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Betamethasone-in-cyclodextrin-in-liposome: The effect of cyclodextrins on encapsulation efficiency and release kinetics

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Abstract

Lipophilic drugs have limited solubility in phospholipid systems, hence maximum entrapment levels in liposomes are known to be low. "Drugs-in-cyclodextrin-in-liposome" systems were previously proposed to overcome this drawback but studies were limited to βCD and HPβCD. In some cases, other cyclodextrins may be more interesting than βCD or HPβCD, such as methylated cyclodextrins. However, these cyclodextrins are known to extract lipid components from the lipid membrane, which may destabilize liposomes.

We tested the influence of several cyclodextrins (βCD, γCD, Dimeb, Trimeb, Crysmeb, Rameb, HPβCD and HPγCD) on the aqueous solubility of betamethasone by phase solubility diagrams and on the encapsulation efficiency in liposomes. The release kinetics of betamethasone was studied using Franz diffusion cells. We showed that release kinetics are directly correlated with encapsulation efficiency, which is closely related to betamethasone concentration in cyclodextrin complex solution. No liposome destruction was observed, even with the testing of methylated cyclodextrins at the highest concentration (40 mM). This can be explained by the fact that these cyclodextrins have a higher affinity for betamethasone than for cholesterol. This was proved by the comparison of phase solubility diagrams of both betamethasone and cholesterol.

Keywords: Liposome; Cyclodextrin; Cholesterol; Betamethasone; Release kinetics

1. Introduction

Encapsulating a sufficient amount of a therapeutic agent is one of the most desirable properties for liposomes. The liposomes encapsulate a hydrophilic drug within an aqueous compartment, while liposomes also entrap the lipophilic drug within the lipid bilayer. Factors affecting the encapsulation efficiency of the drug in the liposomes are various and come from the properties of both liposomes and encapsulated drugs. Concerning the encapsulated drugs, the encapsulation efficiency is affected by hydrophilic or lipophilic properties (Nii and Ishii, 2005). However, accommodation of lipophilic compounds in the lipid phase can be problematic, as some drugs can interfere with bilayer formation and stability, thus limiting the range and amount of valuable drugs that can be associated with liposomes. Accommodation of a poorly water-soluble drug in the lipid bilayer of liposomes is often limited in terms of drug to lipid mass ratio. Entrapping water-soluble drug/cyclodextrin inclusion complexes in the aqueous phase of liposomes has been proposed in order to avoid such drawbacks (McCormack and Gregoriadis, 1994a). "Drugs-in-cyclodextrin-in-liposome" systems have been previously studied by several authors with β-cyclodextrin (βCD) and hydroxypropyl-βCD (HPβCD) (McCormack and Gregoriadis, 1994a,b, 1998; Loukas et al., 1998; Fatouros et al., 2001). The concept, entailing entrapment of water-soluble cyclodextrin–drug inclusion complexes in liposomes, would allow accommodation of insoluble drugs in the aqueous phase of vesicles. This could potentially increase the drug to lipid mass ratio to levels above those attained by conventional drug incorporation into the lipid phase. It could also enlarge the range of insoluble drugs amenable to encapsulation to include, for example, membrane destabilizing agents.

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Furthermore, it could allow targeting of complexes to specific sites and reduce toxicity (McCormack and Gregoriadis, 1994b).

Fatouros showed that the main prerequisite for the drug-in-cyclodextrin-in-liposome approach to be beneficial, is the ability of the specific drug to form an inclusion complex of high drug content with a highly soluble cyclodextrin molecule (Fatouros et al., 2001). However, βCD and HPβCD are not the most solubilizing cyclodextrins and, for some active drugs, higher drug content can be reached with other cyclodextrin derivatives. Chemical modifications of the hydroxyl groups of cyclodextrins often enhance both their solubility in water and their ability to dissolve hydrophobic compounds. α, β and γCD are the less hydrophobic cyclodextrins and HPβCD with the same cavity diameter as βCD is even less hydrophobic. On the other hand, 2,6-dimethyl-βCD (Dimeb) and 2,3,6-trimethyl-βCD (Trimeb) have a deeper cavity and are more hydrophobic than βCD (Nishiho et al., 2000). Cyclodextrins, particularly the methylated derivatives, are known to remove lipid components from cell membranes and liposomes by forming inclusion complexes with the lipids, especially cholesterol. It is thus possible that, during or after entrapment of the drug/cyclodextrin complex solutions, lipids enter the cyclodextrin cavity, replacing the drug. This could destabilize the bilayers to some extent, enabling partial or complete leakage of drug content from vesicles. Rapid release of the drug would eliminate the benefits of liposomal encapsulation, resulting in an efficacy similar to that of an equivalent dose of free drug.

We wanted to enlarge previous studies (McCormack and Gregoriadis, 1994a,b, 1998; Loukas et al., 1998; Fatouros et al., 2001) to study different cyclodextrins currently in use and to increase the understanding of the interaction between drug, cyclodextrin and lipids. The aim of this study was to use different cyclodextrins at different concentrations in order to increase the aqueous solubility of a poorly water-soluble drug and to increase its loading capacity in the aqueous cavity of liposomes. The corresponding objective was to evaluate the liposome retention properties of a model drug encapsulated in the form of cyclodextrin inclusion complexes, according to the type and concentration of the cyclodextrin used.

