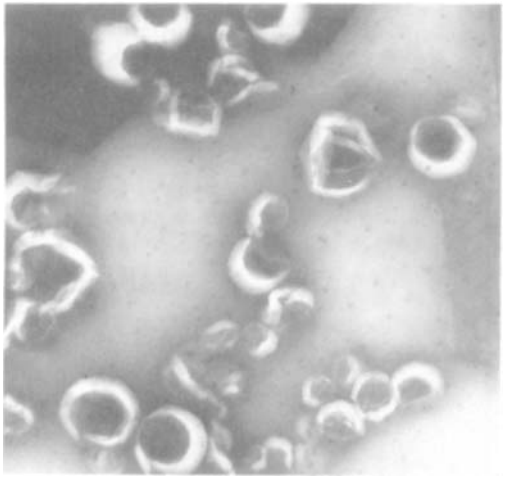

FIGURE 1

Negative Stain Electron Micrographs of Liposomes

in any liposome prepared by using different lipid compositions. Increasing the length of fatty acyl chains of lecithin did not show any enhancement of steroid incorporation into liposomes. When prednisolone palmitate was used for preparing liposomes, increasing the number of carbon atoms of the fatty acyl chains of lecithin resulted in a marked increase in steroid uptake, up to 71%. It appeared that the palmitoyl residue of prednisolone palmitate was compatible with the fatty acyl chains of the liposomal lecithins, causing a marked enhancement of steroid incorporation. To determine the retention of steroids within liposomes in vitro, the liposomes with various lipid compositions and steroids were incubated at 37°C. The results are shown in Table 1.

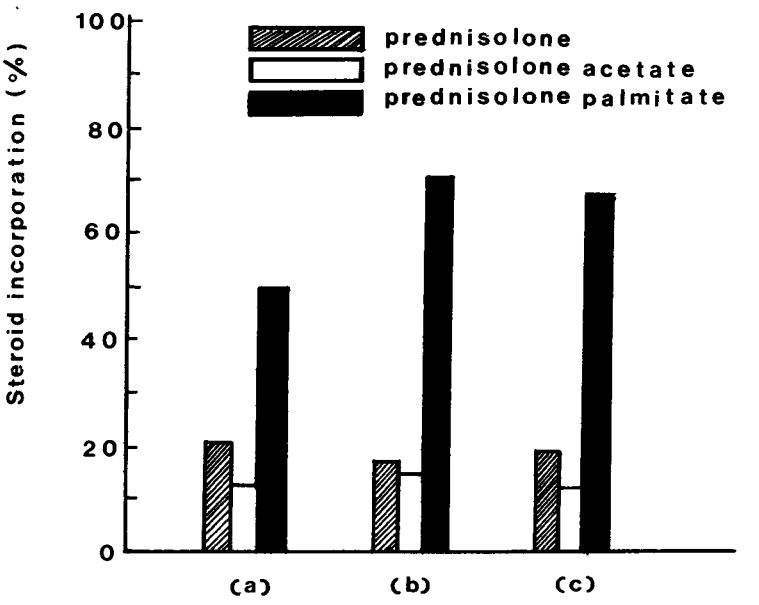

FIGURE 2

Steroid Incorporation With Various Liposomes

(a) DPPC 11 mg/PA 1 mg;

(b) DPPC 9 mg/DHPC 3 mg;

(c) DPPC 9 mg/DSPC 3 mg.

While most of the prednisolone and prednisolone acetate were released from the liposomes, prednisolone palmitate was retained by more than 80.7% within liposomes. This showed that prednisolone palmitate liposomes might be more stable under normal physiological conditions than the liposomes of prednisolone or prednisolone acetate. When the various liposomes were transferred to the analysis pan of DSC after ultracentrifugation at 50,000 x g for 30 min, it was found that liposomes with Tm values near 40°C were:

TABLE 1

Retention of Steroid Within Liposomes

Lipids & Steroid	Retention (%)
DPPC PA prednisolone	10
DPPC PA prednisolone acetate	13
DPPC PA prednisolone palmitate	80.7
DPPC DHPC prednisolone palmitate	86
DPPC DSPC prednisolone palmitate	90

DPPC 11 mg/PA, 1 mg/prednisolone palmitate 5 mg; and DPPC 9 mg/DHPC 3 mg/prednisolone palmitate 5 mg (Fig. 3). However, taking into consideration the incorporation and retention of steroids, the most suitable composition for the liposome system was DPPC 9 mg/DHPC 3 mg/prednisolone palmitate 5 mg.

λ-Carrageenin Induced Rat Paw Edema Test.

