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Preparation and characterisation of liposomes encapsulating ketoprofen–cyclodextrin complexes for transdermal drug delivery

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Abstract

Multilamellar vesicle (MLV) liposomes containing ketoprofen–cyclodextrin complexes intended for drug topical delivery were prepared, with the aim of simultaneously exploiting the favourable properties of both carriers. Drug complexes with β-cyclodextrin (βCyd) and hydroxypropyl-βCyd (HPβCyd), prepared by coevaporation and sealed-heating methods, were characterised by differential scanning calorimetry, hot stage microscopy, scanning electron microscopy and tested for dissolution properties. The coevaporated system with HPβCyd was the most effective, enabling an about 11-fold increase in drug dissolution. Drug and drug-Cyd systems were incorporated in MLV liposomes prepared by the thin layer evaporation technique. All liposomal formulations were characterised for encapsulation efficiency, particle size and morphology, using dialysis, light scattering and transmission electron microscopy techniques, respectively. MLV formation was negatively influenced by the presence of Cyd; nevertheless, it was possible to prepare stable MLVs containing ketoprofen-Cyd complexes. The presence of the Cyd complex affected MLV dimensions but not their lamellar structure. The complex with HPβCyd, in virtue of its greater stability than the βCyd one, allowed higher percentages of encapsulation and gave rise to more stable MLV systems. Permeability studies of drug and drug-Cyd complexes, as such or incorporated in liposomes, performed both across artificial membranes and rat skin, highlighted a favourable effect of Cyd on drug permeation rate, due to its solubilizing action; by contrast, unexpectedly, no skin-permeation enhancer property of liposomes has been evidenced. Confocal laser scanning microscopy studies carried out with the rhodamine-Cyd complex as fluorescent marker, confirmed such results, showing that the label permeated deeper across rat skin layers when it was in solution than when entrapped in liposomes.

Keywords: Ketoprofen; Cyclodextrins; Liposomes; Skin permeation studies

1. Introduction

Ketoprofen is a non-steroidal anti-inflammatory drug, scarcely soluble in water, which is widely used

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as analgesic and for the acute and long-term treatment of rheumatoid arthritis and osteoarthritis. Its short elimination half-life and adverse effects, like gastrointestinal mucosa ulceration, restrict its oral use and make it a good candidate for transdermal administration (Cordero et al., 1997, 2001; Hadgraft et al., 2000). However, due to the excellent barrier function of the skin, the need to use safe and effective enhancers for improving transdermal absorption of drugs is well recognized (Irion et al., 1995; Sinh et al., 1996; Cho and Choi, 1998; Sridevi and Diwan, 2002).

Liposomes have been widely used as safe and effective vehicles for topical drug delivery systems since, in spite of the controversial literature about possible vehicle-mediated phenomena of skin uptake (Alvarez-RománNaik et al., 2004), in several cases they allowed effective drug penetration and enhanced clinical efficacy (Gregoriadis, 2000; Verma et al., 2003a,b). However, the entrapment of poorly water soluble drugs in the lipid bilayers of liposomal membranes is often limited in terms of drug-to-lipid mass ratio and requires the use of organic solvents (McCormack and Gregoriadis, 1994; Gregoriadis, 2000). Furthermore, the carrier functions of liposomes through the skin layers often cannot actually be achieved for lipophilic drugs that, when incorporated in the membrane bilayers rather than entrapped in the aqueous core of the vesicles, are rapidly released from the carriers after administration (Takino et al., 1994).

Cyclodextrins have been widely used to improve solubility and dissolution rate of a number of lipophilic drugs by inclusion complexation in their hydrophobic cavity (Uekama and Otagiri, 1987). The role of cyclodextrins as possible enhancers of percutaneous absorption of drugs has also been investigated (Loftsson et al., 1998; Loftsson and Masson, 2001). It seems that cyclodextrins may improve dermal absorption of drugs mainly by increasing their thermodynamic activity in the vehicles, and favouring their delivery to the skin surface (Loftsson et al., 1998; Matsuda and Arima, 1999; Masson et al., 1999), whereas no carrier functions of cyclodextrins across the skin layers have been clearly demonstrated.

Recently, the entrapment into the aqueous phase of liposomes of lipophilic drugs in the form of water-soluble cyclodextrin inclusion complexes has been proposed as a possible approach for circumventing the problems associated with both systems and combines their relative advantages in a single system. This concept establishes a novel system in drug delivery, by joining liposomes and cyclodextrin complexes of lipophilic drugs and forming drugs-in-cyclodextrins-in-liposomes formulations. (McCormack and Gregoriadis, 1994, 1998; Loukas et al., 1995, 1998).

Therefore, the aim of this work was to prepare liposomes, intended for topical drug delivery, containing ketoprofen–cyclodextrin complexes in the aqueous core, with the purpose of increasing both drug encapsulation stability into the vesicles and its clinical efficacy, by simultaneously exploiting the cyclodextrin solubilizing power towards the drug and the liposome carrier function through the skin layers.

The effectiveness of cyclodextrins in improving solubility and dissolution rate of ketoprofen has already been demonstrated (Funk et al., 1993; Mura et al., 1998). In the present paper, in order to select the most effective cyclodextrin and preparation method for ketoprofen complexation, drug-cyclodextrin complexes with both natural β-cyclodextrin and its hydroxypropyl derivative, prepared by coevaporation and sealed-heating methods, were investigated by differential scanning calorimetry, hot stage microscopy, scanning electron microscopy and tested for dissolution properties. Drug alone and selected drug-cyclodextrin complexes were then incorporated in liposomes prepared by the thin layer evaporation technique. All liposomal formulations were characterised for encapsulation efficiency, particle size and morphology, using, respectively, dialysis, light scattering and Transmission electron microscopy techniques. Permeability properties of these systems were evaluated through both excised rat skin and artificial lipophilic membranes. The liposomal membrane structure and integrity as well as the capacity of the vesicles to penetrate into the rat skin were investigated by confocal laser scanning microscopy using liposomes encapsulating a cyclodextrin complex with rhodamine 6G as fluorescent marker.