Betamethasone was chosen as the model drug. When incorporated into phospholipid films or bilayers, betamethasone, because of its hydrophobic nature, would be expected to be associated with the hydrocarbon chain region of lipid molecules. However, steroids and steroid esters have limited solubility in phospholipid systems (Fildes and Oliver, 1978; Smith et al., 1980), hence maximum entrapment levels are known to be low, with excess drug being present in a crystalline form. Crystalline drugs may be external or internal to the liposome, hence release rates may be partially ascribed to the dissolution of this excess material rather than to efflux from liposomes. The initial rapid release of synthetic steroids from liposomes has also been ascribed to an incompatible fit of the steroid in the liposome bilayer (Shaw et al., 1976; Radhakrishnan, 1991; Batavia et al., 2001). Furthermore, betamethasone is a good model drug, due to its well known ability to form inclusion complexes with various cyclodextrins (Flood et al., 2000; Otagiri et al., 1984).

2. Materials and methods

2.1. Materials

Soybean phosphatidylcholine (SPC), cholesterol (CHOL), stearylamine (SA) and trihydroxymethylaminomethane (trometamol) were purchased from Sigma–Aldrich (Bornem, Belgium). Carbopol® 980 came from Noveon (Brussels, Belgium).

β-Cyclodextrin (βCD, Eur. Pharm., 5th Ed., 7.58% H_2O), hydroxypropylated β-cyclodextrin (HPβCD, Eur. Pharm., 5th Ed., D.S. 0.63, 3.22% H_2O) and Kleptose® Crysmeb (Crysmeb, D.S. 0.5, 4.29% H_2O) were kindly donated by Roquette Frères (Lestrem, France). γ-Cyclodextrin (γCD, 4.25% H_2O), randomly methylated β-cyclodextrin (Rameb) BETA W7 M1.8 (D.S. 1.8, 3.81% H_2O) and hydroxypropylated γ-cyclodextrin (HPγCD, D.S. 0.7, 2.85% H_2O) were a gift from Wacker-Chemie GmbH (Munich, Germany). 2,6-Dimethyl-β-cyclodextrin (Dimeb) and 2,3,6-trimethyl-β-cyclodextrin (Trimeb) were obtained from Cyclolab (Budapest, Hungary). Betamethasone was purchased from Medeva (Braine L'Alleud, Belgium). All other reagents and solvents were of analytical grade.

All experiments were performed using a 0.22 μm-filtered 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (Sigma–Aldrich, Bornem, Belgium), containing 145 mM NaCl and adjusted to pH 7.4 with 0.1N NaOH solution.

2.2. Formation of inclusion complexes

2.2.1. Phase solubility diagrams

Solubility studies were performed as described by Higuchi and Connors (Higuchi and Connors, 1965). Excess amounts of betamethasone were added to various concentrations of cyclodextrins (0, 1, 5, 7.5 and 10 mM for βCD and Trimeb; 0, 5, 10, 20, 50 and 100 mM for other cyclodextrins) in 10 mL of HEPES buffer solutions. The suspensions were shaken in a water bath at 25 °C during 48 h. An aliquot was filtered through a 0.45 μm filter and assayed for betamethasone content by HPLC.

2.2.2. Formation of inclusion complexes for liposome encapsulation

Cyclodextrins were dissolved in an HEPES buffer pH 7.4 in order to obtain a 10 mM βCD or γCD solution and a 10 or 40 mM solution for other cyclodextrins. Betamethasone was added in excess to the cyclodextrin solutions. Water-soluble inclusion complexes were formed after stirring the mixtures for 48 h at 25 °C. The milky solutions were then filtered through a 0.45 μm filter and assayed for betamethasone content by a HPLC method.

2.3. Betamethasone assay

Betamethasone can be determined using a validated HPLC method. HPLC was performed using a system consisting of a LaChrom Merck Hitachi system L-7100 pump, a L-7400 UV detector, a L-7200 autosampler and a D-2500 chromato integrator. Twenty microliters samples were injected on a Lichrocart column (250 mm × 4 mm i.d.) prepared with an octadecylsilane (C18) phase Superspher (Merck) and maintained at 25 °C. The mobile phase consisted of a 50:50 (v:v) mixture HPLC grade acetonitrile and water. The flow rate was 0.8 mL/min. All the samples were analyzed in duplicate. Each sample was diluted with mobile phase before injection in the HPLC system. Betamethasone was detected at 240 nm.

2.4. Liposome preparation

2.4.1. Entrapment of inclusion complexes into liposomes

Small unilamellar vesicles made from SPC:CHOL:SA (60:30:10 mol%) were prepared by hydration of lipid films. SA, due to its charge repulsion effect, avoids sedimentation of liposomes. In practice, the required amounts of lipids were dissolved in chloroform in a round-bottomed flask, using a rotary evaporator and were dried under vacuum. The resulting lipid film was hydrated using 3 mL of the corresponding betamethasone–cyclodextrin complex solution. Suspensions were then extruded through Nucleopore polycarbonate membranes of successive 0.4 and 0.2 μm pore diameters. Free betamethasone complex was separated from liposome-encapsulated betamethasone complexes by three repeated ultracentrifugations at 35,000 rpm for 2 h at 4 °C. The supernatant was removed and the pellet was resuspended in HEPES buffer. Betamethasone and SPC are assayed in purified liposomes.

