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THE EFFECT OF POLYCATION COMPLEXATION ON METHOTREXATE RETENTION IN LIPOSOMES

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ABSTRACT

Methotrexate and a methotrexate-DEAE dextran complex were encapsulated in dipalmitoyl phosphatidyl choline liposomes. In all cases, the degree of encapsulation of methotrexate in the methotrexate-DEAE dextran liposomes was higher than in the plain methotrexate liposomes.

The kinetic permeability of methotrexate from both methotrexate and methotrexate-DEAE dextran dipalmitoyl phosphatidyl choline liposomes was studied at 37°C in pH 7.4 acetate buffer. All liposome systems appeared to show a biphasic first order kinetic release of methotrexate. The initial rapid release probably resulted from the desorption of adsorbed methotrexate, and the subsequent slow release was from the diffusion of the entrapped drug. The desorption kinetics were separated from the diffusion process in the first phase

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by graphing. The methotrexate-DEAE dextran liposome data showed 13% methotrexate bound to the liposome surface compared to 23% methotrexate bound on the plain methotrexate liposome surface.

INTRODUCTION

Methotrexate (MTX) is widely used in the treatment of acute leukemia and a variety of other malignancies[1]. MTX interferes with DNA biosynthesis, thereby preventing the proliferation of malignant cells[2,3]. When infused intravenously, the drug produces pronounced systemic toxicity[4,5]. MTX has been encapsulated in liposomes to improve the selective transport of the drug to cancer cells[6,9]. However, the efficiency of MTX entrapment in MTX-liposomes was low. In addition, the retention of the drug in liposomes was often difficult to control, as it diffused through the phospholipid structure of liposomes. Previous work suggests that MTX binds to polycations such as DEAE-dextran[10]. This study was initiated to examine the feasibility of encapsulating a MTX-DEAE dextran complex in a L- -dipalmitoyl phosphatidyl choline (DPPC) liposome system to improve the efficiency of encapsulation and retention of the drug in liposomes. The results of this study are reported in this paper.

EXPERIMENTAL

Methotrexate, USP (M.W. 454.44, Lederle

Laboratories), DEAE-dextran (M.W. 500,000, Pharmacia Fine Chemicals), L- -dipalmitoyl phosphatidyl choline (synthetic, amorphous 98%, anhydrous M.W. 734.10, Sigma Chemical Company), cholesterol (Eastman Kodak Co.), and stearylamine (anhydrous, M.W. 269.50, Sigma Chemical Company) were used as received.

Preparation of Liposomes

Liposomes were prepared by the chloroform film method, which consisted of the following steps[11,12]. Stearylamine, cholesterol, and DPPC in the molar ratio of 1:2:7, with a total phospholipid mixture weight of 100 mg, were dissolved in 10 ml of chloroform in a flask. Rotary evaporation of the chloroform at 45°C on a Biichi Rotavapor left a thin film on the wall of the flask. Ten milliliters of acetate buffer, pH 7.4, containing various amounts of MTX and DEAE dextran was added and the film was collapsed into multilamellar liposomes by vortexing for 20 minutes at 45°C with the aid of 0.5 mm glass beads. The multilamellar liposome dispersion was sonicated on a Heat System Ultrasonil Sonicator at a setting of 3 for approximately 3 minutes prior to swelling in a water bath at 45°C for two hours.

The resulting liposomes were separated from the unentrapped MTX and DEAE dextran by centrifugation at 45,000 rpm for 30 minutes on a Beckman L2-65B ultracentrifuge. In order to ensure complete removal of the unentrapped MTX and DEAE dextran, the residue was

twice washed with 7.4 pH acetate buffer and recentrifuged. The purified residue was then suspended in acetate buffer and frozen in a dry ice acetone mixture.

Percent Entrapment of MTX in Lipsomes

The separation of MTX or MTX-DEAE dextran liposomes from the unentrapped MTX was achieved by gel filtration chromatography. Only the freshly prepared samples were used in this study and at least two different sample preparations were run for each liposome system.