2. Materials and methods

2.1. Materials

Ketoprofen (keto), l-α-phosphatidylcholine (PC), cholesterol (CH) and rhodamine 6G (Rho) were provided by Sigma-Aldrich (Italy), β-cyclodextrin (βCyd)

and hydroxypropyl-βCyd (HPβCyd) DS 0.6 were a gift from Wacker (Italy). All other reagents were of analytical grade.

2.2. Preparation of drug-cyclodextrin solid systems

Previous phase-solubility studies and C-NMR analyses, performed according to the mole ratio method, accounted for the formation of 1:1 keto-ßCyd and keto-HPßCyd complexes (Mura et al., 1998). Equimolar drug-Cyd physical mixtures (P.M.) were obtained by tumble mixing for 15 min suitable amounts of the 75–150-μm sieve granulometric fractions of the respective simple components. Sealed-heated products (S.H.) were obtained by sealing physical mixtures (1 g) in a 5-mL glass ampoule which was then heated at 80 °C for 3 h. Coevaporated products (COE) were prepared by coevaporation of equimolar drug-Cyd ethanol–water (5:5 v/v) solutions in a rotary evaporator (Büchi R 200/205) at 55 °C. All products were sieved (Retsch, type Vibro) and the 75–150-μm sieve granulometric fraction was used for the following studies.

2.3. Characterization of drug-cyclodextrin solid systems

2.3.1. Differential scanning calorimetry (DSC)

DSC analysis was performed with a Mettler FP85TA apparatus equipped with a Mettler FP80 processor on 5–10 mg samples (Mettler M3 microbalance) scanned in pierced Al pans between 30 and 300 °C at 10 °C min^{-1} under static air.

2.3.2. Hot stage microscopy (HSM)

HSM analysis was performed using an Olympus BH-2 microscope fitted with a Mettler FP-82 hot-stage. A small amount of sample was placed on the sample stage and heated in the 30–300 °C temperature range at a rate of 2 °C min^{-1}.

2.3.3. Scanning electron microscopy (SEM)

SEM analysis was carried out using a Philips XL 30 scanning electron microscope. Briefly, prior to examination, the samples were fixed on a brass stub using double-sided tape and sputter coated with gold–palladium under argon atmosphere (to render them electrically conductive) using a gold sputter module in a high vacuum evaporator. The pictures were then taken using SEM set at an excitation voltage of 20 kV. The magnification selected was sufficient to appreciate in detail the general morphology of the samples under study.

2.3.4. Dissolution studies

Dissolution tests were performed according to the USP 26 basket method, using a Sotax AT7 apparatus (Sotax AG, Switzerland), by adding an excess of drug or its equivalent amount in binary systems with Cyds, to 500 mL of water at 25 ± 0.5 °C, under a constant stirring of 50 rpm. At suitable time intervals, aliquots were withdrawn and spectrometrically assayed for drug content at 260 nm (Hitachi, Mod. 2000 spectrometer). Each test was performed in triplicate (coefficient of variation CV < 3%).

2.4. Liposome preparation and characterization

2.4.1. Liposome preparation

PC-CH liposomes were prepared by the thin layer evaporation technique (Bangham et al., 1965), obtaining multilamellar vesicles (MLV). In brief, the lipid phase (consisting of a mixture of 60 mg PC and 40 mg CH) was dissolved in the minimum amount of an organic solvent (chloroform); it was then removed under reduced pressure using a rotary evaporator (Büchi R 200/205) at 55 °C, thus obtaining a thin film of dry lipid on the wall of the flask. Evaporation was continued for 2 h after the dry residue appeared, to remove the traces of organic solvent. Finally, the film was hydrated by adding 4 mL of distilled water under vigorous stirring in order to favour the formation of vesicles. Drug alone (5 mg) or its equivalent amount as drug-Cyd complex were entrapped in the vesicles in two different ways: the first was dissolved in the organic solvent together with the lipid phase, while the water-soluble complexes were dissolved in the aqueous phase added for the lipidic film hydration. Liposomes were stored at 4 °C.

2.4.2. Determination of encapsulation efficiency

Liposome encapsulation efficiency (EE%) was measured by determining the amount of non-entrapped keto by using the dialysis technique (Trotta et al., 2002; Foco et al., 2005). In a previous paper some

of us demonstrated the suitability of this method, whose results did not show statistically significant differences in comparison with those obtained by the ultracentrifugation technique (López-Pinto et al., 2005). For separating non-entrapped drug from liposomes, 2 mL of drug-loaded liposomal dispersion were placed into a dialysis bag of cellulose acetate (Spectra/Por®, MW cut-off 12000, Spectrum, Canada) which was immersed in 150 mL water at 4 °C magnetically stirred at 30 rpm. Samples (10 mL) taken from the receiver solution at predetermined times were replaced with equal volumes of fresh water and spectrometrically assayed at 260 nm for drug content (Hitachi Mod. 2000 spectrometer). The experiment was stopped when constant drug concentration values were obtained in subsequent withdrawals of the receiver phase (taking into account the progressive dilution of the medium). Encapsulation efficiency was then calculated according to the following equation:

$$\text{EE\%} = \frac{[\text{total drug}] - [\text{diffused drug}]}{[\text{total drug}]} \times 100$$

2.4.3. Determination of liposomal size

The mean particle size of the vesicles, expressed as volumetric diameter, was measured by dynamic light scattering analysis (Mastersizer, Malvern Instruments, Malvern, UK) using a He–Ne laser radiation at a wavelength of 632.8 nm. About 3 mL of liposomal dispersion were suitably diluted in 1 L of distilled water at 25 °C, in order to avoid multiscattering phenomena. Samples were placed in quartz cells and analysed through a 45 mm focus lens by means of a computer-controlled image system. The measurements were carried out at 25 °C and at a scattering angle of 90°, with the following experimental parameters: medium refractive index, 1.330; medium viscosity, 1.0 cps; dielectric constant, 79. The experiments were performed in triplicate and each sample was analysed three times. Correlation functions were performed by a Malvern PCS submicron particle analyzer and a third-order cumulative fitting was used to obtain mean diameter and polydispersity index (Berne and Pecora, 1976).

