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New "drug-in cyclodextrin-in deformable liposomes" formulations to improve the therapeutic efficacy of local anaesthetics

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ABSTRACT

The combined approach of cyclodextrin complexation and entrapment in liposomes was investigated to develop a topical formulation of local anaesthetics. For both benzocaine (BZC) and butamben (BTM), hydroxypropyl-β-cyclodextrin (HPβCD) was a better partner than βCD; drug–HPβCD coevaporated products showed the best solubility and dissolution properties, and were selected for loading into liposomes. Addition of stearylamine to the phosphatidylcholine–cholesterol mixture of the vesicle bilayer allowed obtainment of deformable liposomes with improved permeation and *in vivo* drug anaesthetic effect ($P < 0.05$). Double-loaded deformable liposomes were obtained by adding the drug–HPβCD complex at its maximum aqueous solubility in the vesicles hydrophilic phase, and the remaining amount up to 1% as free drug in the lipophilic phase. The properties of double-loaded liposomes were compared with those of classic single-loaded ones, obtained by adding 1% free drug in the aqueous or lipophilic phase of the vesicles. Size, charge, morphology and entrapment efficiency of the different batches were investigated, respectively, by light scattering, confocal laser scanning microscopy and dialysis, while their therapeutic efficacy was evaluated *in vivo* on rabbits. For both drugs, double-loaded liposomes, exploiting the favourable effects of drug–CD complexation, allowed a significant ($P < 0.05$) enhancement of intensity and duration of anaesthetic effect with respect to those single-loaded.

1. Introduction

Liposomal formulations are extensively investigated in the pharmaceutical field as safe and effective drug carrier systems. In particular, among their several possible pharmaceutical applications, they have been widely applied in topical drug delivery, due to their effectiveness in entrapping drugs and delivering them to the skin, therefore enhancing their clinical efficacy (Margalit, 1995; Fresta and Puglisi, 1996; Gregoriadis, 2000; Verma et al., 2003). In particular, it has been reported that liposomal formulations of various anaesthetics allowed an increase in clinical efficacy in comparison with the plain drugs (Bucalo et al., 1998; Fisher et al., 1998; Grant and Bansinath, 2001; Lim et al., 2005; Cereda et al., 2006; Mura et al., 2007). Interestingly, in the last years, classical liposomes evolved to "highly deformable" liposomes, endowed with enhanced skin penetration ability, and then improved drug skin delivery (El Maghraby et al., 1999; Cevc et al., 2002; Cevc and Blume, 2004; Trotta et al., 2004). These vesicles consist of phospholipids and an edge activator, which is often a single chain surfactant which destabilizes the liposomal lipid bilayers, increasing their elasticity and flexibility (Elsayed et al., 2007). Several studies demonstrated as the penetration across the skin of liposomal vesicles is directly related with their deformability. The high adaptability of such elastic vesicles enable them to squeeze between the cells of the stratum corneum, and thus to penetrate intact to the deep layers of the skin, with an effect comparable to that of a subcutaneous injection (El Maghraby et al., 2008).

Benzocaine (BZC) and butamben (BTM) are ester-type local anaesthetics mostly used in topical, dermal and mucous formulations. Their anaesthetic action is characterized by a rapid but brief effect, compared with the potential duration of pain. Moreover the toxic effect of ester-type local anaesthetics, due to systemic absorption, has been reported (Covino and Vassallo, 1976; Barclay and Vega, 2004). Therefore, the development of a new effective topical delivery system intended to suitably modulate the release rate of these drugs, extending their anaesthetic effect and enhancing their localization in the skin, thus reducing problems of systemic toxicity, could be particularly advisable.

However, the entrapment of lipophilic, poorly soluble drugs in liposomal vesicles requires the use of organic solvents and is lim-

