International Journal of Pharmaceutics, 70 (1991) 1–7

ADONIS 037851739100152X

IJP 02206

Research Papers

Characteristics of mitoxantrone loading on liposomes

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(Received 4 May 1990)
(Accepted 5 June 1990)

Key words: Mitoxantrone; Liposome; Loading efficiency; Dioleoylphosphatidylcholine; Lipid composition; Galactocerebroside

Summary

The characteristics of mitoxantrone loading on liposomes was investigated. It was found that negatively charged liposomes at the ionic strength 0.154 and pH 5.7 gave a high loading efficiency. It should be noted that the surface charges of the liposomes were subject to change from negative to positive by the influence of the interacted mitoxantrone. The neutral liposomes demonstrated a profile of loading efficiency similar to that of the negatively charged liposomes against the added mitoxantrone concentration; however, they had a lower magnitude of the loading efficiency. In the positively charged liposomes the loading efficiency increased with the added mitoxantrone concentration. At a mitoxantrone concentration of 26 μmol/ml, the loading efficiency was similar to that of the negatively charged liposomes. The 'effect of galactocerebroside' on the loading efficiency increased with added mitoxantrone concentration. The liposomes with 1.0 molar ratio galactocerebroside resulted in the highest loading efficiency in high concentrations of mitoxantrone. The loading efficiency had no dependence on the phospholipid concentration used for making liposomal membrane. An almost identical value of the loading efficiency was obtained when ultracentrifuge and dialysis methods were used to separate the mitoxantrone-containing liposomes from free drugs. The effect of vortexed and sonicated liposomes on the loading efficiency gave a similar result although the particle size was different.

Introduction

Mitoxantrone, an anthracenedione derivative, is a novel anticancer chemotherapeutic agent (Zee-Cheng and Cheng, 1978; Johnson et al., 1979; Koeller and Eble, 1988).

Liposomes are widely studied as a potential carrier for anticancer drugs such as methotrexate, bleomycin, ara c, actinomycin D, vinblastine and adriamycin, *cis*-platinum, 6-mercaptopurine and 5-fluorouracil, etc., as they can raise the therapeutic index and lower the side effect of the drugs (Gregoriadis, 1973; Gregoriadis and Neerunjun, 1975; Tsujii et al., 1976; Juliano and Stamp, 1978; Hunt et al., 1979; Patel et al., 1982; Crommelin and von Bloois, 1983; Crommelin et al., 1983; Firth et al., 1984; Ter-Minassian-Saraga and Albrecht, 1985; Khokhar et al., 1989).

It is therefore of particular interest to entrap mitoxantrone into liposomes to investigate whether or not the activity of mitoxantrone can be improved by the liposomes in some modestly active or inactive cancer systems.

In order to evaluate the proper antitumor effect of the mitoxantrone-containing liposomes, it is

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important to obtain a formulation of good loading efficiency of mitoxantrone in liposomes. In the present study, we report the effect of phospholipid composition, added mitoxantrone concentration, pH, ionic strength, phospholipid concentration, methods of preparation and methods of separation of liposomes from free drugs on the loading efficiency for the mitoxantrone-containing liposomes.

Materials and Methods

Materials

Mitoxantrone was obtained from Kingdom Pharmaceutical Co. (R.O.C.). Phosphatidylcholine (from fresh egg yolk, Type XI E), dioleoylphosphatidylcholine, galactocerebroside (from bovine brain, Type II) and cholesterol were purchased from Sigma (U.S.A.). Stearylamine and dicetyl phosphate were obtained from Pharmacia P-L Chemicals (Sweden). General chemicals were of analytical grade.

Methods

Preparation of mitoxantrone-containing liposomes

Multilamellar liposomes were prepared by a method described previously (Law et al., 1988). Phospholipids were dissolved in chloroform in a 50 ml round-bottom flask and dried in a rotary evaporator under reduced pressure at 37 °C to form a thin film on the flask. The desired concentration of mitoxantrone in saline or phosphate-buffered saline of various ionic strengths or pH values was added to the film. Multilamellar liposomes were formed by constant vortexing for 5 min on a vortex mixer (Thermolyne, Syborn, U.S.A.) and sonicated for 1 min in a bath sonicator (Bransonic 220, Smith Kline Co., U.S.A.).