2.4.4. Transmission electron microscopy (TEM)

TEM analysis (Philips CM 10, Philips, USA) was used to examine the ultrastructure of liposomes. To prepare samples, copper grids were coated with a solution of collodion and then a drop of liposomal dispersion was deposited and left for 15 min in contact. Finally, grids were picked up, blotted with filter paper, left to dry for 3 min and then analyzed with TEM (Manosroi et al., 2004).

2.4.5. Confocal laser scanning microscopy (CLSM)

CLSM studies were performed to characterise liposome morphology and lamellar structure directly in solution. Analysis was carried out on a drop of opportunely diluted, freshly prepared liposomal dispersion containing Rho or Rho-Cyd complex (10^{-5} M) as fluorescent marker using a Leica TCS SP II Laser scanning Confocal Imaging System (Leica, Heidelberg, Germany) equipped with a Kr–Ar–He–Ne ions laser and a Leica DM IRE 2 microscope endowed with HC PL Fluotar Leica X10 and X20 dry objectives and HCX PLAN APO Leica X40 multi-immersion objective (numeric aperture 0.85). For excitation of the fluorescent label the 488 nm wavelength was used and the fluorescence emission was detected at 520 nm.

CLSM studies were performed also in order to evaluate the actual carrier functions of liposomes, by visualizing the distribution of the model fluorescent compound in the skin. Rat skin, after a 6-h incubation period in Franz diffusion cells in the presence of a solution of Rho-Cyd complex as such or entrapped in liposomes, was rapidly frozen by liquid nitrogen; sections of skin (50 μm thickness) were then perpendicularly cut with a cryomicrotome and examined to investigate the fluorescent marker distribution in the different skin layers (Verma et al., 2003a).

2.5. Permeation studies

Permeation studies of drug alone or as complex with Cyd, both as such or entrapped in MLV systems were performed for 24 h at 37 ± 1 °C using Franz diffusion cells (Vidrafoc, Barcelona, Spain), with an effective diffusion surface of 2.54 cm^2 and a receiver compartment of 14.5 mL volume consisting of a degassed pH 7.4 phosphate buffer solution (Bosman et al., 1998; Kirjavainen et al., 1999). Permeation experiments were performed in occlusive mode. The donor compartment was filled with 3 mL of aqueous suspension of drug or drug-Cyd complex or liposomal suspension containing

the plain drug or the drug-Cyd complex entrapped, all corresponding to a total drug amount of 5 mg. The solubility of the drug in the receiver medium was 1.45 mg/mL, thus assuring the maintenance of sink conditions for the duration of diffusion experiments. Care was taken to remove any bubbles between the underside of the diffusion membrane and the solution in the receiver compartment. Experiments were performed in sextuple, with both artificial membrane and excised rat skin (see Section 2.5.1). At predetermined intervals, samples (0.55 mL) were withdrawn from the receptor compartment, replaced with an equal volume of fresh medium, and spectrometrically assayed for drug content at 260 nm. It was verified that neither the artificial membrane impregnation agent nor the skin components interfered with the assay.

A correction was calculated for the cumulative dilution of the receptor medium. The cumulative amount of drug transferred into the receptor side was calculated and the results were averaged (CV < 1.5% for permeation experiments with artificial membranes; CV < 4.5% for experiments with rat skin). The results of all these experiments were statistically analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparison post test (Graph Pad Prism, Version 3). The differences were considered statistically significant when $P < 0.01$.

2.5.1. Diffusion membranes

Cellulose nitrate membranes impregnated with lauryl alcohol (membrane weight increase 90–110%) as lipid phase were used as artificial diffusion membranes simulating the epidermal barrier (Mura et al., 1993).

Abdominal skin of Wistar rats stemming from the Animalary Service of University of Seville (aged 17–22 weeks, weight 250–300 g) was used for permeation studies. Rats were killed by diethylic ether inhalation. After depilation and washing, abdominal skin was excised, thoroughly washed with the pH 7.4 buffer solution, dried and carefully cleaned from the subcutaneous fat, and then preserved at −25 °C. Before using, the skin was thawed, pre-hydrated for 1 h with the pH 7.4 buffer solution and then mounted in the diffusion chamber of the Franz cell with the stratum corneum facing the donor compartment and the dermal side toward the receptor fluid, which was stirred with a magnetic bar at 50 rpm.

3. Results and discussion

3.1. Characterization of drug-Cyd complexes

The interaction in solution between keto and both βCyd and HPβCyd has already been investigated in depth by some of us by phase-solubility and C-NMR studies (Mura et al., 1998). Therefore, in the present work, we devoted our research to the solid-state characterization of the different drug-Cyd solid binary systems prepared.