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ited by possible problems of bilayer destabilization (Gregoriadis, 2000). Moreover, drugs incorporated in the membrane bilayers rather than in the aqueous core of the vesicles, are more rapidly released after administration, thus not fully exploiting the carrier functions of liposomes through biological membranes. A possible means to overcome these drawbacks is to add the lipophilic drug, dissolved in a suitable water–miscible organic solvent, such as ethanol, to the hydrophilic phase of the vesicles (Maestrelli et al., 2009). The entrapment of hydrophobic drugs in the aqueous core of liposomes as soluble inclusion complexes with cyclodextrins has been proposed as an interesting alternative to avoid the use of organic solvents, thus obtaining drug-in cyclodextrin-in liposome systems (McCormack and Gregoriadis, 1994, 1998). This approach can be useful to both increase drug solubility and stability (Loukas et al., 1998) and better control the *in vivo* fate of poorly soluble drugs, avoiding the rapid release observed after conventional incorporation into the liposome lipid phase (McCormack and Gregoriadis, 1994, 1998). Moreover, complexation with cyclodextrins showed to be able to increase drug solubility and permeation across the skin, thus improving drug bioavailability through topical route (Matsuda and Arima, 1999; Loftsson and Masson, 2001). The effectiveness of such a combined approach, which simultaneously exploits the cyclodextrin solubilizing power towards the drugs and the liposome carrier function through the skin, has been recently demonstrated by using both classic (Fatouros et al., 2001; Maestrelli et al., 2005, 2006; Lira et al., 2009) and deformable (Gillet et al., 2009) liposomes. Further advantages can be obtained by the use of a double-loading technique, i.e. by preparing liposomes loaded with the plain drug in the lipophilic phase and its cyclodextrin complex in the aqueous phase of the vesicles, so that to obtain both a fast onset action and a prolonged effect (Bragagni et al., 2010). All these studies pointed out the importance of preformulation studies aimed to the selection of the most suitable cyclodextrin type and of the most effective complex preparation method, in order to can take the maximum of benefit from such a combined strategy.

Therefore in this work, we thought it worthy of interest to prepare and characterize deformable liposomes bearing butamben or benzocaine cyclodextrin complexes and characterize them for their performance *in vitro* and *in vivo*. With this aim, we evaluated the solubilizing and complexing power towards these drugs of native β-cyclodextrin and its hydrophilic derivative hydroxypropyl-β-cyclodextrin. Solid drug–cyclodextrin systems were prepared according to different techniques and characterized for both solid-state and dissolution properties. The most effective systems were then selected for preparation of double-loaded liposomes. For comparison purposes, we also prepared liposomes with the lipophilic plain drug all loaded in the lipophilic phase of the vesicles or in the hydrophilic one, using in the second case a water–ethanol mixture to dissolving the drug.

Liposomes were prepared by using a mixture of phosphatidylcoline, cholesterol and stearylamine, where the ionic surfactant was purposely added to the composition of the liposomal membrane in order to increase the bilayer deformability (Trotta et al., 2004; Bragagni et al., 2010). Moreover, since the epithelial cells in the various tissues, including the skin, carry a negative charge, the use of a positively charged delivery system should result in better drug permeability and prolonged pharmacological action (Piemi et al., 1999).

The liposomal batches were characterized for morphological properties using confocal laser scanning microscopy (CLSM), while their particle size, Zeta potential, and entrapment efficiency were determined by using, respectively, light scattering, and dialysis techniques. The anaesthetic efficacy of the different drugs liposomal dispersions was tested *in vivo* on rabbits.

2. Materials and methods

2.1. Materials

Butamben (BTM) (butyl-4-aminobenzoate, pK_a = 2.5, log P = 2.61 (Grouls et al., 1997)), benzocaine (BZC) (ethyl-4-aminobenzoate, pK_a = 2.8, log P = 1.44 (Grouls et al., 1997)), cholesterol (CH), l-α-phosphatidylcholine (PC), stearylamine (SA) and rhodamine 6G were from Sigma–Aldrich (Italy). β-Cyclodextrin (βCD) and hydroxypropyl-β-cyclodextrin (HPβCD, average molecular weight 1399, MS 0.65) were a generous gift of Roquette Pharma (France). Carbopol® 940 (polyacrylic acid) was kindly supplied by Noveon, Inc (Cleveland, OH, USA). All other reagents were of analytical grade.