Phosphatidylcholine or dioleoyl phosphatidylcholine was the main lipid component for the construction of liposomes and a molar ratio of 1.6 was used. Cholesterol was added to stiffen the liposomal membrane in a molar ratio of 1.0. Liposomes with a positive or negative charge were composed of a 0.15 molar ratio of stearylamine or dicetyl phosphate, respectively. The negatively charged liposomes showed a mobility of 1.31 $\mu m\ s^{-1}\ V^{-1}\ cm^{-1}$ which approximates that of human red blood cells. Galactocerebroside was incorporated into the liposomal membrane in a molar ratio of 0.2, 0.4, 0.6 and 1.0, respectively.

Sonicated liposomes were prepared by sonication with a probe type sonicator (Heat Systems-Ultrasonics Inc., Model W-220). The vortexed liposomes were cooled in ice-water and sonicated at 125 W for 2 min at 20-s intervals under an atmosphere of nitrogen.

Separation of mitoxantrone-containing liposomes from free mitoxantrone

The mitoxantrone-containing liposomes were separated from the unentrapped mitoxantrone by ultracentrifugation at $2.8 \times 10^{-5} \times g$ for 20 min (Beckman TLA-100, U.S.A.) and washed with saline or phosphate-buffered saline three times. Dialysis (Spectra/por 2, MW 12 000–14 000 cutoff, Spectrum Medical Industries, U.S.A.) was also performed to separate the mitoxantrone-containing liposomes from unentrapped mitoxantrone. 5 ml of the sample was dialysed against 40 l of saline or phosphate-buffered saline over 24 h to remove the free drug completely.

Assay of the entrapped mitoxantrone

The entrapped mitoxantrone concentration was determined by lysis of the liposomes with absolute alcohol. One volume of liposomes was mixed well with 3 volumes of absolute alcohol to obtain a clear solution so as to measure the absorbance at 242 nm on a spectrophotometer. No interference by the absolute alcohol was found with the assay. The entrapped mitoxantrone concentration in liposomes was expressed as loading efficiency in μmol mitoxantrone per μmol phospholipid.

Particle size analysis

The particle size of the liposomes was estimated by a Laser Particle Analyzer system (LPA-3000, Photal, Otsuka Electronics, Japan).

Mobility determination

Mobility determinations on the liposomes were carried out at 25 °C using a Rank MK II Micro-

electrophoresis Apparatus (Rank Bros., U.K.). The liposomes were made in 10^{-3} M sodium chloride solution in order to provide a medium of suitable conductance and to keep the ionic strength constant. The pH of the dispersions was adjusted to the required value by using hydrochloric acid and/or sodium hydroxide solution. A flat cell assembly and platinum electrodes were used. Ten particles were timed in both directions of the electric current to minimize the polarization of electrodes. The mobility is expressed by $U = V/E$, where V is the velocity of the migrating particle and E is the field strength calculated from the applied voltage divided by the distance between the electrodes.

Results and Discussion

Effect of lipid composition on loading efficiency

The loading efficiency against added mitoxantrone concentration on negatively charged liposomes formed using dioleoylphosphatidylcholine/cholesterol/dicetyl phosphate, positively charged liposomes consisting of dioleoylphosphatidylcholine/cholesterol/stearylamine, and neutral liposomes comprising dioleoylphosphatidylcholine is shown in Fig. 1. Regarding the liposomes with negative or neutral charge, the loading efficiency increased with mitoxantrone concentration in the low concentration range, but in the high concentration region the influence of the added mitoxantrone concentration reached a plateau entrapment value. The liposomes with a positive charge showed that, in the concentration range studied, the loading efficiency increased with added drug concentration. The characteristics of charge on the loading efficiency showed, in the low concentration range, the order of negatively charged liposome > neutral liposome > positively charged liposome. In the high concentration range, the loading efficiency of negatively charged liposomes was also greater than that of neutral liposomes. However, the loading efficiency of positively charged liposomes increased with concentration and was similar to that of the negatively charged liposomes at a mitoxantrone concentration of 26 μmol/ml.

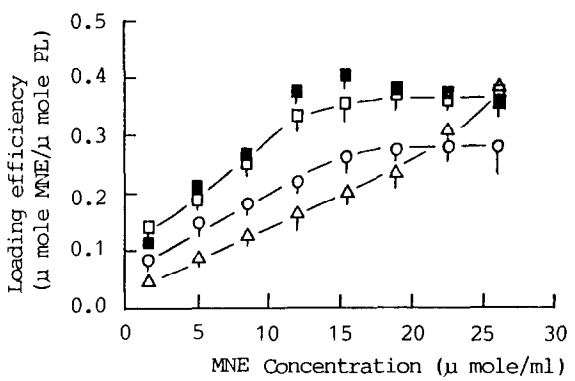

Fig. 1. Effect of lipid composition on loading efficiency. (□) Negatively charged liposomes (DOPC/C/DP 1.6:1:0.15); (○) neutral liposomes (DOPC); (△) positively charged liposomes (DOPC/C/S 1.6:1:0.15); (■) negatively charged liposomes (PC/C/DP 1.6:1:0.15). DOPC, dioleoylphosphatidylcholine; C, cholesterol; DP, dicetyl phosphate; S, stearylamine; MNE, mitoxantrone; PL, phospholipid; PC, phosphatidylcholine.