The thermal curves of pure keto, βCyd, HPβCyd, and their respective equimolar P.M., COE and S.H. products are reported in Fig. 1. The thermal curve of the drug indicated its crystalline anhydrous state, exhibiting a sharp endothermal peak at 94.5 °C, while both Cyds showed a broad endothermal effect, in the 70–140 °C range, associated with water loss. The drug melting peak was still detectable in P.M. with both Cyds, while it completely disappeared in both COE and S.H. products, indicating drug complexation and/or amorphization. The DSC curve of the P.M. with βCyd was characterized by another small endothermic peak at about 170 °C. A similar effect was also observed in mixtures of βCyd with naproxen and attributed to a reversible transformation of βCyd (Bettinetti et al., 1989). This small endothermic effect

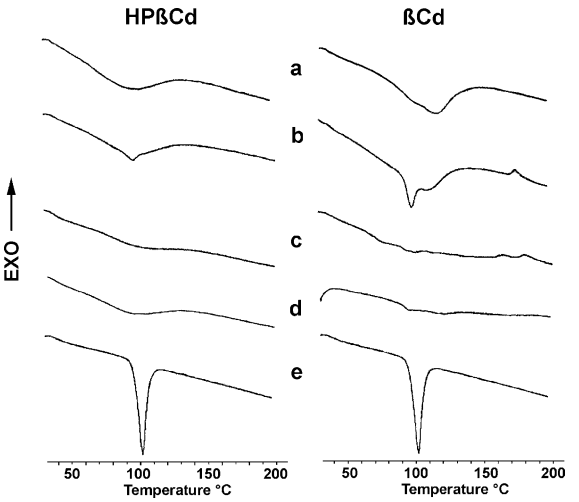

Fig. 1. DSC curves of equimolar keto-HPβCyd and keto-βCyd systems prepared with different techniques: pure Cyd (a), physical mixture (b), coevaporated system (c), sealed heated system (d) and pure ketoprofen (e).

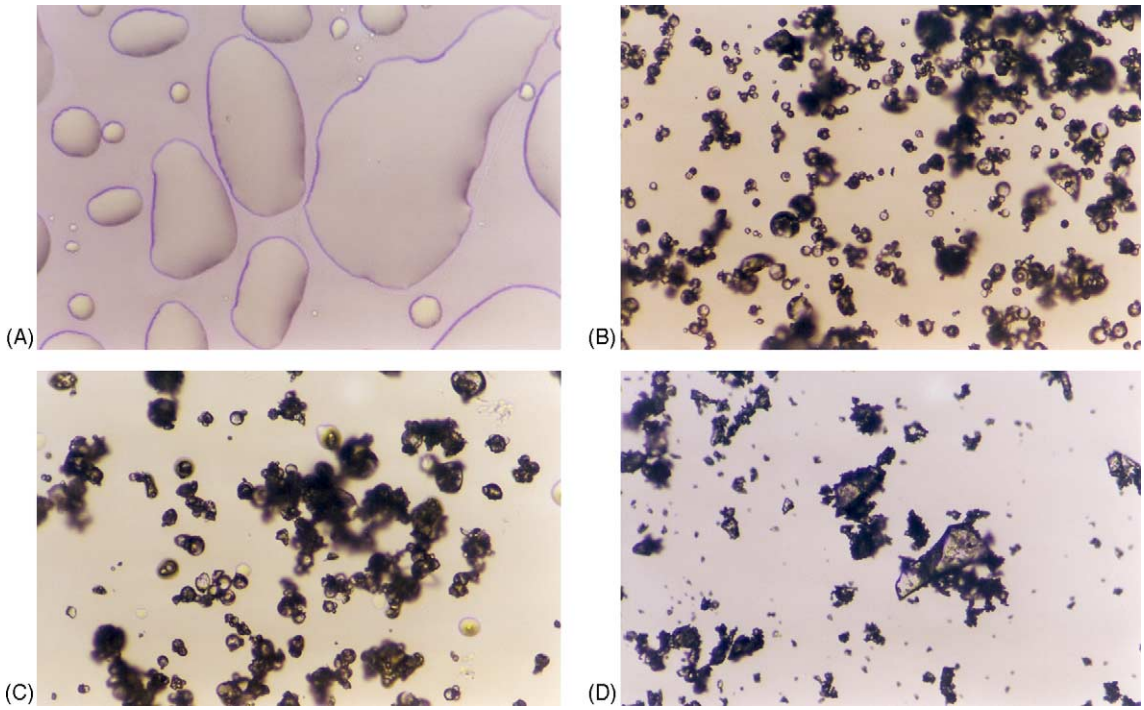

Fig. 2. Photomicrographs of ketoprofen (A), HPβCyd (B), and their equimolar physical mixture (C) and coevaporated product (D) taken at 95 °C during HSM analysis.

was not detectable at the full scale limits of the heat flow adopted for curve a (right column) in Fig. 1. HSM analysis (Fig. 2) supported the interpretation of DSC results, making it possible to detect in both simple blends, at around 94–95 °C, the melting process of the characteristic drug polyhedric crystals; on the contrary, no fusion phenomena were observed at this same temperature in either COE or S.H. products.

Results of SEM analysis, performed to investigate the morphologies of pure drug and carriers and their combinations, were consistent with these findings, showing that the typical polyhedron-shaped drug crystals, still recognizable in both P.M., were no longer detectable in the COE and S.H. systems (Fig. 3), where the formation of amorphous aggregates was instead observed.

Dissolution studies (Fig. 4) indicated that HPβCyd was in all cases more effective than the natural βCyd in improving drug dissolution properties and, for both Cyds, COE and S.H. products exhibited rather similar behaviours, being clearly more efficacious than the corresponding physical mixtures. COE with HPβCyd was the most effective system, with an about 11-fold increase in dissolved drug amount after 60 min with respect to drug alone. COE systems were thus selected for entrapment in liposomes, also taking into account the greater ease for a possible scaling-up of the coevaporation method than the sealed-heating one.

3.2. Characterization of liposomes entrapping keto or keto-Cyd complexes

The mean particle size and the encapsulation efficiency (determined, respectively, by light scattering analysis and the dialysis method) of MLV vesicles loaded with keto alone or as COE with βCyd or HPβCyd are summarised in Table 1.