2.4.2. Entrapment of betamethasone into liposomal bilayers

Lipid vesicles SPC:CHOL:SA (60:30:10 mol%) containing betamethasone were prepared by hydration of lipid films. In practice, the required amount of lipids was dissolved in chloroform in a round-bottomed flask. Two milliliters of a betamethasone solution (1 mg/mL in absolute ethanol) were added. The solution was evaporated under vacuum using a rotary evaporator. The resulting lipid film was hydrated using 3 mL of HEPES buffer. Suspensions were then extruded through polycarbonate membranes of successive 0.4 and 0.2 μm pore diameters. Free betamethasone was separated from liposome-encapsulated betamethasone by three repeated ultracentrifugations at 35,000 rpm for 2 h at 4 °C. The supernatant was removed and the pellet was resuspended in HEPES buffer. Betamethasone and SPC are assayed in purified liposomes.

2.5. Liposome characterization

2.5.1. Measurement of liposome size

Liposome dispersions were sized by photon correlation spectroscopy (PCS) (HPPS, Malvern Instruments). Measurements were made at 25 °C with a fixed angle of 90° and sizes quoted are the average mean for the liposomal hydrodynamic diameter (nm).

2.5.2. Freeze-fracture electron microscopy

Freeze-fracture replicas of liposome suspensions and liposomes dispersed within 1.5% (w/v) Carbopol® gel were examined in transmission electron microscopy. A drop of liposome suspension or liposomes dispersed within gel, containing 20% glycerol as a cryoprotectant, was deposited in a small gold cup and rapidly frozen in liquid nitrogen. Fracturing, freeze etching and shadowing with Pt-C were performed at −100 °C in a shadowing equipment (Balzers® BAF-400) fitted with a freeze-fracture and etching unit. The replicas were examined with a JEOL (JEM-100SX) transmission electron microscope, operating at 80 kV accelerating voltage.

2.5.3. Quantification of SPC

Total lipid concentrations were calculated by measuring SPC by enzymatic method (phospholipids B, Wako, Neuss, Germany) and by assuming that the lipid composition was unchanged during the different steps of liposome preparation. The principle of this enzymatic assay is the following: this assay consists of the cleavage by phospholipase D of SPC in choline, which is oxidized in betaine with the simultaneous production of hydrogen peroxide. The hydrogen peroxide that is produced quantitatively, oxidatively couples four-aminoantipyrine and phenol. Peroxydation results in the generation of a colored compound, quinoneimine, quantified by spectrophotometry at 505 nm (spectrophotometer Perkin-Elmer Lambda 11).

Encapsulation efficiency corresponds to the drug to lipid mass ratio or the amount of betamethasone in purified liposomes (C_B) compared to the total lipid concentration (C_L) in purified liposomes:

$$\text{encapsulation efficiency} = (C_B/C_L) \times 100$$

2.6. Gel studies

2.6.1. Gel preparation

Appropriate amounts of Carbopol® 980 and trometamol were ground in a mortar to obtain a homogeneous mixture. Liposome dispersions and HEPES buffer were then added to Carbopol® in order to obtain a final betamethasone concentration of 150 μg/mL and a final Carbopol® concentration of 1.5% (w/v). A reference gel containing free betamethasone was prepared by solubilization of betamethasone in ethanol. The alcoholic solution and HEPES buffer were then added to Carbopol® in order to obtain a final betamethasone concentration of 150 μg/mL and a final Carbopol® concentration of 1.5% (w/v).

2.6.2. In vitro release of betamethasone from gels

Analyses were carried out using a Franz type glass diffusion cell. This consists of two compartments with a membrane clamped between the donor and receiver chambers, assembled with a polycarbonate membrane (pore size: 0.1 or 0.8 μm). The cell body was filled with 8 mL of a receptor phase consisting of HEPES buffer solution pH 7.4. About 300–350 mg of gel were then weighed exactly and were placed into the donor cell compartment and tamped down on the membrane, previously moistened with receptor phase. Samples of receptor phase (1 mL) were withdrawn after predetermined time intervals (0.5, 1, 2, 3, 4, 6 and 24 h) and the betamethasone concentration was measured by HPLC. Each sample removed was replaced by an equal volume of fresh receptor phase. The calculated betamethasone concentrations were plotted as a function of time. All the permeation studies obtained were determined six times in two independent experiments and the mean values ± standard deviations were calculated.

2.7. *Phase solubility diagram of CHOL and SPC*

As described in Section 2.2.1, excess amounts of CHOL or SPC were added to various concentrations of HPγCD, Crysmeb, Rameb and HPβCD (0, 5, 10, 20, 50 and 100 mM) in 3 mL of HEPES buffer solution. The suspensions placed under inert atmosphere (nitrogen) were shaken in a water bath at 25 °C during 48 h. After filtration through a 0.45 μm filter, SPC was assayed by the enzymatic method described previously and CHOL by the following validated HPLC method: HPLC was performed using the same system as described in Section 2.3. Fifty microliters samples were injected on a Lichrocart column (250 mm × 4 mm i.d.) prepared with an octylsilane (C8) phase LiChrospher (Merck) and maintained at 35 °C. The mobile phase consisted of a 20:31:32:17 (v:v) mixture of methanol, acetonitrile, tetrahydrofuran and water HPLC grade. The flow rate was 1.7 mL/min. All the samples were analyzed in duplicate. Each sample was diluted with mobile phase before injection in the HPLC system. CHOL was detected at 214 nm.

