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Characterization, stability and in vivo targeting of liposomal formulations containing cyclosporin

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Abstract

Cyclosporin A (CSA) is a potent immunosuppressive drug that was recently encapsulated into different liposomal formulations. Optimization of CSA formulated liposomes preparation was the goal of this study. Liposomes composed of dipalmitylphosphatidylcholine (DPPC) containing CSA were prepared and characterized by differential scanning calorimetry (DSC). In vitro characterization of the formulated model liposomes including the entrapment efficiency and stability in the presence of mono- and divalent ions at different temperatures (5, 21, 37°C) and in the absence and presence of cholesterol (Chol) was carried out. Furthermore, in vivo targeting of CSA to mouse livers from liposomal preparations was investigated and compared with a non-liposomal formulation. A slight decrease in transition temperature (T_m) of the liposomes formed was noted with increase in CSA content. Entrapment of CSA in the liposomal vesicles was found to be dependent to some extent on the Chol level. The release rate of CSA from liposomes was enhanced in the presence of the divalent ions, Ca^{2+} and Mg^{2+}, indicating low stability in the presence of these ions compared with Na^{+}. The release rate was affected by storage temperature and depended on the existence of Chol. In the absence of Chol, the release rate decreased with increasing temperature. On the other hand, in the presence of Chol, the rate of release was directly proportional to the temperature. In vivo study showed that a higher CSA content which lasted for more than 11 days was achieved in mouse livers from liposomal compared with non-liposomal preparations.

Keywords: Cyclosporin; Liposome; Stability; Liver targeting

1. Introduction

Liposomes are microscopic vesicles composed of a bilayer of phospholipids or any similar amphipathic lipids. They can encapsulate and effectively deliver both hydrophilic and lipophilic substances (Fielding, 1991; Akbarieh et al., 1993) and may be used as a non-toxic vehicle for insoluble drugs (Lidgate et al., 1988). The stability of liposomes and their ability for drug delivery as well as targeting are dependent on the type of lipid used and the method of preparation (Taylor et al., 1990; Moghimi et al., 1991; Betageri and Burrell, 1993). The use of an appropriate liposomal system as a carrier for a given drug can be effective in concentrating this drug into the target tissue (Kim et al., 1993) and enhancing its stability. Generally, the phospholipid DPPC has been used for preparation of liposomes in numerous

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experiments and is recognized as a pertinent model (Bauer et al., 1990). It has been shown that the incorporation of cholesterol into lipid bilayer membranes enhances the stability of liposomes in serum and reduces the clearance rate from the blood circulation (Senior and Gregoriadis, 1982).

Cyclosporin A (CSA), a potent immunosuppressive drug, is extensively used in organ transplantation to prevent tissue rejection. The drug was recently encapsulated into different liposomal formulations (Vadiei et al., 1989; Stuhn-Sekalec and Stanaav, 1991) in order to overcome the serious side effects as well as the wide inter-subject pharmacokinetic variability that CSA exerts upon its administration by conventional methods. Advantage in this regard was taken of the natural tendency of liposomes to be preferentially targeted into the reticuloendothelial system in the liver and spleen and in reducing the concentration of the drug in other potential sites.

The purpose of this research was to study the characteristics of CSA liposomal formulations. The entrapment efficiency as well as the stability of the formulated liposomes at different temperatures in the presence of various ions was studied in the absence and presence of cholesterol. Furthermore, the effect of cholesterol on drug targeting into the liver of mice was also studied.

2. Materials and methods

2.1. Materials

L-α-Dipalmitoylphosphatidylcholine (DPPC) and cholesterol (Chol) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A). Cyclosporin A (CSA) was a gift from Sandoz Pharm. Ltd (Switzerland). Solvents used for chromatographic analysis were HPLC grade. All other materials and solvents were of analytical grade.