The dimensions of liposomes entrapping the plain drug were similar to those of empty liposomes, whereas, on the contrary, larger vesicles were obtained in the presence of keto-Cyd complexes. Literature data concerning the influence of Cyd presence on the liposomal vesicle dimensions are controversial. In fact some authors did not find significant differences in the mean

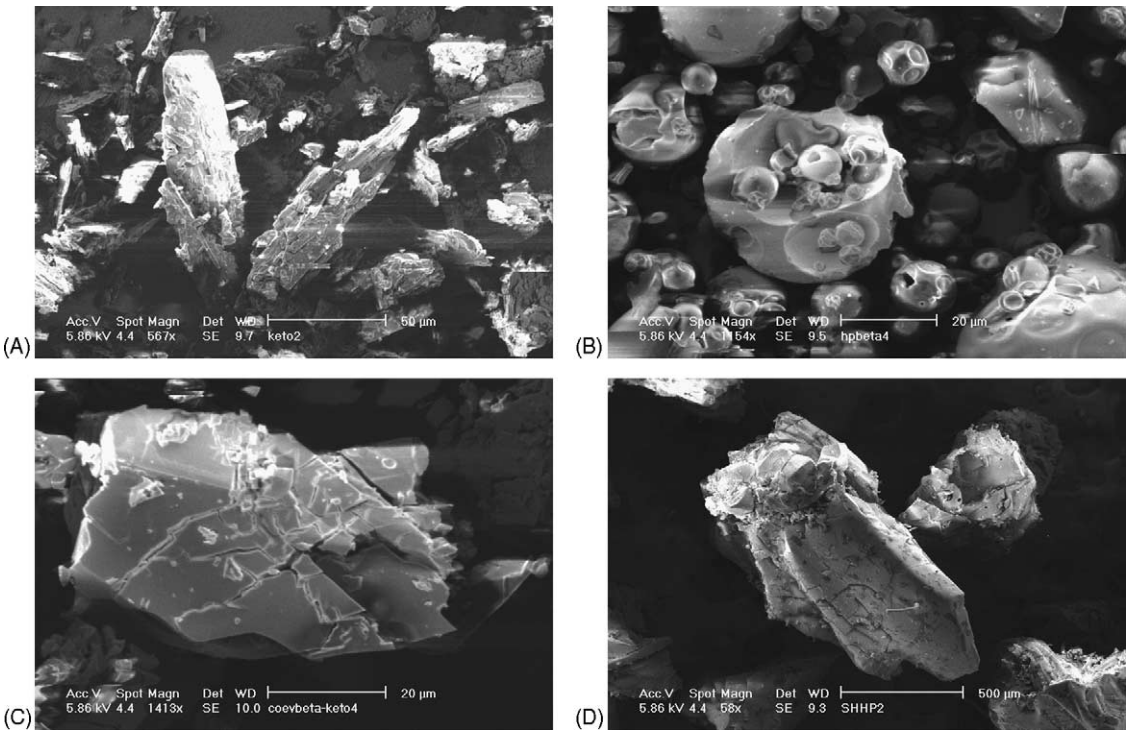

Fig. 3. SEM micrographs of ketoprofen (A), HPβCyd (B), and equimolar keto-HPβCyd complexes obtained by sealed heating (C) and coevaporation (D) methods.

size of liposomes containing the drug-Cyd complexes in comparison with empty liposomes or liposomes containing drug alone (Skalko et al., 1996; Fatouros et al., 2001), whereas other authors reported the opposite (Becirevic-Lacan and Skalko, 1997; Oommen et al., 1999). Probably, these contrasting results depend on the different experimental conditions and/or methods utilised by the authors to prepare and/or analyze empty and loaded liposomes. Therefore, adequate systematic studies should be performed to obtain greater insight about this, as yet, not well assessed phenomenon.

MLV containing keto-Cyd complexes exhibited reduced encapsulation efficiency in comparison with

Table 1
Mean particle size, polidispersity index and efficiency of encapsulation (EE%) of empty MLV liposomes or encapsulating ketoprofen (keto) alone or as complex with βCyd or HPβCyd

Entrapped guest	MLV particle size (μm)	Polidispersity index	MLV EE%
–	1.58 ± 0.08	1.2 ± 0.1	–
keto	1.52 ± 0.05	1.3 ± 0.2	56.0 ± 3.2
keto-βCyd	3.05 ± 0.07	1.1 ± 0.3	26.8 ± 1.6
keto-HPβCyd	3.16 ± 0.10	1.1 ± 0.8	33.8 ± 2.0

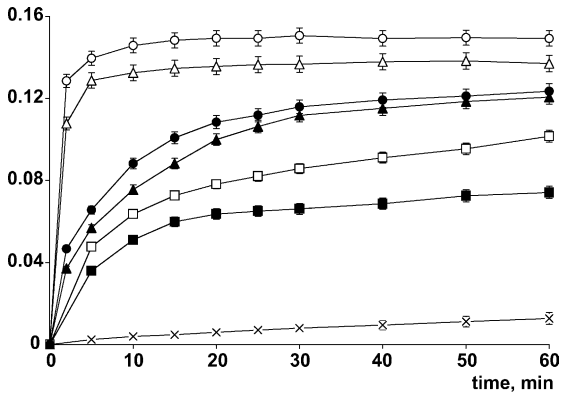

Fig. 4. Dissolution curves of ketoprofen alone (×) and from its physical mixture (■, □), sealed heated (△, ▲) and coevaporated (◯, ●) systems with βCyd (filled symbols) and HPβCyd (open symbols).