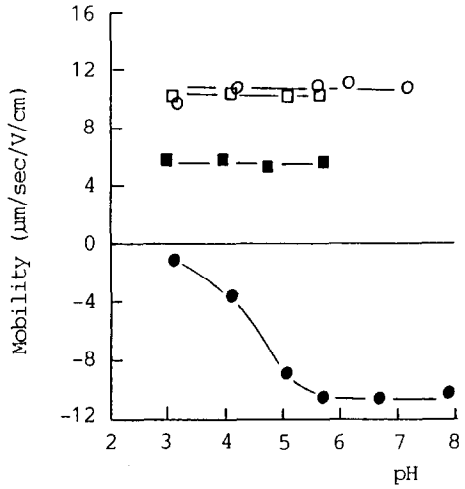

Fig. 2. The pH-mobility profiles of liposomes. (■) Negatively charged liposomes with mitoxantrone (MNE); (●) negatively charged liposomes without MNE; (□) positively charged liposomes with MNE; (○) positively charged liposomes without MNE.

The mobility profiles of positively charged and negatively charged liposomes in the presence or absence of mitoxantrone against pH are shown in Fig. 2. The negatively charged liposomes resulted in negative mobility. It increased with pH from 3 to 5 due to the negative charges on the surface of the liposome becoming ionized as the pH of the

medium increased. The mobility of the negatively charged liposomes levelled off from pH 5 to 8 because of the complete ionization of the charges on the surface of the liposome. The addition of mitoxantrone to the negatively charged liposomes caused a dramatic change in the mobility-pH profile which indicated a positive mobility in the pH range studied (Fig. 2). It is likely that in this pH range, mitoxantrone is in an ionized form and is positively charged. When mitoxantrone is incorporated into the negatively charged liposomes, some of the ionized mitoxantrone molecules bind to the charged head groups of the liposomes through electrostatic interaction. An excess of mitoxantrone molecules bound to the liposomes leads to the occurrence of a reversed charge of the liposomes which results in a positive mobility. Therefore, charge interaction between mitoxantrone and the negatively charged liposomes would be one of the mechanisms responsible for the entrapment of mitoxantrone in the liposomes. However, for the positively charged liposomes, the mobility showed no effect in the presence or absence of mitoxantrone in the pH range studied. The charge interaction for positively charged liposome encapsulation of mitoxantrone seems unlikely to occur.

The mechanisms for the loading of the drugs into the liposome include entrapping in the aqueous compartment of the liposome, binding to the hydrophilic head groups of the phospholipids of the liposomal membrane or interaction with the hydrophobic regions in the bilayers (Gregoriadis, 1976). It is likely that all three of the above mechanisms can be applied to the entrapment of mitoxantrone in liposomes. From the above results, the binding of mitoxantrone to the hydrophilic head groups of the phospholipids probably occurs via electrostatic interaction. Since the properties of the surface charge of the liposomes determine the pharmacokinetic behavior of the entrapped drugs (Jonah et al., 1975; Scherphof et al., 1980; Abraham et al., 1984), the above data regarding the surface charge altered from negative to positive on the liposomal membrane and unchanged on the positively charged liposomes after the entrapment of mitoxantrone would be of great value for future in vivo studies.

The loading efficiency of the negatively charged liposomes composed of phosphatidylcholine as shown in Fig. 1 indicates a result similar to that of the negatively charged liposomes composed of dioleoyl phosphatidylcholine. The alternation of the phospholipids of the liposomal membrane from synthetic dioleoylphosphatidylcholine to natural egg phosphatidylcholine did not alter the loading efficiency.