Phase solubility diagrams were also performed in the presence of both betamethasone and CHOL: excess amounts of both components were added to increasing concentrations of cyclodextrins. After shaking and filtration in the same manner as described previously, both CHOL and betamethasone were assayed. It was previously checked that HPLC methods were selective.

3. Results and discussion

Fig. 1 shows the phase solubility diagrams obtained for betamethasone and the different cyclodextrins tested. This figure shows that HPγCD, Crysmeb and Dimeb increased the aqueous solubility of betamethasone to the highest degree. A_L diagrams were obtained with βCD, Trimeb, HPβCD, HPγCD, Crysmeb and Dimeb, while Rameb and γCD showed A_N and B type diagrams, respectively. In presence of low γCD concentrations, the solubility of betamethasone increases (up to 10 mM) and then decreases at higher γCD concentrations. A linear regression was applied for all cyclodextrins, except γCD, in order to obtain the $K_{1:1}$ affinity constant. The following equation was applied: $K_{1:1} = \text{slope}/S_o(1 - \text{slope})$, where S_o corresponds to the intrinsic solubility of betamethasone. Table 1 indicates the $K_{1:1}$ constant values obtained. The results show that betamethasone has a very high affinity for HPγCD, Crysmeb, βCD and Dimeb. Interaction with Trimeb was the lowest. Due to its weak complexation capacity of betamethasone, Trimeb was subsequently abandoned. Dimeb was also abandoned due to the difficulty of obtaining sufficient amounts of pure 2,6-Dimeb.

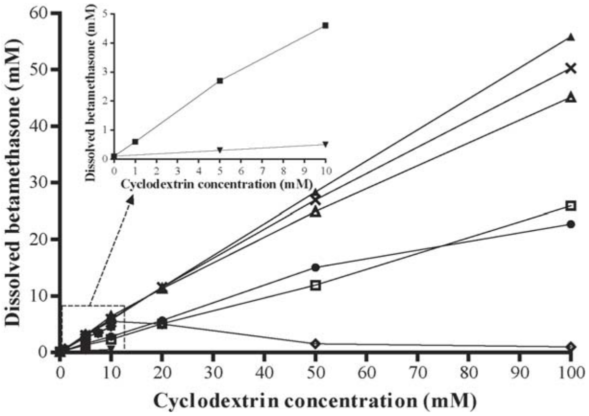

Fig. 1. Phase solubility diagram of betamethasone (water, 25 °C, 48 h): βCD (■), γCD (◊), Dimeb (△), Trimeb (▼), Crysmeb (×), Rameb (●), HPβCD (□), and HPγCD (▲).

Phase solubility studies allowed us to select effective cyclodextrins. HPβCD, Rameb, Crysmeb and HPγCD were selected and tested at 10 and 40 mM because of their high complexation capacity. βCD and γCD were also selected but were only tested at the 10 mM concentration: βCD because of its limited aqueous solubility and γCD because of the B type solubility profile obtained with this cyclodextrin. As a matter of fact, the solubility of the betamethasone–γCD complex decreases at higher γCD concentrations. The corresponding betamethasone complex solutions were prepared in an HEPES buffer for encapsulation in liposomes. Betamethasone concentrations of these solutions are indicated in Table 1. HPγCD and Crysmeb were shown to be particularly effective for obtaining high betamethasone concentrations.

SPC:CHOL:SA (60:30:10) liposomes containing betamethasone and betamethasone–cyclodextrin complexes were prepared by the film hydration method. Morphology and size analyses were performed by freeze-fracture electron microscopy and PCS. Liposomes are characterized by mean hydrodynamic diameters of between 181 ± 22 and 319 ± 42 nm (Table 2). Two major observations may be drawn from the PCS measurements. Firstly, the polydispersity index was always lower than 0.2, indicating that liposome populations were homogeneous in size. Secondly, no significant difference was found between the sizes of liposomes encapsulating betamethasone and liposomes encapsulating betamethasone–cyclodextrin inclusion complexes (10 or 40 mM), except for the betamethasone–Rameb

Table 1
Stability constants of the different complexes and concentrations of betamethasone solubilized in 10 and 40 mM cyclodextrin solutions

System	$K_{1:1}$ (M^{-1})	Solubilized betamethasone (mg/mL)	
		In 10 mM CD solution	In 40 mM CD solution
Betamethasone–βCD	8049	1.29	–
Betamethasone–γCD	–	2.05	–
Betamethasone–HPβCD	3013	0.94	2.84
Betamethasone–HPγCD	12606	2.03	6.83
Betamethasone–Dimeb	8018	–	–
Betamethasone–Rameb	2947	1.17	3.91
Betamethasone–Trimeb	417	–	–
Betamethasone–Crysmeb	10011	2.13	7.09

Table 2
Size ± S.D. (nm) and encapsulation efficiency ± S.D. (%) of liposomes containing free betamethasone or betamethasone–cyclodextrin complexes

