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LIPOSOMAL DELIVERY SYSTEM FOR THE TARGETING AND CONTROLLED RELEASE OF PRAZIQUANTEL

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ABSTRACT

The targeting of Praziquantel in a liposomal delivery system can protect its uptake by non-diseased tissues, reduce its metabolism and facilitate its absorption by parasites for a long time period. This paper describes the critical parameters controlling the formation and stability of Praziquantel-encapsulated liposomes with ways of optimizing entrapment. The *in vivo* study of drug release indicates that Praziquantel, as a racemic mixture or stereoisomer, is present in the mouse liver ten days after its administration in liposomal form. Molecular configuration has no effect on deposition of the drug in the liver or on its concentration. Targeting of the (-)-stereoisomer of Praziquantel in liposomal form could eventually lead to the chemoprophylactic treatment of schistosomiasis.

INTRODUCTION

Praziquantel is a broad-spectrum antihelminthic drug that has been shown to be highly effective against all known species of Schistosomes infecting humans[1,2]. The failure of mass treatment to control schistosomiasis has been attributed to the fact that therapy is not sufficiently long-lasting[3]. The apparent half-life of Praziquantel and its metabolites is only a few hours when it is administered intravenously or orally. Only traces of the drug remain in serum after 24 hours[4]. As currently produced, Praziquantel is a racemic mixture. However, its anti-schistosomal activity is mainly concentrated in the (-)-stereoisomer[1,5-7]. In fact, the (-)-isomer is about twice as effective as the racemic drug while the (+)-isomer is much less potent[4].

The schistosomiasis parasite has a high affinity for phospholipids, and ingested lipids are found to be incorporated into stable parasite structures rather than utilized by the host for degradative energy-yielding metabolism[8]. On the other hand, the schistosomiasis parasite resides in the sinusoids of the liver as it matures for two weeks following infection[9], and it is assumed that the presence or slow release of Praziquantel in the liver will prevent its development. For these reasons, the targeting of Praziquantel in a liposomal delivery system is expected to protect host tissues, reduce drug metabolism and improve its uptake by the parasite over a long period of time. In fact, it has been reported that antimonial drugs incorporated into liposomes have a much greater effect in reducing leishmaniasis parasite counts in the liver than equivalent drug quantities injected alone[10]. Recent results from our laboratory in mice have revealed that the liposomal form of tartar emetic could have potential applications in the chemoprophylaxis of schistosomiasis[11].

A prerequisite for the successful introduction of liposomes in therapy is the stability of the formulation. Freezing the liposome dispersion is one approach that can be taken to achieve prolonged liposome shelf-life[12].

Lyophilization and rehydration, which include a freezing and thawing cycle, represent another method used by many laboratories for better stability of liposomal formulations[12-17].

The purpose of this work was to study the physico-chemical characteristics of Praziquantel-liposomes and their impact on *in vitro* and *in vivo* drug release. The specific distribution of Praziquantel in the mouse liver was also investigated as a function of its molecular configuration.

MATERIALS AND METHODS

Materials

All chemicals, Dipalmitoyl phosphatidylcholine (DPPC), dibehenoyl phosphatidylcholine (DBPC), cholesterol and Praziquantel were purchased from the Sigma Chemical Co., St. Louis, MO. Levo and dextro enantiomers of Praziquantel were a gift from Dr. Ming-Xin Qian, Chongqing University of Medical Sciences, Chongqing, China. All solvents used for chromatographic Praziquantel analysis were of HPLC grade and purchased from Fisher Scientific, Fair Lawn, NJ. Sterile sodium chloride solution was obtained from Abbott Laboratories, Montreal, Canada.

Preparation of Liposomes

Praziquantel, a lipophilic drug with very low water solubility, was incorporated in the lipid phase. In this work, we used a racemic mixture and two enantiomers [R-(-) and S-(+)] of Praziquantel. The liposomes were composed of DPPC and/or DBPC, cholesterol and Praziquantel at different molar ratios, e.g., 1.2:1.2:1.2:1 for cholesterol-poor liposomes and 1.2:1.2:2.4:1 for cholesterol-rich liposomes.