A 0.5 ml sample of the liposome preparations was loaded on the colum (1.6 x 20 cm) which was previously packed with sephadex G-50 fine. The column was eluted with distilled water. About thirty fractions of 80 drops per tube with a flow rate of one drop every 3 seconds were collected with an automatic fraction collector. The relative fluoresence of each fraction was measured on a spectrofluorometer with the 2 nm bandpass, 375 nm excitation wavelength and 460 nm emission wavelength. The relative fluorescence of each fraction was plotted against the number of tubes or fractions.

In all cases, the elution of the "cloudy" fractions containing liposome-entrapped drug was followed by the clear fractions of unentrapped drug, which was shown by the presence of two distinct peaks of relative fluorescence. The areas under the curve of the liposome

peak and the free MTX peak were used to determine the percent of MTX entrapped in the liposomes.

The Kinetic Permeability Study of the Release of MTX from the Liposome Systems

The liposomes were separated from the free MTX solution by ultracentrifugation. Ten milliliters of the dispersion was centrifuged at a speed setting of 45,000 rpm for 30 minutes at 4°C. The resulting pellet was washed with pH 7.4 ± 0.05 acetate buffer to remove any free MTX and again centrifuged. The final liposome pellet was resuspended in 10 ml of pH 7.4 ± 0.05 acetate buffer. The dispersion was then divided into 14 individual samples of 0.7 ml each. The fourteen sample tubes were placed in a shaking water bath at 37 ± 0.5°C. Two samples were taken at each time interval from 0 to 110 hours. For each sample, the MTX remaining in the liposomes was separated from the free released MTX in the system by gel column chromatography. The relative fluorescence of each tube from the gel chromatography was measured on a spectrofluorometer and the percent MTX remaining in the liposomes at various sampling time intervals was calculated.

RESULTS AND DISCUSSION

The separation of MTX or MTX-DEAE dextran liposomes from the unentrapped MTX by gel filtration column chromatography resulted, in all cases, in two distinct peaks of relative fluorescence. Liposomes were eluted

first and were followed by free MTX. The area under the peaks corresponds to the total amount of liposome entrapped drug (first peak) and unentrapped drug (second peak). The percent entrapment of MTX as a free or complexed drug in DPPC liposomes was calculated from the areas under the two curves. The MTX-DEAE dextran DPPC system provided about 64% higher percent entrapment than the MTX-DPPC system, 18% vs. 11% entrapment. This may be due to the large molecular size and the special configuration of DEAE-dextran polymer drug complex, which may serve to increase the spacing of the aqueous phase between the phospholipid bilayer of the liposomes.

The Kinetic Permeability Study of Lipsome Systems

It has been suggested a two-compartment, first order kinetic model can be used to describe the diffusion of a drug from a multilamellar liposome system where there is fast diffusion from the outer layer and slower diffusion from the inner layers[13]. This model can also be applied to a small or unilamellar liposome system where surface adsorbed drug and the drug entrapped in the liposome particle represents the two compartments. There is evidence that adsorption of the materials on the liposome surface may occur[14].

The kinetics of MTX release from MTX-DPPC and MTX-DEAE dextran-DPPC liposomes is shown in Figure 1. Both MTX-DEAE dextran liposome and MTX-liposome systems appear to have a biphasic first order kinetic release of