In our experiment, the anti-inflammatory activity of prednisolone palmitate encapsulated with liposomes of DPPC and DHPC was compared with free prednisolone hemisuccinate in 40% DMSO solu-

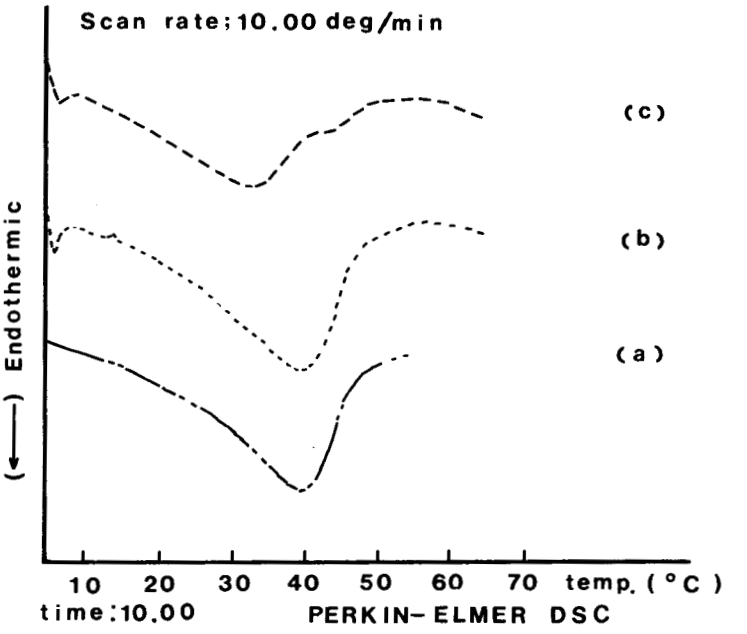

FIGURE 3

Thermotropic Phase Transition of Lipid Vesicles

(a) DPPC 11 mg/PA 1 mg/prednisolone palmitate 5 mg;

(b) DPPC 9 mg/DHPC 3 mg/prednisolone palmitate 5 mg;

(c) DPPC 9 mg/DSPC 3 mg/prednisolone palmitate 5 mg.

tion. The liposomal prednisolone palmitate was approximately 2 times as potent as prednisolone hemisuccinate (Fig. 4).

DISCUSSION

We have investigated a liposomal steroid system for intravenous use. Through a series of in vitro tests, we set up a steroid delivery system--DPPC 9 mg, DHPC 3 mg, and prednisolone palmitate 5 mg--following considerations of incorporation, stability, size,

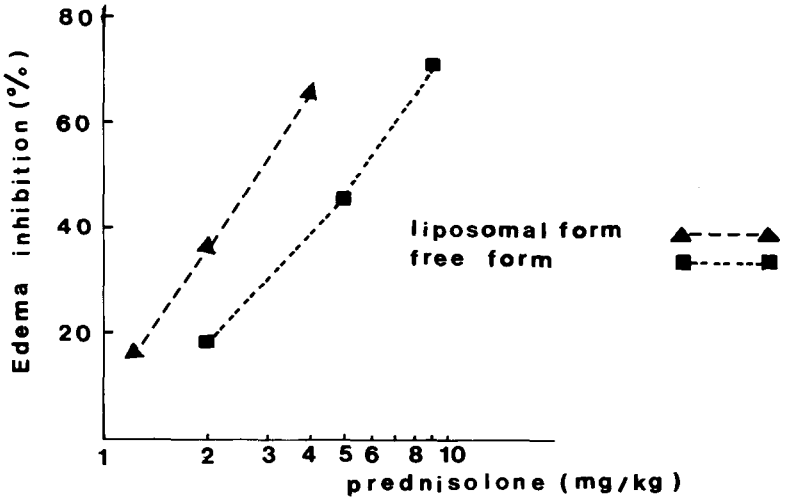

FIGURE 4

Edema Inhibition of Liposomal Prednisolone Palmitate and Free Prednisolone Hemisuccinate

and Tm value. These parameters may enable a liposome to be changed into a fluid state at the inflamed region and maximize the release of liposomal content. Regarding incorporation and stability, this system showed a greater improvement over other previously published results [3,8].

Although the potency increase was not as large when compared to the study of cortisol palmitate [4], it still showed a two-fold increase in potency by the carrageenin paw edema test via intravenous injection. This improvement in anti-inflammatory activity might be partly explained by efficient phagocytosis of the cells involved in the inflammatory process and facilitation of liposomal lysis near 40°C, as suggested by Yatvin et al. [11].

From the results of this study, it is indicated that incorporation efficiency of steroids could be improved and the resultant liposome having stability and the desired Tm value might be obtained employing proper lipid compositions. It is possible that a liposomal anti-inflammatory steroid may be a means of reducing severe dose-related toxic side effects by decreasing the dosage required for therapy and increasing the therapeutic index.

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