Chloroform solutions of the lipid mixtures and drug contained in 200 ml pear-shaped flasks were dried under reduced pressure. The flasks were rotated by a rotary evaporator to aid the formation of uniformly thin lipid

films on the flask walls. Each dried film was then hydrated in 10 ml 0.9% sodium chloride (USP) containing 18 mg per 100 ml Praziquantel by swirling at a temperature higher than the transition temperature of the lipid mixture until all the lipids were dispersed. Aliquots of multilamellar liposomes thus formed were diluted four-fold with saline and filtered through a 0.2 μm Gelman membrane filter. By this procedure, multilamellar liposomes are retained by the filter, and only non-associated Praziquantel, if any, will pass through. The filtrate was dried under nitrogen flow at 37°C, redissolved in ethanol and analyzed by HPLC to determine the free drug concentration. The liposomes retained by the filter were re-dispersed in saline. These liposomal suspensions were then centrifuged at 20000 g for 60 min to spin the multilamellar liposomes down to a pellet, and the supernatant was carefully separated. The pellet was re-suspended in fresh saline, 0.3 M calcium chloride or 0.3 M magnesium chloride solution (100 mg lipid/ml). From each suspension thus prepared, 3 aliquots of 10 μl were dissolved in ethanol and analyzed for total Praziquantel content. Similarly, the supernatant was dried, redissolved in ethanol and examined for free drug concentration. From these data, the percent amount of Praziquantel associated with liposomes was determined.

In vitro Drug Release Kinetics

An aliquot of each liposomal suspension was diluted five-fold with saline and distributed in 18 test tubes (10 ml in each). To determine the effect of bivalent ions (Ca^{2+} and Mg^{2+}) at the same concentration (0.3 M) on drug release, suspensions were prepared and diluted in either 0.3 M calcium chloride or 0.3 M magnesium chloride solution. Three test tubes were employed for each storage condition (static and stirring) and for each temperature (4°C, 25°C and 37°C). To establish the same stirring condition at these 3 temperatures, we used three new single-speed orbital shakers (Adams Nutator, Clay Adams, Parsippany, NJ). Every 24 h, after gentle homogeni-

zation, 250 μl of each suspension were transferred to an Eppendorf tube for drug release and size distribution studies. For size determination, 10 μl were diluted with 3 ml saline, and the rest was centrifuged at 20000 g for 60 min. The supernatant was analyzed by HPLC to examine drug release. The pellet was also dissolved in ethanol and examined for drug incorporation. These experiments were repeated three times and the values presented are the means of the data obtained.

Size Characteristics

An aliquot of multilamellar liposomes was diluted ten-fold with saline and analyzed for size with a Coulter counter (Coulter TA-II, Coulter Electronics, Montreal, Que. Canada). Particle size was between 1 and 4 μm. To obtain multilamellar vesicles small in size and homogeneous in nature, aliquots of multilamellar liposomes were sonicated at 4°C (in an ice bath), using an ultrasonic processor (Model W-350) equipped with a 1/2-inch flat tip at 200 w/cm2 (Heat Systems Ultrasonics, Farmingdale, NY). The size distribution of the liposomes thus prepared was assessed with a Nanosizer (N4SD, Coulter Electronics).

The growth in vesicle size of sonicated Praziquantel-containing liposomes was determined from changes in particle diameter after storage at 4°C, 25°C and 37°C for 3 weeks under static and stirring conditions. The effect of bivalent ions such as Ca^{2+} and Mg^{2+} at the concentration of 0.3 M on size changes was also studied for the same time period under different conditions.

HPLC Analysis

Praziquantel concentration in all cases was measured with a Gilson UV detector (Model 116) and a Gilson pump (Model 302, Gilson Medical Electronics, Villiers-le-Bel, France). Praziquantel was separated on a 25 cm x 4.6 mm 5-μm Alltech C18 column (Alltech, Deerfield, IL). The mobile

phase was a mixture of 40% HPLC grade water and 60% acetonitrile (v/v) buffered with phosphoric acid at pH 3.5, flowing at a rate of 1.5 ml/min. The eluent was monitored at 214 nm, and the results were recorded on an IBM-AT computer equipped with Gilson 714 HPLC System Controller Software.

In vivo Drug Release Kinetics

In this investigation, five groups of 20 female Swiss mice with a mean weight of 19±2.5 g were used. Four groups received Praziquantel by subcutaneous injection (dose: 50 mg/kg body weight). For the first group, Praziquantel was suspended in 1% carboxy methylcellulose aqueous solution, while the three other groups received the liposomal form of the drug, namely, levo-, dextro- and racemic Praziquantel-encapsulated liposomes. The fifth group served as controls. On the first, third, fifth, seventh and tenth days, 4 mice from each group were sacrificed and their livers were homogenized in 5 ml of phosphate buffer (0.05 M at pH 7). As an internal standard, 2 μg of clonazepam were added to the aqueous phase of homogenized samples. The organic phase was extracted twice by 5 ml of a hexane/ethylacetate (2:1) mixture and evaporated under nitrogen flow at 37°C. The residue after extraction was dissolved in 250 μl acetonitrile for HPLC analysis.