2.2. Phase-solubility studies

Phase-solubility studies were performed by adding an excess of drug to 10 mL of pH 5 phosphate buffer solution containing increasing amount of βCD (0–12.5 mM) or HPβCD (0–25 mM). The vials were sealed and electromagnetically stirred (750 rpm) at constant temperature (25 °C) until equilibrium (72 h). An aliquot of solution was then withdrawn with a filter syringe (pore size 0.45 μm) and spectrometrically assayed (UV/VIS 1600 Shimadzu spectrophotometer, Tokyo, Japan) for BZC or BTM concentration at 280 and 287 nm, respectively. The presence of CD did not interfere with the spectrophotometric assay of both drugs. Each experiment was performed in triplicate (C.V. < 3.5%). The apparent 1:1 stability constants (Ks) of the complexes were calculated from the slope of the straight lines of the phase-solubility diagrams and the drug solubility in the medium (Higuchi and Connors, 1965).

2.3. Preparation and characterization of drug–CD binary systems

Equimolar systems of BZC and BTM with each CD were obtained by physical mixing, co-grinding and coevaporation techniques. Physical mixtures (P.M.) were prepared by homogeneous mixing of previously weighted powders in a mortar with a spatula for 15 min. Co-ground (GR) products were prepared by ball-milling P.M. in a high energy vibrational micro-mill (Retsch, GmbH, Dusseldorf, Germany) at a frequency of 24 Hz for 30 min. Coevaporated (COE) products were prepared by coevaporation of equimolar drug–CD 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 granulometric fraction was used for the following studies. The single components and the different drug–CD binary systems were characterized by X-ray powder diffractometry (Bruker D8, Cu Kα radiation, 40 kV, 40 mA, 5–36° 2θ range, scan rate 0.05° s^{-1}), DSC analysis (Mettler TA4000 Star^e System, 10 °C min^{-1} in the 30–120 °C temperature range under static air), FT-IR spectrometry (Perkin-Elmer Mod. 1600, nujol dispersion in the 4000–600 cm^{-1} region) and scanning electron microscopy (Philips XL 30, excitation voltage of 20 kV, samples sputter coated with gold–palladium under Ar atmosphere to render them electrically conductive).

2.4. Dissolution rate studies

Dissolution rate of each drug, both alone and from the different drug–CD binary systems, was determined according to the dispersed amount method, by adding 600 mg of drug or drug-equivalent in a 300 mL beaker containing 100 mL of water thermostated at 37 °C and stirred at 100 rpm with a glass three-blade propeller immersed in the beaker 25 mm from the bottom. At time intervals, samples were withdrawn with a filter-syringe (pore size 0.45 μm) and spectrometrically analysed for drug content as described above. The same volume of fresh medium was added and

the correction for the cumulative dilution calculated. Each test was repeated three times (coefficient of variation < 5%).

2.5. Preparation of liposomes

Multi-lamellar vesicles (MLV) liposomes consisting of mixtures of PC, CH and SA in different molar ratios (1:1:0; 6:1:1; 5.5:1:1.5) as lipid phase were obtained by thin layer evaporation (TLE) (Bangham et al., 1965). Briefly, the lipid mixture was dissolved in chloroform which was then removed under vacuum at 58 °C, thus obtaining a thin film of dry lipid on the flask wall. Evaporation was continued for 2 h after the dry residue appeared, to completely remove all the traces of solvent. The film was then hydrated by adding the hydrophilic phase under vigorous mechanical shaking with a vortex mixer until vesicle formation. Drug-loaded liposomes were prepared following three different ways: (a) 1% free drug was dissolved in the lipophilic phase, and water was used as hydrophilic phase; (b) 1% free drug was dissolved in the hydrophilic phase (consisting in this case of a water:ethanol 60:40, v/v mixture, to allow drug solubilization); (c) the drug–CD complex was dissolved in water, as hydrophilic phase, up to its maximum solubility (0.5%, w/v for BZC and 0.3%, w/v for BTM), and the remaining amount to reach 1% was added as free drug in the lipophilic phase (double-loading).

2.6. Characterization of liposomes

2.6.1. Determination of encapsulation efficiency

Liposomes encapsulation efficiency was indirectly determined, separating the non-entrapped drug from drug-loaded liposomes by dialysis experiments. According to a previously developed method (Mura et al., 2007), 3 mL of drug-loaded liposomes was placed into a dialysis bag of cellulose acetate (Spectra/Por®, MW cut-off 12000, Spectrum, Canada) immersed in a closed vessel containing 150 mL of water at 20 °C, magnetically stirred at 30 rpm. Samples, withdrawn at time intervals, were replaced with equal volumes of fresh solvent and spectrometrically analysed (UV 1601 Shimadzu). The experiment was stopped when constant drug concentrations were obtained in subsequent withdrawals from the receiver phase (taking into account the progressive dilution of the medium). The percent of encapsulation efficiency (*EE*%) was calculated according to the following equation:

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

Each result is the mean of at least three separate experiments.