those containing drug alone. This can be explained by the different preparation method of liposomes used in the two cases. In fact, the vesicles containing keto alone, due to the very low water solubility of the drug, were prepared by dissolving it in the lipophilic phase, together with PC and CH. By contrast, the vesicles containing the drug as hydrosoluble complex were prepared by dissolving it in the water used for hydration of the lipidic film. Therefore, since in the case of MLV liposomes the volume occupied by the aqueous phase is smaller than that occupied by the lipidic phase, when keto is added to the organic phase, it is entrapped in greater amounts in the multi-layer liposomal membrane, with respect to the hydrophilic complex into the aqueous core. On the other hand, liposomes containing the keto-HPβCyd complex showed higher encapsulation efficiency than those containing the keto-βCyd one. Considering the drug loading procedure used in the case of drug-Cyd complexes, this result was attributed to the higher hydrophilicity of the hydroxypropylated βCyd, which made its entrapment into the aqueous core of the vesicles more favourable, as well as to the greater solubility and stability of its complex with Keto (Mura et al., 1998). In fact, the higher amount of free lipophilic drug present in the aqueous solution in the case of the less stable complex with βCyd could not be included in the structure of the already formed lipid bilayers during the lipid film hydration phase, and, probably, it was simply adsorbed (and then easily released) on the vesicle surface, and/or it partially precipitated in the aqueous phase during MLV formation. An analogous result, as far as the different encapsulation efficiency is concerned, was obtained for prednisolone complexes with βCyd and HPβCyd included in liposomes: the authors ascribed this to the higher amounts of drug interacting with the hydroxypropyl-derivative as a consequence of its more expanse hydrophobic cavity (Fatouros et al., 2001).

TEM analysis (Fig. 5) showed that MLV formation was negatively influenced by the presence of Cyd. In fact when Cyd was added alone to the water used for lipidic film hydration, it did not allow the formation of stable spherical liposomal vesicles but gave rise to the formation of unstable asymmetric aggregates (Fig. 5B). It was verified that this finding was not an artefact of the technique which requires sample dehydration and could consequently enhance possible Cyd-liposome interactions. Indeed, the presence in such systems of aggregation phenomena, rather than of regular vesicles, was confirmed by CLSM analysis, a technique widely used to characterise various colloidal systems, including liposomes (Lamprecht et al., 2000). Such an effect, which was not observed in the case of

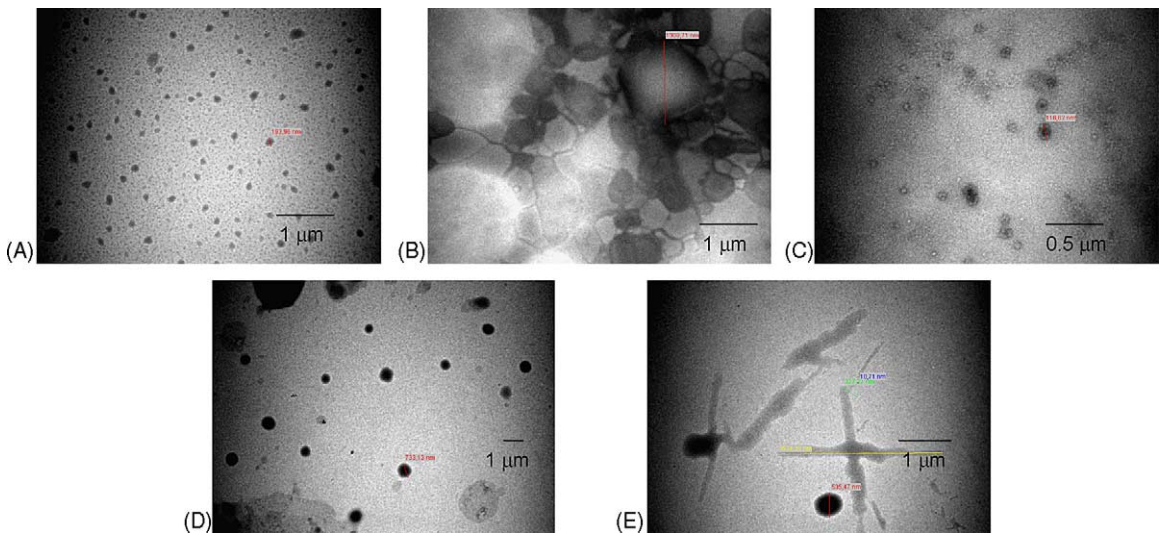

Fig. 5. TEM micrographs of MLV empty (A), or containing Cyd alone (B), ketoprofen alone (C) and ketoprofen complexes with HPβCyd (D) and βCyd (E).

drug-Cyd complexes, could be reasonably attributed to a reduced integrity and stability of the bilayer structure of liposomes as a consequence of both the surfactant and complexing properties of Cyds towards CH, whose presence is known to make vesicles more rigid and firmer (Hartel et al., 1998; Fatouros et al., 2001). The liposome destabilizing effect due to the presence of cyclodextrins has recently been confirmed (Bouldemarat et al., 2004; Piel et al., 2004); the effect has been found to be dependent on both the lipid and Cyd type and concentration and has been attributed to a fluidization of the vesicles induced by Cyd as a consequence of CH extraction from the phospholipids bilayer. On the contrary, it was possible to prepare stable MLV containing keto-Cyd complexes. Probably, the presence of keto, which has higher affinity than CH for the inclusion into the Cyd hydrophobic cavity, hampered interactions of the macrocycle with the liposomal membrane constituents, thus allowing MLV formation. The type of Cyd used affected the MLV properties: in fact, liposomes containing keto-HPβCyd complexes were of spherical shape and homogeneous aspect, whereas precipitation of drug crystals was observed in MLV with keto-βCyd systems (Fig. 5E). This was probably due to the lower stability of the complex of keto with native Cyd (Mura et al., 1998): part of the drug, less strongly complexed with βCyd, recrystallized in the aqueous solution during the MLV preparation. Such a finding was in agreement with the lower encapsulation efficiency obtained with keto-βCyd than with keto-HPβCyd complex, as discussed above.