RESULTS AND DISCUSSION

Trapping Efficiency

Table 1 summarizes the various lipid compositions and trapping efficiencies of the liposomal systems prepared in this work. The results clearly show that the highest drug/lipid ratios (up to 5% wt/wt) and trapping efficiencies (up to 61%) were achieved when DBPC and DPPC were present in the same molar ratio. Although the amount of drug dissolved in the liposome aqueous phase slightly increases entrapment because of the very low

TABLE 1

Characteristics of Praziquantel-encapsulated Liposome Preparations

No.	Liposome Composition	Ratio	Lipid/Drug (mol/mol)	Trapping Efficiency
1	DBPC/DPPC/C/Pz	1.2:1.2:1.2:1	8.5:1	61%
2	DBPC/DPPC/C/Pz	1.2:1.2:2.4:1	12:1	42%
3	DBPC/C/Pz	1.2:1.2:1	22:1	12%
4	DBPC/C/Pz	2.4:1.2:1	21:1	18%
5	DPPC/C/Pz	1.2:1.2:1	24:1	11%
6	DPPC/C/Pz	2.4:1.2:1	23:1	16%
7	DPPC/C/Pz	2.4:1.6:1	30:1	13%
8	DPPC/C/Pz	2.4:0.8:1	44:1	9%

trapped volume, evaluation of trapping efficiency is based only on the amount of drug used in the lipid phase. The quantity of Praziquantel encapsulated was strongly dependent on the lipid composition of the liposomes. As we can see, trapping efficiency did not seem to change significantly when DPPC was substituted by a longer acyl chain lipid such as DBPC (formulations 3, 5 and 4, 6). However, drug encapsulation reached its highest level when both DBPC and DPPC were present at an equimolar ratio. This is in agreement with studies on other lipophilic agents[18,19]. In fact, the ability of the bilayer to form a "solvent" for lipophilic drug molecules is limited by its dimensions and molecular structure[20].

Coevaporation of the lipid and drug from chloroform in a round-bottom flask was found to produce maximum Praziquantel entrapment in liposomes. This is consistent with other work on liposome preparations, suggesting that coevaporation of lipids and lipophilic compounds from organic solvents generates practically the highest incorporation[21,22].

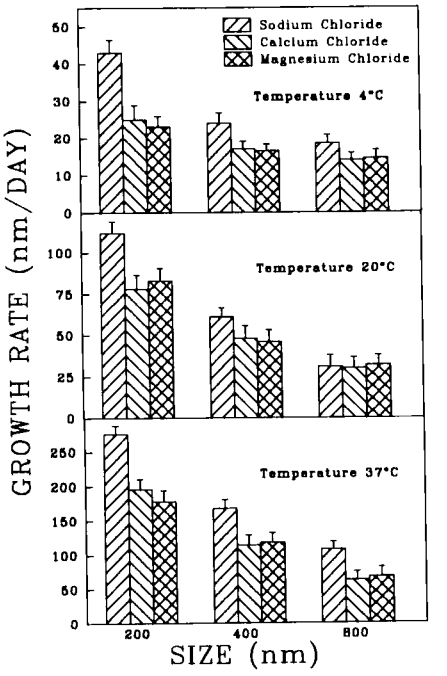

Figure 1. Effect of different ions (Na^+, Ca^{2+}, Mg^{2+}) on the growth rate of Praziquantel-containing liposomes at different temperatures under static conditions. Values are the means obtained for cholesterol-poor and cholesterol-rich formulations.

Size Characteristics and Particle Stability

The growth rate of Praziquantel-containing liposomes was determined in nm/day from changes in particle diameter after storage at 4°C, 25°C and 37°C for 3 weeks under static and stirring conditions. Three different size ranges of liposome suspension were examined, i.e. 200 nm, 400 nm and 800 nm of cholesterol-poor and cholesterol-rich preparations. The effect of different ions at 0.3 M concentration on growth rate was also studied.

No remarkable difference was observed between cholesterol-poor and cholesterol-rich liposomes. The divalent ions (Ca^{2+} and Mg^{2+}) stabilized the liposomes and significantly inhibited the growth rate (Figure 1). For these liposomal formulations, growth inhibition was dependent on vesicle size.