Liposomes containing	Cyclodextrin concentration (mM)	Size ± S.D. (nm)	Encapsulation efficiency ± S.D. (%)
Betamethasone	0	206 ± 62	0.39 ± 0.17
Betamethasone–βCD complex[a]	10	–	–
Betamethasone–γCD complex[a]	10	219 ± 23	1.18 ± 0,18
Betamethasone–HPβCD complex[a]	10	181 ± 22	0.56 ± 0.28
Betamethasone–HPβCD complex[a]	40	248 ± 28	1.05 ± 0.28
Betamethasone–HPγCD complex[a]	10	187 ± 25	1.12 ± 0.07
Betamethasone–HPγCD complex[a]	40	192 ± 31	2.21 ± 0.30
Betamethasone–Rameb complex[a]	10	204 ± 34	0.58 ± 0.14
Betamethasone–Rameb complex[a]	40	319 ± 42	1.13 ± 0.43
Betamethasone–Crysmeb complex[a]	10	221 ± 29	1.16 ± 0,16
Betamethasone–Crysmeb complex[a]	40	206 ± 27	2.78 ± 0,28

[a] The corresponding drug concentrations in the cyclodextrin solutions are indicated in Table 1.

40 mM complex, for which the size was significantly greater ($p < 0.05$). This observation could not be explained at the present time.

To confirm the results by an imaging method, a freeze-fracture technique with subsequent transmission electron microscopy was used. Betamethasone-loaded vesicles can be compared to the betamethasone inclusion complex-loaded ones (Fig. 2). These pictures show the presence of ≈200 nm small unilamellar vesicles of homogeneous size. Thus, the results from PCS and freeze-fracture electron microscopy measurements are in good agreement, confirming that liposomes encapsulating betamethasone–Rameb 40 mM complex are greater in size.

Betamethasone encapsulation efficiencies are reported in Table 2. It must be noted that encapsulation efficiencies represent the drug to lipid mass ratio, which explains the relatively low values obtained compared to those reported in other papers expressing the encapsulation efficiencies in function of the total drug concentration. Liposomes containing 10 mM betamethasone–βCD complex were rapidly abandoned because of betamethasone precipitation during liposome preparation. A first view of the results reveals that the amount of betamethasone entrapped was higher when encapsulated in the form of an inclusion complex, whatever the cyclodextrin used. This observation confirms that betamethasone has limited solubility in phospholipid systems, giving rise to low maximum entrapment levels. Results also show that encapsulation efficiency increased with the concentration of cyclodextrin. γCD also seems effective, since a 10 mM betamethasone–γCD complex allowed to reach an encapsulation efficiency as high as a 40 mM betamethasone–Rameb or HPβCD complex. However, γCD

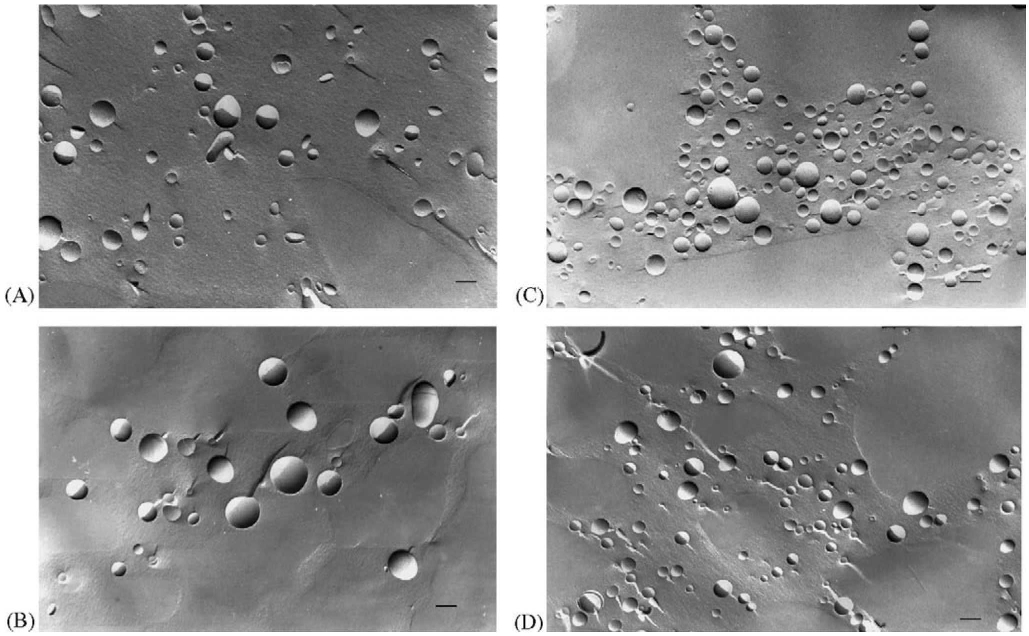

Fig. 2. Transmission electron micrographs of freeze-fractured samples. SPC:CHOL:SA vesicles including free betamethasone (A), SPC:CHOL:SA vesicles including betamethasone–Rameb 40 mM complex (B), SPC:CHOL:SA vesicles including betamethasone–HPβCD 40 mM complex (C), and SPC:CHOL:SA vesicles including betamethasone–γCD 10 mM complex (D). Bar represents 200 nm.

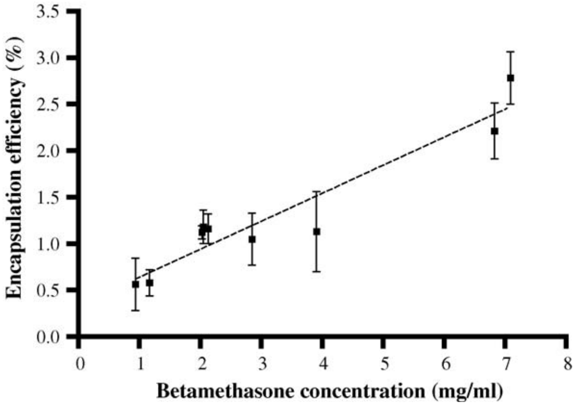

Fig. 3. Correlation between encapsulation efficiency (%) and betamethasone concentration of complex solution (mg/mL).