On the other hand, the presence of Cyd did not affect the MLV lamellar structure, as put in evidence by CLSM analysis. Bidimensional and tridimensional fluorescent and transmission images of liposomes containing Cyd complexes with the fluorescent marker Rho (Fig. 6) showed that the complex was actually included

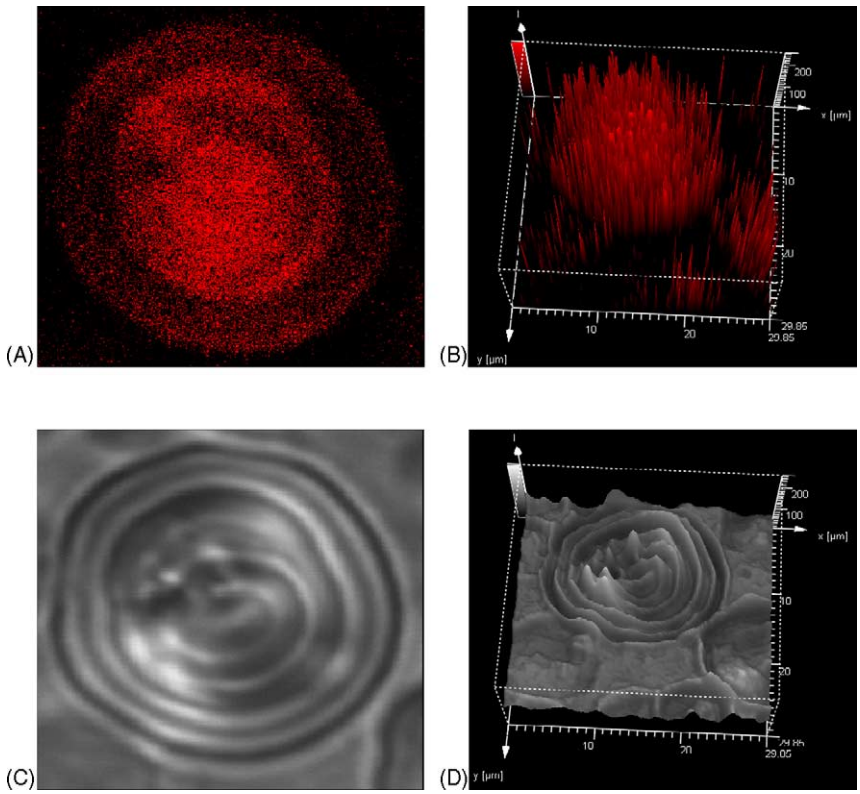

Fig. 6. Fluorescent (top) or transmission (bottom) bidimensional (A, C) or tridimensional (B, D) CLSM images of liposomes containing rhodamine-HPβCyd coevaporated system.

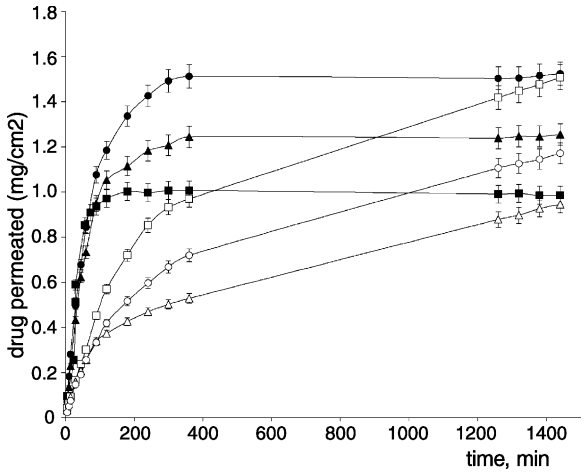

Fig. 7. Ketoprofen permeation profiles across artificial membrane of suspensions (filled symbol) and liposomes (open symbol) containing pure drug (■, □), or drug-βCyd (▲, △) or drug-HPβCyd (●, ○) complexes.

in the vesicles and made it possible to demonstrate that the multilamellar structure of MLV was maintained.

3.3. Permeation studies

Permeability profiles across artificial membranes and rat skin of keto and keto-Cyd complexes as such or incorporated in liposomes are reported, respectively, in Figs. 7 and 8.

In the experiments with artificial membrane, a rapid permeation rate was observed from suspensions containing either plain drug or its complex with Cyd, and a plateau was reached after only 120–150 min in the case of drug alone and after about 350 min in the case of its Cyd complexes. This longer time could be explained by the dynamic equilibrium existing in solution in the donor compartment between complexed and free drug, that progressively supplies new diffusible drug molecules, as the free drug permeates through the membrane. The amount of permeated drug was in the order keto-HPβCyd > keto-βCyd > keto ($P < 0.001$). The best performance shown by the system with HPβCyd is attributable to the greater solubilizing power towards the drug of such Cyd (Mura et al., 1998) which allowed a greater amount of drug in solution, available for permeation.

When keto (as such or as complex with Cyd) was entrapped in liposomes, a slow and almost linear diffusion profile was instead observed during the entire 24 h period. The prolonged release effect of liposome formulations in comparison with simple drug solutions or suspensions is well known (Gregoriadis, 1988). Moreover, contrary to that observed in the case of suspensions, keto permeated faster from liposomes containing plain drug than from those containing drug-Cyd complexes, and the following trend was observed: keto > keto-HPβCyd > keto-βCyd ($P < 0.001$). This finding was attributed to the different preparation method of the liposomes, as illustrated above. The drug alone, incorporated in the phospholipidic membrane bilayer, can be released rapidly, whereas the complexed drug, entrapped in the internal aqueous core, permeated more slowly, since it had first to overcome the lipidic barrier of the vesicle