2.2. Preparation of liposomes

Multilamellar vesicles (MLVs) were prepared following the film method (Bangham et al., 1965). The liposomes were composed of DPPC, CSA and Chol at molar ratios of 1:0.2:0 (Chol-free), 1:0.2:0.5 (low-Chol) and 1:0.2:1 (high-Chol). The required amount of drug, DPPC and Chol, if needed, were dissolved in the least amount of chloroform which was then slowly evaporated under reduced pressure using a rotary evaporator at 40°C. A thin film was deposited on the inner wall of a quick-fit 100 ml pear-shaped glass flask. Traces of chloroform were removed by a jet of dry nitrogen. The dried film was then hydrated in 10 ml of 0.9% sodium chloride (USP) by swirling at a temperature higher than the transition temperature of the lipid (60°C) and in the presence of four or five small glass beads, until all the lipid was dispersed (about 1 h).

2.3. DSC analysis

Liposomal preparations of 10% w/v lipid dispersion were used to determine the transition temperature (T_m) for each sample using a differential scanning calorimeter (Dupont Co., Model 9900, CT, U.S.A.). Pure indium was used to calibrate both the temperature scale and quantitative heat changes. The samples were heated from 20 to 80°C at a scanning rate of 5°C/min.

2.4. Drug entrapment

The prepared liposomal dispersions were centrifuged at $50000 \times g$ (Sorvall ultracentrifuge, Dupont Co., Model OTD65B, CT, U.S.A.) for 60 min at 4°C in order to separate the multilamellar vesicles from the aqueous solution which contained the non-associated CSA. Free drug concentration in the aqueous solution was determined by an high-performance liquid chromatographic (HPLC) assay method (Waters Associates, U.S.A.) that was developed in our laboratory. An appropriate volume of the aqueous solution was directly injected in the HPLC system. HPLC separation was achieved with a U-Bondapak C_{18} column (Waters Associates; 10 μm, 30 cm $\times$ 3.9 mm) heated at 70°C using a temperature control module (Waters Associates, U.S.A.). The mobile phase consisted of a mixture of 75% acetonitrile and 25% HPLC grade water buffered at pH 6.0 with 0.3% phosphoric acid, the flow rate being 1.5 ml/min. The eluant was monitored at

214 nm using a UV detector (Waters Associates, Model 481, U.S.A.).

The amount of entrapped CSA was determined by subtracting the amount of free drug from the total and was expressed as a percentage of drug entrapped (trapping efficiency).

2.5. Effect of temperature and type of ions on in vitro release of CSA from liposomes

An aliquot from each of the Chol-free, low Chol and high Chol liposomal suspensions was divided into three portions and then centrifuged at 50 000 × g at 4°C. The pellets obtained were redispersed in an equal volume of saline, $CaCl_2$ (0.3 M) or $MgCl_2$ (0.3 M) to study the effect of monovalent (Na^+) and divalent (Ca^{+2} and Mg^{+2}) ions on drug release. Each of the prepared dispersions was further divided into three portions and stored at 5, 21 (room temperature) or 37°C (total of 27 tubes). At predetermined time intervals an aliquot of 25 μl from each dispersion was diluted with 10 ml saline and then centrifuged at 50 000 × g at 4°C for 60 min. The supernatent was assayed for content of released CSA using an HPLC method and the pellet obtained was dissolved in methanol and examined for retained drug. Each experiment was triplicated and the mean values were recorded.

2.6. Cyclosporin targeting

Adult male Swiss mice weighing 29–33 g were used in the study. The mice were divided into five groups, each of 20 mice. Three groups received CSA (15 mg/kg) liposomal preparations containing no Chol, low Chol, or high Chol, as a subcutaneous injection. One group received the same dose of CSA subcutaneously as a suspension in 50% propylene glychol. The fifth group was left as control. After 2, 4, 6, 8 and 11 days four mice from each group were killed and their livers were homogenized in 5 ml phosphate buffer, pH 7.4. Samples were kept at −20°C pending analysis. An aliquot of each homogenized liver was analyzed for CSA content using a radioimmunoassay method (Cyclo. trac SP, I-125 RIA kit, Drug International Inc., NJ, U.S.A.). Each point is represented by the average amount of CSA in four livers.