Fig. 4. Transmission electron micrographs of freeze-fractured samples. SPC:CHOL:SA vesicles including betamethasone–HPβCD 40 mM complex, dispersed within a 1.5% Carbopol® gel. Bar represents 200 nm.

could not be tested at 40 mM due to the B type phase solubility diagram obtained with this cyclodextrin. Fig. 3 shows that there is a significant correlation between the betamethasone concentration of the complex solution and encapsulation efficiency (Pearson test, p-value <0.0001, $r^2 = 0.9133$). These results confirm the previous observations of Fatouros (Fatouros et al., 2001), who stated that, if a high encapsulation of active substance is needed and if the drug-in-cyclodextrin-in-liposome approach is chosen, a very important step is to select a cyclodextrin which will have the highest solubilizing efficiency. However, in the case of betamethasone, the most solubilizing cyclodextrins are HPγCD and Crysmeb, which may also interact with lipid components of the liposome membrane, particularly the methylated derivative of βCD.

The release of betamethasone from liposomes containing uncomplexed betamethasone or from liposomes containing betamethasone–cyclodextrin complexes was evaluated after dispersion of the vesicles in a 1.5% Carbopol® gel, in order to obtain a final betamethasone concentration of 150 μg/g. As shown by freeze-fracture electron microscopy, no morphological changes of liposomes were observed after dispersion in the Carbopol® gel (Fig. 4). Franz cells were used to evaluate the release kinetics of betamethasone from liposomes dispersed within the gel. Test conditions were chosen in order to respect sink conditions in the receiver compartment. It was shown at first that the release kinetics of betamethasone are not dependent on the pore size of the membrane. Encapsulation of betamethasone within liposomes led to a slower release compared to a free betamethasone hydroalcoholic reference gel, due to the well known reservoir effect of liposomes (Fig. 5A and B). Release rates observed for betamethasone may be partially ascribed to the dissolution of an excess material present in a crystalline form and to the efflux from liposomes (Radhakrishnan, 1991). In vitro release of betamethasone from liposomes containing inclusion complexes with Rameb, HPβCD and γCD at 10 mM concentration led to a slower release compared to betamethasone encapsulated liposomes (Fig. 5A). The release rate increases in the order: HPβCD ≈ γCD < Rameb < HPγCD ≈ without cyclodextrin < Crysmeb. Except for γCD, the rank order observed for betamethasone release corresponds to the rank order observed for encapsulation efficiencies (Table 2) or for betamethasone complex concentration (Table 1): HPβCD < Rameb < HPγCD < Crysmeb. The release profile obtained with γCD is not related to the encapsulation efficiency.

At the highest cyclodextrin concentration tested (40 mM, Fig. 5B), betamethasone release rate increased. Betamethasone retention within the gel was lower with increasing cyclodextrin concentration. Only HPβCD allows a slower release of betamethasone than that from betamethasone liposomes without cyclodextrins. As observed at 10 mM, the rank order observed for betamethasone release corresponds to the rank order observed for encapsulation efficiency (Table 2) or for betamethasone complex concentration (Table 1): HPβCD < Rameb < HPγCD < Crysmeb. Fig. 6 shows that whatever the cyclodextrin and its concentration, the percentage of betamethasone released after 24 h may be related to encapsulation efficiency by a dose–response curve ($r^2 > 0.9$). The correlation between the percentage of betamethasone released and encapsulation efficiency is statistically significant (Pearson test, $p = 0.017$). This observation leads to the conclusion that betamethasone release kinetics depend on the encapsulation efficiency and thus on the concentration of the complex solution. In the case of this study, the release kinetics only depend on the cyclodextrin ability to complex the active substance.

It seems that betamethasone release kinetics are not due to interactions between cyclodextrins and the lipid components of the liposome membrane. In order to have unambiguous proof of