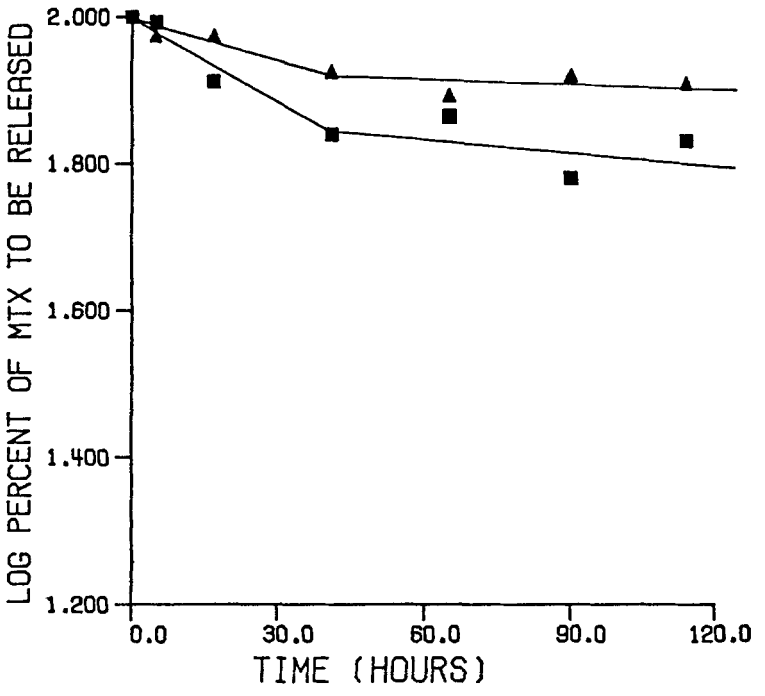

Figure 1 . The Characteristic Release of MTX from Non-lyophilized DPPC (Dipalmitoyl Phosphatidyl Choline) Liposome Systems in 0.02 M Acetate Buffer, pH 7.4 ± 0.05, at 37 ± 0.5° C. Symbol ■ represents MTX-liposome system, and symbol ▲ represents MTX-DEAE dextran complex liposome system.

Table 1. Half lives for MTX release from DPPC liposome systems.

Liposome System	Half-Life (hours) First Phase	Second Phase
MTX, Methotrexate	75	666
Methotrexate-DEAE dextran	186	4682

Table 2. Comparison of Adsorbed MTX and Entrapped MTX Remaining To Be Released for MTX-liposomes and MTX-DEAE-dextran liposomes.

Time (hrs.)	MTX-Liposome Adsorbed MTX	MTX-Liposome Entrapped MTX	MTX-DEAE Dextran Liposome Adsorbed MTX	MTX-DEAE Dextran Liposome Entrapped MTX
0	23.1	76.9	13.5	86.5
5	14.3	76.5	10.3	86.5
17	4.5	75.6	5.4	86.3
41	0.5	73.7	1.5	86.0

MTX from the liposomes. The first rapid release is probably due mainly to the desorption of the adsorbed MTX on the surface of the liposome and, to a lesser extent, to the diffusion of the entrapped MTX. The second slower release, which occurs after about 40 hours, is assumed to be solely from the diffusion of the entrapped drug through the lipid bilayer. The amount of MTX remaining to be released at the end of the first phase was found to be 69% for the in MTX-liposomes and 85% for the complex liposomes.

The half life of MTX release from both the MTX and MTX-DEAE dextran DPPC liposomes reported in Table 1. The half life for the MTX-DEAE dextran-DPPC system was approximately 2.5 times longer than that of the MTX-liposome system in the first phase and approximately 7 times longer in the second phase.

As mentioned earlier, the release of MTX from liposomes in the first phase is probably due mainly to a surface desorption process and, to a lesser extent, to a diffusion process. The second phase, however, is due primarily to diffusion of entrapped MTX through the lipid membrane. The amount of surface bound MTX *vs.* entrapped MTX can be approximated quite easily with the amount of entrapped MTX determined by extrapolation of the second phase curve to zero time and surface bound MTX obtained by difference. The results are shown in Table 2.

From an analysis of the above data, it would appear that the MTX-DEAE dextran complex liposome system is superior to the free MTX-liposomes system. The presence of the polycation polymer, DEAE-dextran, not only increases the entrapment efficiency of MTX in liposomes but also significantly increases the half-life of the delivery system. A comparison of the cytotoxic behavior of the two systems will be reported in a separate communication.

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