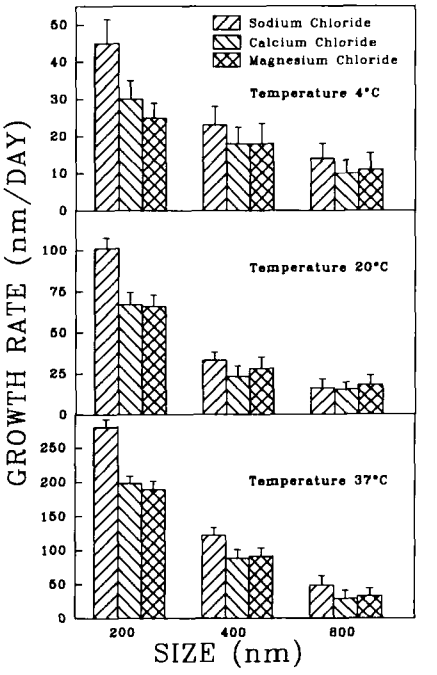

Figure 2. Effect of different ions (Na^+, Ca^{2+}, Mg^{2+}) on the growth rate of Praziquantel-containing liposomes at different temperatures under stirring conditions. Values are the means obtained for cholesterol-poor and cholesterol-rich formulations.

Bilayer stability was more pronounced at higher vesicle sizes. For example, for larger liposomes, at 800 nm, Ca^{2+} and Mg^{2+} stabilized particle size. At 200 nm and less, vesicle-vesicle fusion occurred, apparently because of the excess surface energy produced during sonication. Although the growth rate increased with storage temperature, it was significantly lower under stirring conditions (Figure 2). In fact, the growth rate decreased with agitation. This decrease was more significant for larger vesicles and less marked for liposomes of 200 nm. Since liposomal membrane integrity is process- as well as formulation-dependent, the optimization of formulation variables and preparation conditions is of great importance in obtaining meaningfully reproducible data.

***In vitro* Drug Release Kinetics**

The long-term stability of liposomes is essential for their successful introduction into therapeutic regimens. In this study, we examined the drug leakage of homogeneous liposome suspensions of 0.8-1.0 μm at 3 different temperatures (4°C, 20°C, 37°C). Figures 3 and 4 present the results obtained with respect to Praziquantel latency after 3 week storage. Although the trapping efficiency of cholesterol-poor liposomes was higher than that of cholesterol-rich liposomes, drug latency was much lower after 3 weeks under different storage conditions. These findings clearly show that the proper selection of bilayer components, e.g., inclusion of a high cholesterol ratio and longer acyl chain phosphatidylcholine into the bilayer which yield a more solid membrane, is essential for the development of stable liposome preparations for Praziquantel delivery. This observation concurs with studies performed on Doxorubicin-containing liposomes, which demonstrated that bilayer stability is strongly dependent on the lipid composition of liposomes[23].

***In vivo* Drug Release Kinetics**

Figure 5 charts the hepatic drug concentrations in mice receiving 50 mg/kg of the racemic mixture of Praziquantel incorporated in cholesterol-poor and cholesterol-rich liposomes. The latter presented a lower release rate and protected the drug for at least 10 days. This was consistent with recent work on liposome stability which revealed that inclusion of a high cholesterol ratio into the liposome structure prevented phospholipid loss when the liposomes were exposed to serum[24].

Because it is likely that configuration of the Praziquantel molecule could to some extent affect entrapment, drug release characteristics and eventually deposition and metabolic transformation in the liver, we studied the behaviour of the two enantiomers of Praziquantel and its racemic mixture. The results obtained (Figure 6) clearly showed that the two stereoisomers as well as the racemic mixture were present in the liver at a concentration of 5 μg/g after 10 days. Configuration of the Praziquantel molecule had no

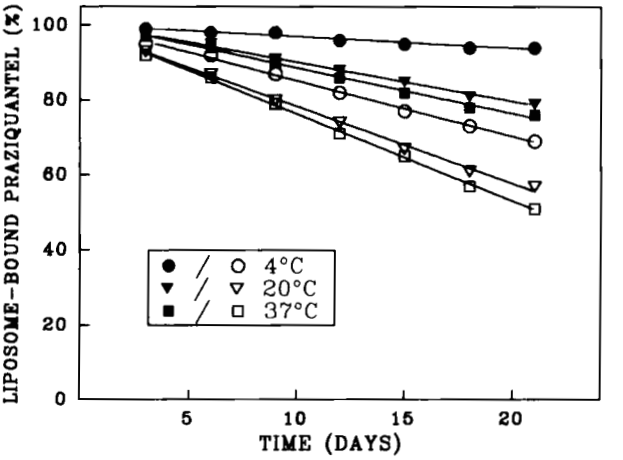

Figure 3. Praziquantel latency of cholesterol-poor (open symbols) and cholesterol-rich (filled symbols) liposomes under static conditions at different temperatures as a function of time.