Effect of galactocerebroside on loading efficiency

Liposome containing galactocerebroside was found to enhance the uptake of entrapped drugs in liver (Jonah et al., 1978; Bachhawat et al., 1984; Naoi and Yagi, 1984). In this study, galactocerebroside was incorporated into the liposomal membrane to evaluate the loading efficiency of the liposomes. This preparation will be useful in the specific delivery of the mitoxantrone into the liver to increase the drug activity against liver cancers. A molar ratio of 0.2, 0.4, 0.6 and 1.0 of galactocerebroside was incorporated into the negatively charged liposomes, respectively. This is to examine the loading efficiency in correlation with the ad-

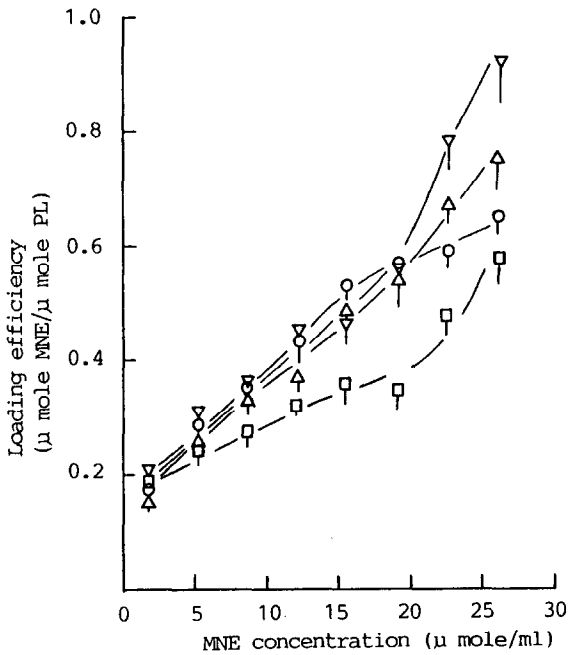

Fig. 3. Effect of galactocerebroside on loading efficiency. (□) 0.2; (○) 0.4; (△) 0.6; (▽) 1.0 molar ratio of galactocerebroside.

ded mitoxantrone concentration (Fig. 3). As the mitoxantrone concentration increased, the loading efficiency of the galactocerebroside-incorporated liposomes rose. In low concentrations of the added mitoxantrone, it demonstrated no significant change in the loading efficiency with different molar ratio of galactocerebroside. The 0.2 molar ratio galactocerebroside fraction showed a lower loading efficiency which gradually deviated from that obtained in the other fractions. In high concentrations of added drug, the liposomes with 1.0 molar ratio galactocerebroside had the highest loading efficiency, followed by the liposomes with 0.6 molar ratio galactocerebroside, those with 0.4 molar ratio galactocerebroside and those with 0.2 molar ratio galactocerebroside.

As galactocerebroside incorporates into the liposomal bilayers, the hydrophilic head group of the galactose will orient toward to the aqueous phase with the hydroxyl functional groups of the galactose exposed outside. Therefore, during the entrapment process, the mitoxantrone molecules may bind to the galactose head groups by hydrogen bonding which is one of the most important mechanisms for the entrapment of mitoxantrone into galactocerebroside-containing liposomes. This may cause an increase in loading efficiency as the molar ratio of the galactocerebroside increases with the hydroxyl groups for hydrogen bonding. This binding effect is quite significant in high concentrations of the added mitoxantrone since more mitoxantrone molecules will participate in the interaction.

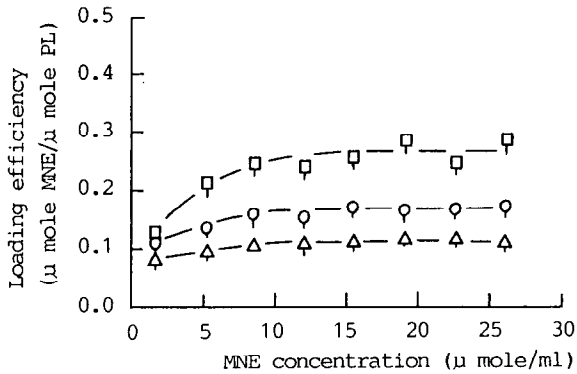

Fig. 5. Effect of pH on loading efficiency at an ionic strength of 0.067. (□) pH 5.7; (○) pH 5.1; (△) pH 4.5.

Effect of pH and ionic strength on loading efficiency

Fig. 4 represents the profiles of loading efficiency against added mitoxantrone concentration at an ionic strength of 0.154 and in pH 4.5, 5.1 and 5.7 for negatively charged liposomes. The results indicate that the loading efficiency increased with pH. Fig. 5 shows the same profiles as Fig. 4 at an ionic strength of 0.067. This demonstrates the same results against pH as those at ionic strength 0.154. However, the loading efficiency seemed to be higher at high ionic strength condition.