2.6.2. Determination of particle size and Z potential

The average particle size and charge of the liposomes were calculated with a Zetamaster apparatus (Malvern Instruments, Malvern, UK) at a temperature of 25 ± 1 °C. Liposomal preparations were analysed 24 h after preparation. For particle size measurements, liposomal suspensions were properly diluted with distilled water in order to avoid multiscattering phenomena. The intensity of the laser light scattered by the samples was detected at an angle of 90° with a photomultiplier. At least three independent samples were taken, each of which was measured four times. For each specimen, 10 autocorrelation functions were analysed using a cumulative analysis. From this analysis, the *z*-average value was obtained, which approximates the diameter of the liposomes.

For surface charge determination, liposomal dispersions, suitably diluted with distilled water, were dropped into the Zetamaster electrophoretic cell and the *Z* potential determined by electrophoretic mobility measurement. At least 5 independent samples were taken for each batch, each of which analysed at least three times.

2.6.3. Liposome morphology

Confocal laser scanning microscopy (CLSM) analyses were performed to characterize liposome morphology directly in solution. Towards this aim, some liposomal dispersions were prepared by adding a hydrophobic fluorescent probe, i.e. rhodamine 6G, in the internal phase (Maestrelli et al., 2009). Analysis was carried out on a drop of suitably diluted, freshly prepared liposomal dispersion using a Leica TCS SP II Laser Scanning Confocal Imaging System (Leica, Heidelberg, Germany); the apparatus was equipped with a Kr–Ar–He–Ne ion laser and a Leica DM IRE 2 microscope endowed with HC PL Fluotar Leica X10 and 20× dry objectives and HCX PLAN APO Leica 40× multi-immersion objective on its oil position (numeric aperture 0.85). Samples were analysed by using the transmitted light (López-Pinto et al., 2005). For excitation of the fluorescent label the 488 nm wavelength was used and the fluorescence emission was detected at 520 nm. Each experiment was performed in sextuple.

2.7. Gel preparation

Liposomal suspensions were formulated as Carbopol gel for performing *in vivo* studies in the rabbit model, since gel formulation allowed deposition of a more constant and reproducible amount of the drug with respect to liquid formulations.

A Carbopol gel base was prepared by suspending 0.5 g Carbopol 940 in 99.5 mL of bidistilled water, stirring for 24 h at room temperature and then adding triethanolamine up to pH 7.0 for gelification. The resulting gel was stored in capped glass containers, at 4 °C, in the dark. Gels loaded with the drug were prepared just before the use according to a previously established procedure (Mura et al., 2007). Briefly, Carbopol gel base was carefully mixed, using a spatula, with a 1% hydro-alcoholic solution or liposomal suspension (directly used as obtained) of each drug in a 50/50 (w/w) ratio, thus obtaining a final drug concentration in the gel of 0.5% (w/w). The maintenance of morphology and dimensions of vesicles in liposomal gel formulations was evaluated by optical microscopy (Microscope Hund medicus, AFL Bonn, Germany) with camera (Kodak "Easy Share DX4530" 5.0 mega pixels) under 1000× magnification.

2.8. In vivo studies

The anaesthetic activity of BZC and BTM formulated in Carbopol gel, as such or entrapped in the different liposomal formulations, was assayed *in vivo* in albino rabbits according to the conjunctival reflex test (Ghelardini et al., 2001). Male albino rabbits (2.5–3.0 kg) from Morini (San Polo d'Enza, Italy) were used. One rabbit was housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were kept at 23 ± 1 °C with a 12 h light/dark cycle, fed with a standard laboratory diet and tap water *ad libitum*. All experiments were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory animals. All efforts were made to minimize animal suffering and to limit the number of animals used. Animals were divided into as many groups (each formed by six rabbits) as the number of formulations to test. A fixed amount of each sample was instilled in the conjunctival sac of the left eye of the rabbit, whereas a corresponding blank formulation (without drug) was simultaneously instilled in the right eye as control. The external sides of rabbit eyes were then stimulated at interval times with a cat whisker to induce the conjunctival reflex and, consequently, the closure of the palpebrals. The local anaesthetic activity of the drug is evidenced by the necessity of a higher number of stimuli to provoke the reflex. The

significance of the differences between different formulations was tested using the 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 $P < 0.05$.