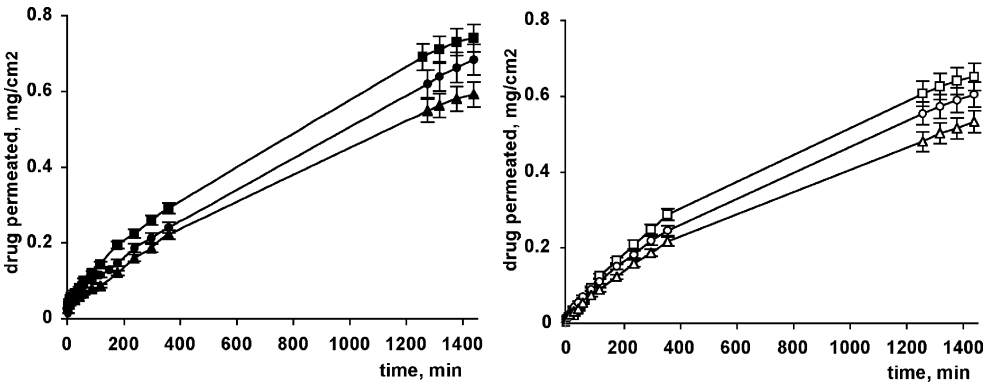

Fig. 8. Ketoprofen permeation profiles across rat skin of suspensions (filled symbol) and liposomes (open symbol) containing pure drug (■, □), or drug-βCyd (▲, △) or drug-HPβCyd (●, ○) complexes.

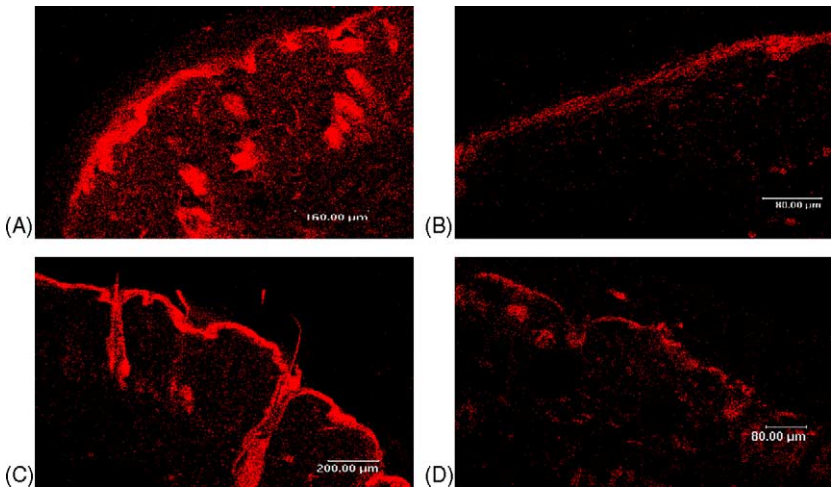

Fig. 9. CLSM images of cross sections of abdominal rat skin after 6-h incubation in Franz diffusion cell with suspension (on the left) and liposomes (on the right) containing rhodamine-HPβCyd (A, B) and rhodamine-βCyd (C, D) complexes.

membrane. Therefore, entrapment of the hydrosoluble complex in the internal aqueous core of the liposomes seems to actually allow a more stable encapsulation of the drug.

Experiments across rat skin (Fig. 8) showed, as a general trend, a noticeably slower drug permeation rate from all the examined formulations with respect to the tests with artificial membrane. This was expected, as a consequence of the stronger barrier effect due to the presence of the stratum corneum. As in the previous series of experiments, drug permeation rate was significantly ($P < 0.001$) higher from suspensions than from the corresponding liposomal formulations, which then confirmed their prolonging effect on drug release. Moreover, the same trend was observed as for the drug amount permeated from liposomal formulations (i.e. keto > keto-HPβCyd > keto-βCyd, $P < 0.001$). This finding corroborated the importance of the different mode of drug incorporation into the vesicles (dissolved in the lipid or aqueous phase) in determining its release rate, as well as the possibility of better modulating it by Cyd complexation. However, differently from the experiments with artificial membrane, this same trend was found also for the corresponding suspensions ($P < 0.01$), which also exhibited almost linear profiles as well, without any plateau effect. Evidently, in this case, the lower drug permeability rate through the skin annulled the favourable effect due to the enhanced drug solubility given by Cyd complexation. Therefore, these results confirmed that the presence of Cyd improved drug permeation only by increasing its solubility in the vehicle (Loftsson et al., 1998; Matsuda and Arima, 1999) and not by enhancing its permeability across the skin.

Unexpectedly, however, liposomes only exhibited a controlling effect on drug release but they did not show any drug carrier function through the skin layers. In fact the drug amount permeated after 24 h from liposomal formulations was about 20% less than from the corresponding suspensions.

This result was further confirmed by skin penetration studies performed by CLSM analysis with the Rho-Cyd complex. CLSM images taken after 6 h incubation of rat skin with the label complex (Fig. 9) clearly showed that it permeated deeper when it was in solution than when entrapped in liposomes.

4. Conclusion

The possibility of using the combined approach of Cyd complexation and liposome entrapment in order to increase the skin permeation properties of the anti-inflammatory drug ketoprofen has been investigated in the present work. Drug complexation with Cyd resulted in a significant improvement of drug dissolution properties. In particular, coevaporated systems with HPβCyd gave rise to an 11-fold increase in

dissolved drug amount. Entrapment in MLV of Keto-Cyd complexes was successfully obtained, in spite of the destabilizing effect of Cyd due to its complexing capacity toward the vesicle membrane components, such as cholesterol (Hartel et al., 1998). The enhanced water solubility of the drug-Cyd complex allowed its entrapment in the internal aqueous phase of the vesicle, instead of in the external bilayers, thus assuring a more stable drug encapsulation in the carrier and a better control of drug release. However, differently from expectations (Verma et al., 2003a), entrapment of keto-Cyd complex in liposomes did not improve the drug permeability properties through the skin.

Further studies will be therefore necessary to investigate in depth the role of the preparation method, the lipid phase composition, the lamellar membrane structure and the liposome dimensions (Verma et al., 2003b) on their skin-permeation enhancer properties, in order to adequately improve their effectiveness.

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