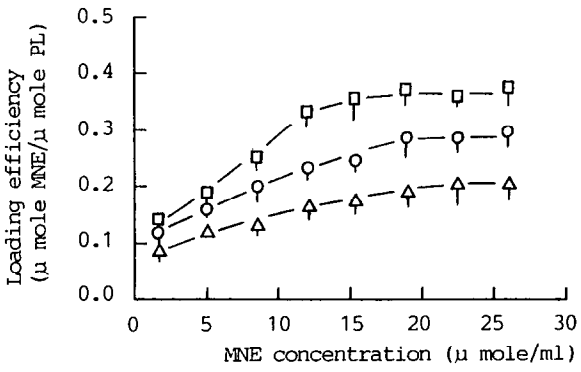

Fig. 4. Effect of pH on loading efficiency at an ionic strength of 0.154. (□) pH 5.7; (○) pH 5.1; (△) pH 4.5.

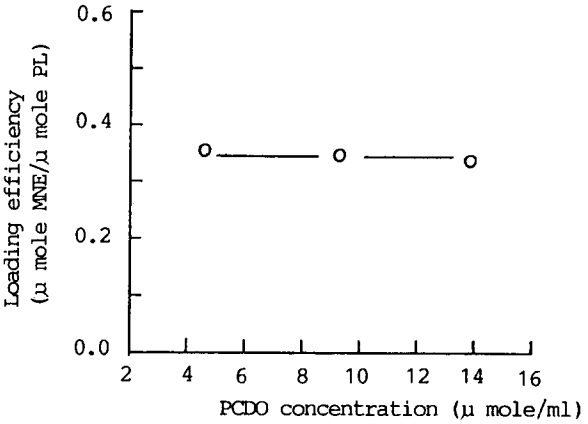

Fig. 6. Effect of phospholipid concentration on loading efficiency.

Effect of phospholipid concentration on loading efficiency

The effect of the dioleoylphosphatidylcholine concentration for the construction of negatively charged liposomes on the loading efficiency is illustrated in Fig. 6. In the concentration range studied, the loading efficiency of mitoxantrone in liposomes showed no significant change with the dioleoylphosphatidylcholine concentration used for making the liposomal membrane. It has been reported that, in the preparation of doxorubicin-containing liposomes (Crommelin et al., 1983), the capacity of the loading of doxorubicin in the positively charged liposomes does not depend on the film thickness of the phospholipid, i.e. on the added phospholipid concentration for film making.

Effect of methods to separate mitoxantrone-containing liposomes from free mitoxantrone on loading efficiency

Several methods can be used to separate drug-containing liposomes from free drugs, such as ultracentrifugation, dialysis and gel filtration. Ultracentrifugation is the fastest method for liposome separation and it is easy to reconstitute the precipitated liposomes with the medium in a suitable volume to obtain the desired concentration. However, it has the disadvantage that rupture, aggregation or caking of the precipitated liposomes occurs if centrifugation is conducted at too rapid a velocity. It is therefore necessary to carry out centrifugation at a slower speed or for a shorter time. Also, its application to submicron particles should be limited. Dialysis is time-consuming. Nevertheless, it would not alter the concentration of the liposomes after dialysis. Gel filtration takes as much time as ultracentrifugation, yet since the increased volume of the eluate leads to the dilution of the concentration of liposomes, a large volume of the liposomes for administration will cause problems during in vivo studies. In this study, we chose ultracentrifugation and dialysis methods to examine the effect of the separation of the mitoxantrone-containing liposomes from free mitoxantrone on loading efficiency. Both were found to be equally effective (Table 1).

TABLE 1

The effect of dialysis and ultracentrifugation methods for separation of mitoxantrone-containing liposomes from free mitoxantrone on loading efficiency

	Dialysis	Ultracentrifugation
Loading efficiency (μmol mitoxantrone/ μmol phospholipid)	0.360 ± 0.034	0.34 ± 0.040

TABLE 2

The effect of sonicated and vortexed liposomes on loading efficiency

	Sonicated	Vortexed
Loading efficiency (μmol mitoxantrone/ μmol phospholipid)	0.380 ± 0.040	0.360 ± 0.034

Effect of methods of preparation for liposomes on loading efficiency

The purpose of preparing sonicated liposomes in this study was to reduce the particle size of the multilamellar liposomes resulting from the vortexing method rather than to sonicate the vortexed multilamellar liposomes to obtain single unilamellar liposomes. The particle size obtained for the vortexed liposomes was 14 μm and for the sonicated liposomes 5 μm. Table 2 shows that both vortexed and sonicated liposomes gave a similar loading efficiency in spite of the particle size difference.

Acknowledgements

This study was supported by the National Science Council R.O.C. (NSC 77-0204-B075-04 and NSC 79-0412-B075-03).

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