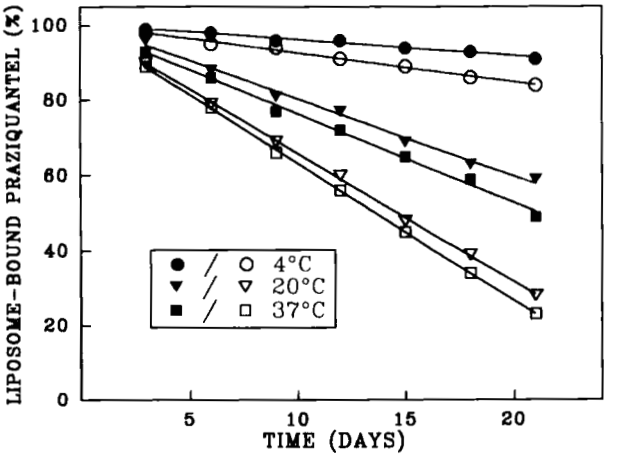

Figure 4. Praziquantel latency of cholesterol-poor (open symbols) and cholesterol-rich (filled symbols) liposomes under stirring conditions at different temperatures as a function of time.

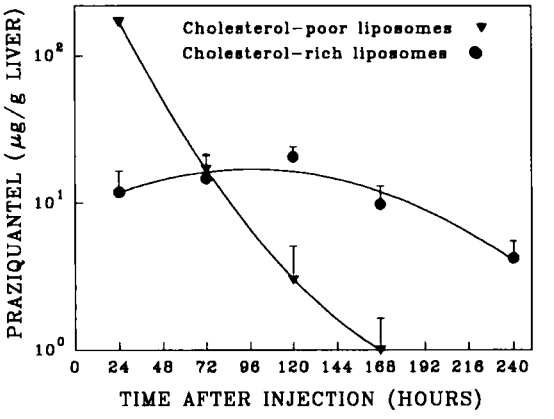

Figure 5. Praziquantel concentration in the liver after injection of the liposomal form of the drug (50 mg/kg body weight). Each point represents the mean value ± standard deviation of the mean.

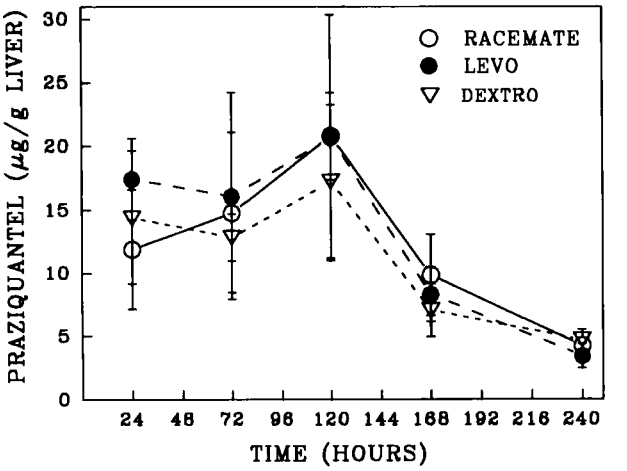

Figure 6. Hepatic drug concentration after injection of the liposomal form of the racemic mixture and two enantiomers of Praziquantel (50 mg/kg body weight). Each bar represents the mean value ± standard deviation of the mean.

significant effect on drug concentration and its deposition in the liver. If we bear in mind that antischistosomal activity is present only in (-)-stereoisomer, its entrapment in liposomes may increase the effectiveness of Praziquantel. Evaluation of the different forms of Praziquantel in liposomes and their dose-dependent kinetics in infected mice will furnish a definitive answer if this strategy is to be successful in the chemoprophylaxis of schistosomiasis.

CONCLUSIONS

This study describes different variables that are crucial for the entrapment, stability and *in vivo* release of Praziquantel encapsulated in liposomes. The results obtained demonstrated that Praziquantel, which disappeared within 1 hour after administration to mice, could be found after 10 days in the liver if given in a liposomal form. There were no significant differences between the (-)-stereoisomer, (+)-stereoisomer and racemic mixture of Praziquantel in terms of drug concentration and distribution in the liver. Targeting of the racemic mixture and (-)-isomer of Praziquantel to the liver, using a liposomal delivery system, could lead to an effective application of this drug for the treatment of schistosomiasis.

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