3. Results and discussion

The thermotropic behavior of the phospholipid DPPC, forming the cholesterol free liposomes, was checked by DSC scanning. Liposomes containing different molar ratios of DPPC/CSA, namely, 1:0, 1:0.05, 1:0.1 and 1:0.2, gave transition temperature (T_m) values equal to 43.0, 41.02, 40.73 and 40.03, respectively. It was clear that the increase in CSA ratio resulted in a decrease in T_m, indicative of the incorporation of CSA in liposomal vesicles. This is consistent with the findings of others (Bauer et al., 1990), who concluded that the penetration of hydrophobic drugs into the DPPC bilayer is accompanied by lowering in T_m, an expression of destabilization of the phospholipid assemblies.

The small change in T_m with increase in CSA ratio may be explained by the ability of the drug molecules to be adsorbed on the phospholipid/water interface besides the deep partitioning into the phospholipid bilayer. Partitioning is expected to have high capacity for CSA and may resemble the behavior of CSA in some micellar systems (Khidr, 1987).

3.1. Entrapment of CSA in liposomes

The coevaporation of the lipid and CSA, a lipophilic drug, from organic solvents was used for liposomal preparation in order to obtain practically the highest drug incorporation (Juliano and Daoud, 1990). The entrapment of CSA in the liposomal vesicles was found to be dependent on the Chol level. The trapping efficiencies were 59.7, 64.5 and 68.4% for liposomes containing no Chol, low- and high-Chol, respectively. The small increase in entrapment with Chol content could be due to the increase in DPPC bilayer thickness.

3.2. Release of CSA from DPPC liposomes

The release of CSA from the liposomal preparations was studied in the presence of various ions at different temperatures. CSA release from

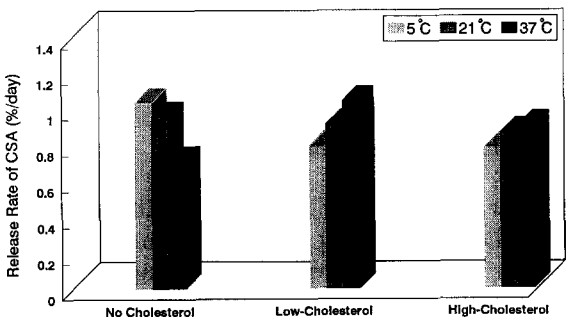

Fig. 1. Effect of temperature and Chol content on release rate of CSA from DPPC liposomes in the presence of sodium ions.

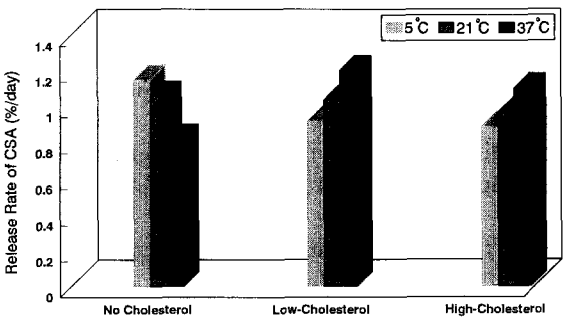

Fig. 3. Effect of temperature and Chol content on release rate of CSA from DPPC liposomes in the presence of calcium ions.

the prepared liposomes was found to be steady after 2 days in all cases and for up to 30 days of storage.

The leakage of CSA was examined in the presence of monovalent ions (Na^+) as well as divalent ions (Ca^{+2} and Mg^{+2}). Fig. 1–3 reveal that divalent ions provide an increase in CSA release compared with that of the monovalent ions with a greater effect for Ca^{+2} than Mg^{+2}. It was also clear that the rate of release of CSA decreased with increse in Chol content of liposomes in the presence of different ions (at 5 and 21°C). This may indicate that Chol decreases the bilayer disorder induced by ions. The inclusion of cholesterol may increase the ordered structure lipid bilayer with a condensing effect that tends to stabilize the liposomes and retards drug leakage (Taylor et al., 1990). Accordingly, a high Chol content as well as presence of monovalent rather than divalent ions is essential for the development of stable liposomal preparations for the delivery of CSA.