3. Results and discussion

3.1. Phase-solubility studies

The solubility of both drugs linearly increased with increasing the concentration of both CDs, giving in all cases A_L-type phase-solubility diagrams, indicative of the formation of soluble complexes of probable 1:1 mol:mol stoichiometry (Higuchi and Connors, 1965) (Fig. 1a and b). The apparent 1:1 stability constant values, calculated from the straight lines of the diagrams, were 1315 and 352 M^{-1}, respectively, for the complexes of BZC with βCD and HPβCD, suggesting that the presence of hydroxypropyl substituents can hamper the inclusion of BZC into the CD cavity, because of the partial covering of its opening. On the contrary, the apparent 1:1 stability constant for the complexes of BTM with βCD and HPβCD were 290 and 273 M^{-1}, respectively, indicating in this case a similar complexing power of both CDs. On the other hand, it should be pointed out that the solubilizing efficiency of the β-derivative, calculated as the ratio between drug solubility in 25 mM aqueous solution and in pure water, was for both drugs clearly greater (about 12 for BZC and 26 for BTM) than that of the parent compound (about 0.5 for BZC and 3.8 for BTM) at its highest aqueous concentration (12.5 mM).

3.2. Solid-state characterization of drug–CD binary systems

It has been widely proved that the method used for the preparation of drug–cyclodextrin solid inclusion complexes can significantly affect the physicochemical and dissolution properties of the obtained solid systems which have to be properly evaluated (Mura et al., 1999; Al-Marzouqi et al., 2007). Therefore, the different equimolar drug–CD solid systems, prepared by physical mixing, co-grinding and coevaporation, were carefully characterized by DSC, X-ray powder diffraction, FTIR and SEM analyses, and for dissolution behaviour.

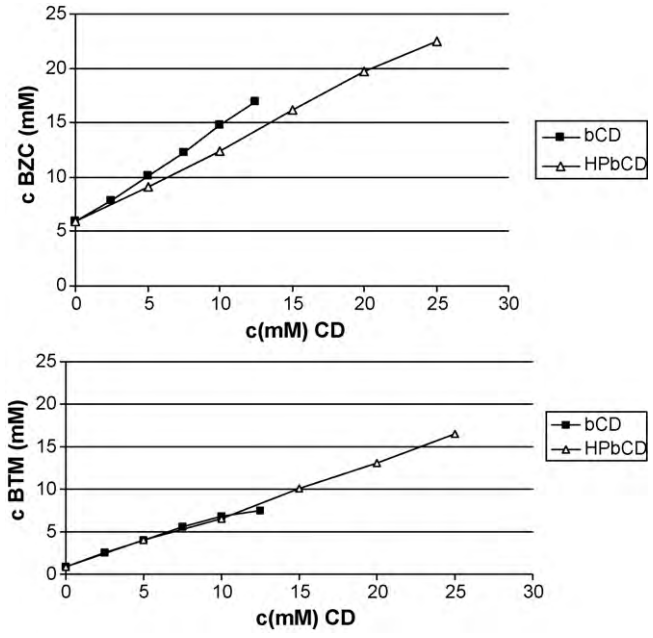

Fig. 1. Phase-solubility diagrams of benzocaine (BZC) and butamben (BTM) in the presence of increasing concentrations of βCD and HPβCD in pH 5 phosphate buffer solution at 25 °C.

The thermal curves of pure BZC and BTM, pure βCD and HPβCD and the corresponding drug–CD equimolar systems prepared with the different techniques are reported in Fig. 2a and b, respectively. The DSC curves of pure drugs were typical of anhydrous crystalline substances, exhibiting a flat thermal profile followed by a sharp melting endotherm at about 94° C (BZC) and 60 °C (BTM), respectively. On the contrary, both CDs exhibited in the examined range of temperature a broad endothermal effect attributed to the sample dehydration. The DSC curves of all equimolar drug–CD physical mixtures were practically the simple superimposition of the thermal profiles of the corresponding pure components, suggesting the absence of drug–CD interactions. The thermal curves of co-ground products of both drugs with βCD showed a marked reduction in intensity of the drug melting peak in comparison with the related physical mixture, as well as a concomitant shift at lower temperature. Both these effects are indicative of drug–carrier interactions and of loss of drug crystallinity. Finally, the complete disappearance of BZC and BTM melting peak was observed in their co-ground products with HPβCD, and in all binary systems obtained by coevaporation. This phenomenon, attributable to both inclusion complex formation and/or complete drug amorphization, was however undoubtedly an index of stronger drug–carrier interactions in these products.