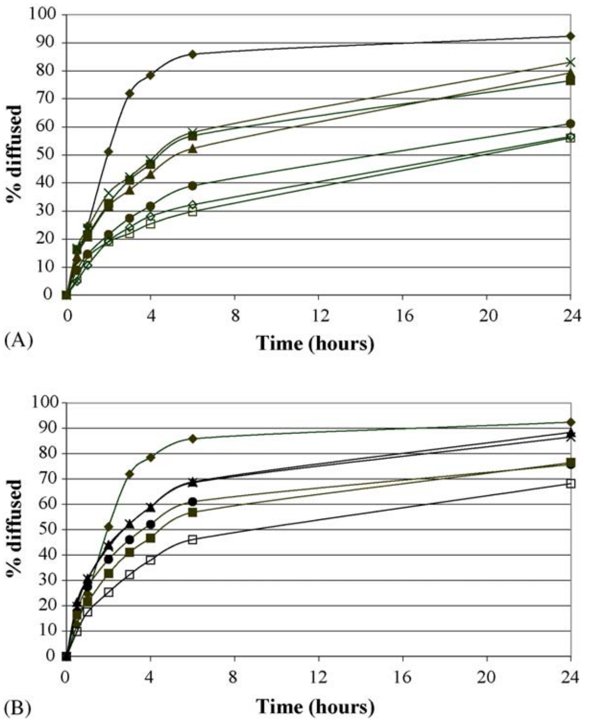

Fig. 5. (A) Comparison of betamethasone release kinetics from gels containing free betamethasone (♦) and SPC:CHOL:SA liposomes containing: betamethasone (■), betamethasone–γCD 10 mM (◊), betamethasone–Crysmeb 10 mM (×), betamethasone–Rameb 10 mM (●), betamethasone–HPβCD 10 mM (□), betamethasone–HPγCD 10 mM (▲). (B) Comparison of the betamethasone release kinetics from gels containing free betamethasone (♦) and SPC:CHOL:SA liposomes containing: betamethasone (■), betamethasone–Crysmeb 40 mM (×), betamethasone–Rameb 40 mM (●), betamethasone–HPβCD 40 mM (□), and betamethasone–HPγCD 40 mM (▲).

the latter hypothesis, the corresponding phase solubility diagram of cyclodextrin–betamethasone and of cyclodextrin–lipid components were realized in the same conditions. For all cyclodextrins tested, solubilization of SPC was negligible in comparison with that of CHOL. Fig. 7 compares the diagrams obtained for CHOL and betamethasone. It can be seen that, at both concentrations evaluated, 10 and 40 mM, all cyclodextrins tested had a higher affinity for betamethasone than for CHOL. This was confirmed by a phase solubility diagram in presence of both betamethasone and CHOL in a Rameb solution of increasing concentration. The diagram shows (Fig. 8) that the solubility of betamethasone in Rameb solutions is not influenced by the presence of CHOL. On the contrary, the aqueous solubility of CHOL in Rameb solutions is decreased due to the presence of betamethasone in solution. CHOL is displaced from the cyclodextrin cavity due to the presence of betamethasone.

In the presence of both betamethasone and CHOL, the cyclodextrin can form complexes with both molecules and each complex may be characterized by a stability constant (K_β and K_{CHOL}). In our case, the affinity of cyclodextrins was higher for betamethasone than for CHOL ($K_\beta > K_{\mathrm{CHOL}}$). This means that in presence of betamethasone, interactions between cyclodextrins and CHOL will be low. However, some kind of instability must occur as betamethasone leaks out and as cyclodextrins become empty. Equilibrium is then displaced. Empty cyclodextrins

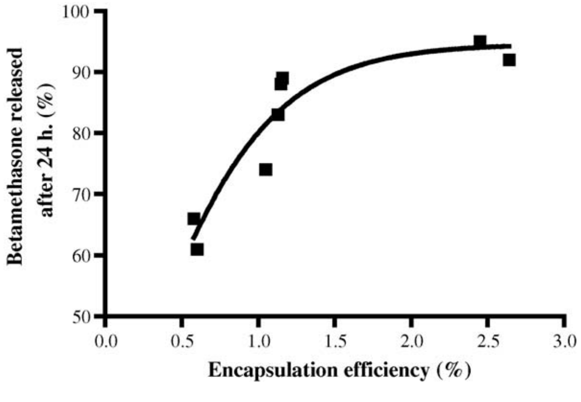

Fig. 6. Correlation between the percentage of betamethasone released after 24 h and encapsulation efficiency (%).

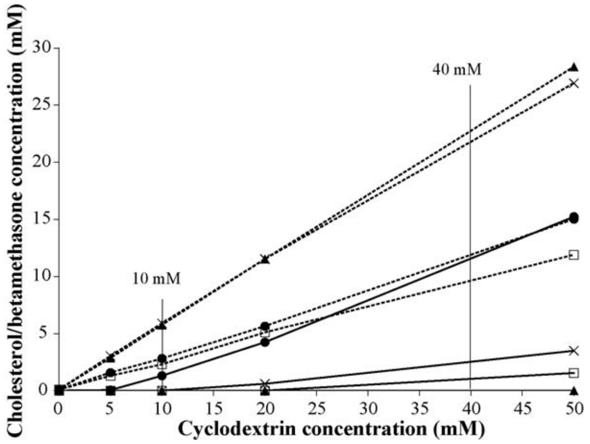

Fig. 7. Comparison of the phase solubility diagram of betamethasone (dotted line) and cholesterol (unbroken line) (water, 25 °C, 48 h): Crysmeb (×), Rameb (●), HPβCD (□), and HPγCD (▲).

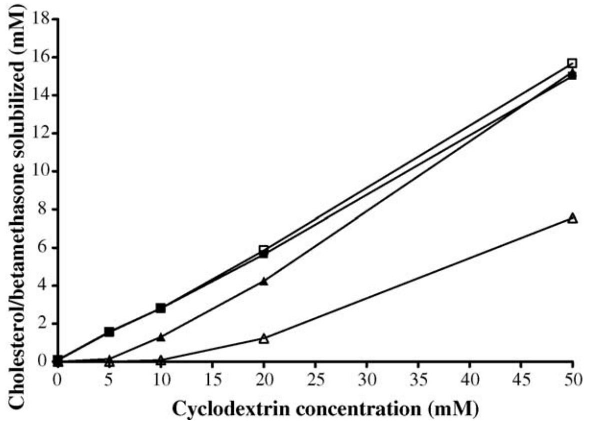

Fig. 8. Influence of cholesterol/betamethasone on the phase solubility diagram of betamethasone/cholesterol in presence of Rameb (water, 25 °C, 48 h): betamethasone (■) and betamethasone in presence of cholesterol (□), cholesterol (▲) and cholesterol in presence of betamethasone (△).

complex more and more cholesterol. Extraction of cholesterol will not immediately result in lysis and dissolution of the liposomes. Lipid bilayers losing cholesterol will largely remain intact but become more fluid and/or permeable for betamethasone release.