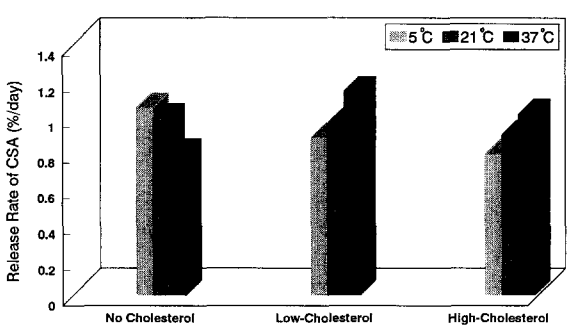

Fig. 2. Effect of temperature and Chol content on release rate of CSA from DPPC liposomes in the presence of magnesium ions.

Fig. 1–3 also show the effect of temperature on the release rate from liposomes formulated with and without Chol. In the presence of Chol, the leakage rate increased with rise in storage temperature. For instance, in the presence of Na^+, the release rates were 0.79, 0.91 and 1.03% per day for low-Chol liposomes and 0.76, 0.86 and 0.90% per day for high-Chol liposomes at 5, 21 and 37°C, respectively. However, in the absence of Chol the release rates decreased with increase in temperature and were 1.02, 0.95 and 0.70% per day at 5, 21 and 37°C, respectively. The same behavior was noted in the presence of Ca^{+2} and Mg^{+2} ions. These observations may reveal that in the absence of Chol the rate-determining step for CSA release is the dissolution of the drug, where solubility determination of CSA showed a decrease with increase in temperature. On the other hand, in the presence of Chol the release of CSA may depend on diffusion through the condensed liposomal bilayer which is directly proportional to temperature. From the above results and for optimum storage temperature, it is recommended to store Chol-free CSA liposomes on the shelf while, for liposomal preparations containing Chol, they should be stored at low temperature.

3.3. *In vivo targeting of CSA*

Fig. 4 shows the quantity of CSA in mouse livers as a function of time after a subcutaneous

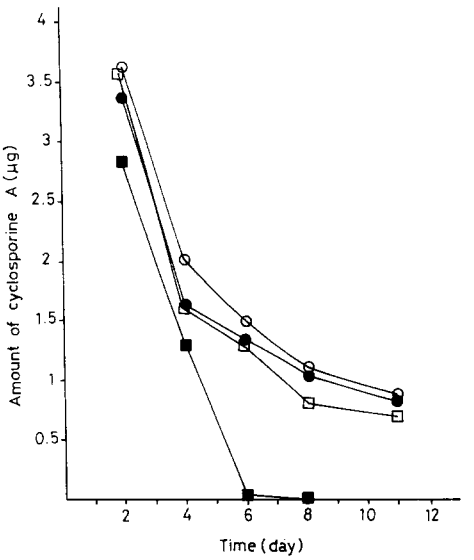

Fig. 4. Amount of CSA (μg) in mouse liver as a function of time. Each point represents the average amount of CSA in four livers. Standard error of the mean was within 3.5%. Non-liposomal (■); liposomes with no Chol (○); liposomes with low Chol (●); liposomes with high Chol (□).

injection of 15 mg/kg of drug formulated in liposomes compared with that of non-liposomal formulations. In the case of non-liposomal formulations, liver drug content decreased rapidly and disappeared from the livers within 6 days. On the other hand, liposomal preparations provided higher liver drug content that lasted for a longer period (more than 11 days). The above results demonstrated that liposomal formulations containing CSA, a drug of long half-life (16 h, Lemaire et al., 1986), are stable in vivo, especially at high Chol content with the slowest rate of disappearance from liver.

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