The X-ray powder diffraction patterns of pure BZC and BTM and of their various binary products with βCD and HPβCD are presented in Fig. 3a and b, respectively. The diffraction patterns of BZC, BTM and βCD displayed several sharp peaks, indicative of their crystalline nature, while HPβCD exhibited the halo pattern typical of amorphous products. The crystallinity peaks of both drugs were still detectable in the respective physical mixtures with βCD and they emerged from the halo pattern of the βCD-derivative. Some drug crystallinity loss observed in physical mixtures with HPβCD can be attributed to the homogeneous blending with the amorphous carrier. On the contrary, a completely amorphous pattern was obtained for co-ground products of both drugs with both CDs, and for both coevaporated products with HPβCD, while some crystallinity peaks were observed in coevaporated products with βCD. These results were apparently in partial disagreement with DSC findings. Evidently, the traces of residual crystalline drug, evidenced by DSC analysis in co-ground products, were not detectable by X-ray analysis. On the contrary, the presence in drug–βCD coevaporated systems of some crystallinity peaks, some of which were at different 2θ values with respect to those of the corresponding physical mixture, could be explained by the formation of a new solid phase induced by complexation, which is prone to be brought to an amorphous state by the thermal energy supplied in a DSC scan. DSC curves and X-ray spectra of both pure drugs submitted to the same evaporation process (data not shown) did not present any change with respect to those of the corresponding untreated drugs, thus excluding the possible formation of a polymorphic form during the treatment and further confirming the presence in coevaporated products of a new solid phase, due to the drug–CD complex formation.

The FTIR spectra of all drug–CD physical mixtures did not differ from those of the corresponding drug alone, not even in the areas of their main absorption bands in the 1600–1800 cm^{-1} spectral zone (data not shown). Comparing the FTIR spectra of coevaporated systems and co-ground systems of both drugs with both CDs with those of the corresponding physical mixtures, no appreciably changes or shifts of these bands were detectable, a part from a marked reduction in intensity, indicative of drug–CD solid-state interactions and/or sample amorphization.