4. Conclusions

This study shows that liposomes may conserve their reservoir effect when encapsulating cyclodextrin complexes, even with methylated cyclodextrins. During complex solution entrapment, included betamethasone is partially displaced from the cyclodextrin cavity to a degree that is dependent on the stability constant of the complex. It was shown that betamethasone forms very stable complexes with HPγCD, Crysmeb, HPβCD and Rameb and, at both concentrations tested, 10 and 40 mM, these cyclodextrins had a higher affinity for betamethasone than for CHOL. This high affinity allows the entrapment of highly concentrated betamethasone inclusion complex solutions without or with minimal interactions with the lipid components of the liposome membrane. The release kinetics observed were directly correlated with encapsulation efficiency. The betamethasone content will be released, because of the permeability of the liposome, depending on the gradient concentration. The presence of betamethasone in the cyclodextrin cavity significantly delays the interaction between the cyclodextrin and the lipid molecules even if some kind of instability occurs as betamethasone leaks out and cyclodextrins become empty. Lipid bilayers losing cholesterol will largely remain intact but become more permeable for betamethasone release. Liposomes have a true membrane diffusion barrier effect on the release kinetics of betamethasone.

As the release kinetics are directly correlated with encapsulation efficiency, the choice of the final formulation will be a compromise between the reservoir effect with relatively slow release kinetics and high encapsulation efficiency in order to have concentrated preparations. In this case the encapsulation of betamethasone–HPγCD 10 mM or of betamethasone–Rameb 40 mM seems particularly interesting as these combinations allow to obtain the same release profile as that from liposomes encapsulating betamethasone without cyclodextrins but with higher encapsulation efficiencies. In vivo studies will help us to confirm this hypothesis.

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References

Batavia, R., Taylor, K., Craig, D., Thomas, M., 2001. The measurement of beclomethasone dipropionate entrapment in liposomes: a comparison of a microscope and an HPLC method. Int. J. Pharm. 212, 109–119.

Fatouros, D., Hatzidimitiou, K., Antimisiaris, S., 2001. Liposomes encapsulating prednisolone and prednisolone-cyclodextrin complexes: comparison of membrane integrity and drug release. Eur. J. Pharm. Sci. 13, 287–296.

Fildes, F., Oliver, J., 1978. Interaction of cortisol-21-palmitate with liposomes examined by differential scanning calorimetry. J. Pharm. Pharmacol. 30, 337–342.

Flood, K., Reynolds, E., Snow, N., 2000. Characterization of inclusion complexes of betamethasone-related steroids with cyclodextrins using high-performance liquid chromatography. J. Chromatogr. A 903, 49–65.

Higuchi, T., Connors, K., 1965. Phase solubility techniques. Adv. Anal. Chem. Instrum. 4, 127–212.

Loukas, Y., Vraka, V., Gregoriadis, G., 1998. Drugs, in cyclodextrins, in liposomes: a novel approach to the chemical stability of drugs sensitive to hydrolysis. Int. J. Pharm. 162, 137–142.

McCormack, B., Gregoriadis, G., 1994a. Drugs-in-cyclodextrins-in-liposomes: a novel concept in drug delivery. Int. J. Pharm. 12, 249–258.

McCormack, B., Gregoriadis, G., 1994b. Entrapment of cyclodextrin-drug complexes into liposomes: potential advantages in drug delivery. J. Drug Target. 2, 449–454.

McCormack, B., Gregoriadis, G., 1998. Drugs-in-cyclodextrin-in-liposomes: an approach to controlling the fate of water insoluble drugs in vivo. Int. J. Pharm. 162, 59–69.

Nii, T., Ishii, F., 2005. Encapsulation efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method. Int. J. Pharm. 298, 198–205.

Nishiho, J., Shiota, S., Mazima, K., Mizuno, H., Yoshida, J., 2000. Interactions of cyclodextrins with dipalmitoyl, distearoyl, and dimyristoyl phosphatidyl choline liposomes. A study by leakage of carboxyfluorescein in inner aqueous phase of unilamellar liposomes. Chem. Pharm. Bull. 48, 48–52.

Otagiri, M., Fujinaga, T., Sakai, A., Uekama, K., 1984. Effects of beta- and gamma-cyclodextrins on release of betamethasone from ointment bases. Chem. Pharm. Bull. 32, 2401–2405.

Radhakrishnan, R., 1991. Novel liposome composition for the treatment of interstitial lung diseases. US Patent 5,049,389.

Shaw, I., Knight, C., Dingle, J., 1976. Liposomal retention of a modified anti-inflammatory steroid. Biochem. J. 158, 473–476.

Smith, D., Kappes, J., Bentley, P., Bennet, R., 1980. Intraarticular therapy with liposomal steroids: a comparative study in rabbits. Arthritis Rheum. 23, 748–749.