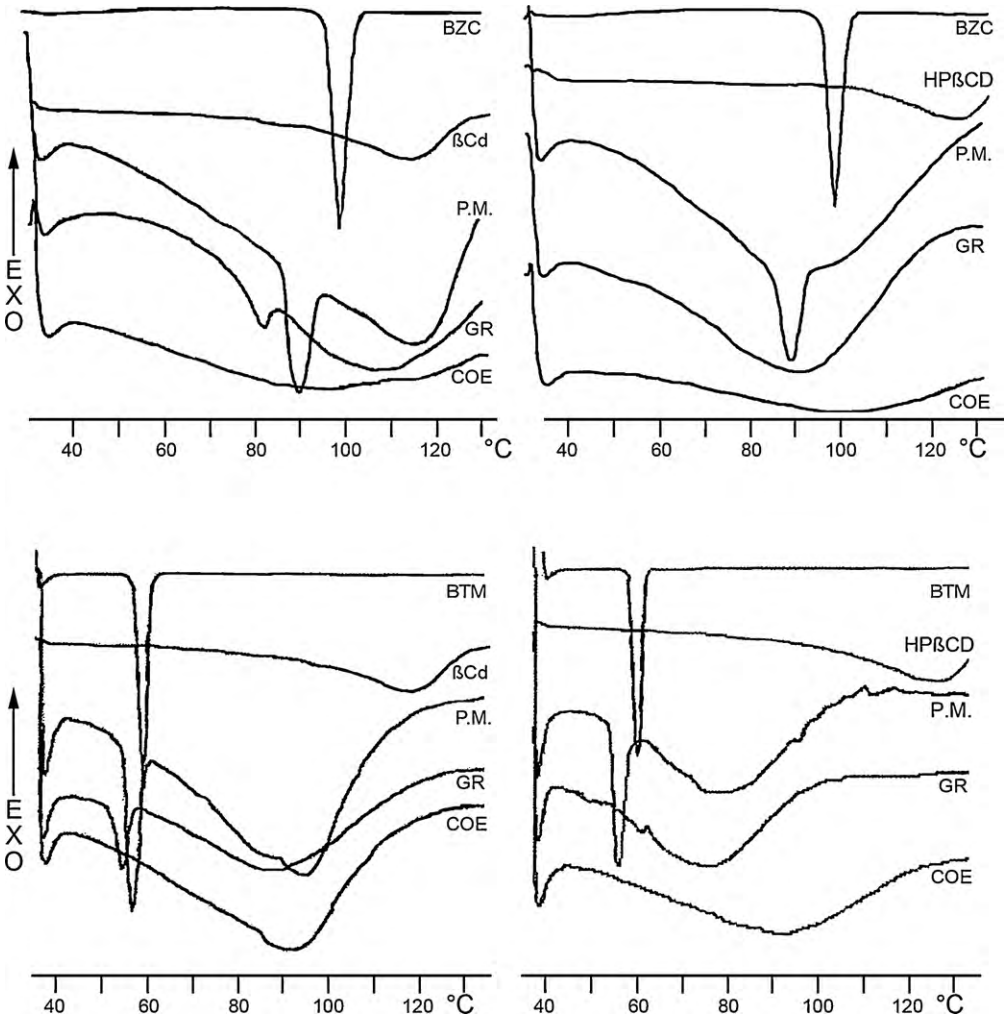

Fig. 2. DSC curves of pure benzocaine (BZC), butamben (BTM), βCD and HPβCD, and of equimolar drug–CD systems obtained by physical mixing (P.M.), co-grinding (GR) or coevaporation (COE).

SEM analysis showed that the preparation methods of drug–CD systems led to a drastic change in shape and aspect with respect to the original components and their simple blending. Selected micrographs are shown in Fig. 4. BTM particles appeared as oblong polyhedric crystals with smooth surfaces, while BZC particles looked as irregular lamellar crystals; on the other hand, βCD consisted of large crystals with a parallelogram shape, whereas HPβCD was seen as amorphous spherical or pieces of spherical particles. The characteristic drug crystals, mixed with CD particles or adhered to their surface, were clearly detectable in all physical mixtures (data not shown). On the contrary, the original morphology of both drugs and CDs disappeared in coevaporated and co-ground products, where only amorphous aggregates (co-ground products) or flakes of irregular size (coevaporated products) were observed and it was no longer possible to differentiate the initial components, as is shown for the series of products with HPβCD.

3.3. Dissolution studies of drug–CD binary systems

Dissolution profiles of both drugs, alone and from their different binary systems with the examined CDs, are presented in Fig. 5a and b for BZC and BTM series, respectively. As can be seen, all dissolution curves achieved a plateau after about 30 min in the case of BZC systems and even in a lower time in the case of BTM systems. The presence of CD gave rise in all cases to an improvement of drug dissolution properties. HPβCD was for both drugs a more effective partner than the natural CD in improving their dissolution properties. This is probably due to the higher wettability and hydrophilicity as well to the greater solubilizing efficiency towards BZC and BTM of this βCD derivative. As for the influence of the preparation technique, dissolution tests revealed that, for both drugs, coevaporation was clearly the most effective one in improving their dissolution behaviour, followed by co-ground products and finally by the simple physical mixtures. This behaviour is attributable to the greater effectiveness of the coevaporation technique than the co-grinding one in establishing stronger interactions between drug and host molecules and promoting drug amorphization and/or inclusion complex formation, as emerged from solid-state studies.

In conclusion, coevaporated products with HPβCD were the most effective systems. The achieved drug aqueous concentration at equilibrium from these systems were 0.5% (w/v) for BZC and 0.3% (w/v) for BTM, with a relative about 5-fold and 14-fold increase with respect to drugs alone. Therefore, coevaporated products with HPβCD were selected for entrapment in liposomes.

3.4. Development of liposomal formulations

The presence in the liposomal bilayer structure of a cationic surfactant such as stearylamine, can play an important role in modifying the *Z* potential, as well in increasing the bilayer deformability and in promoting interaction with the negatively charged epithe-

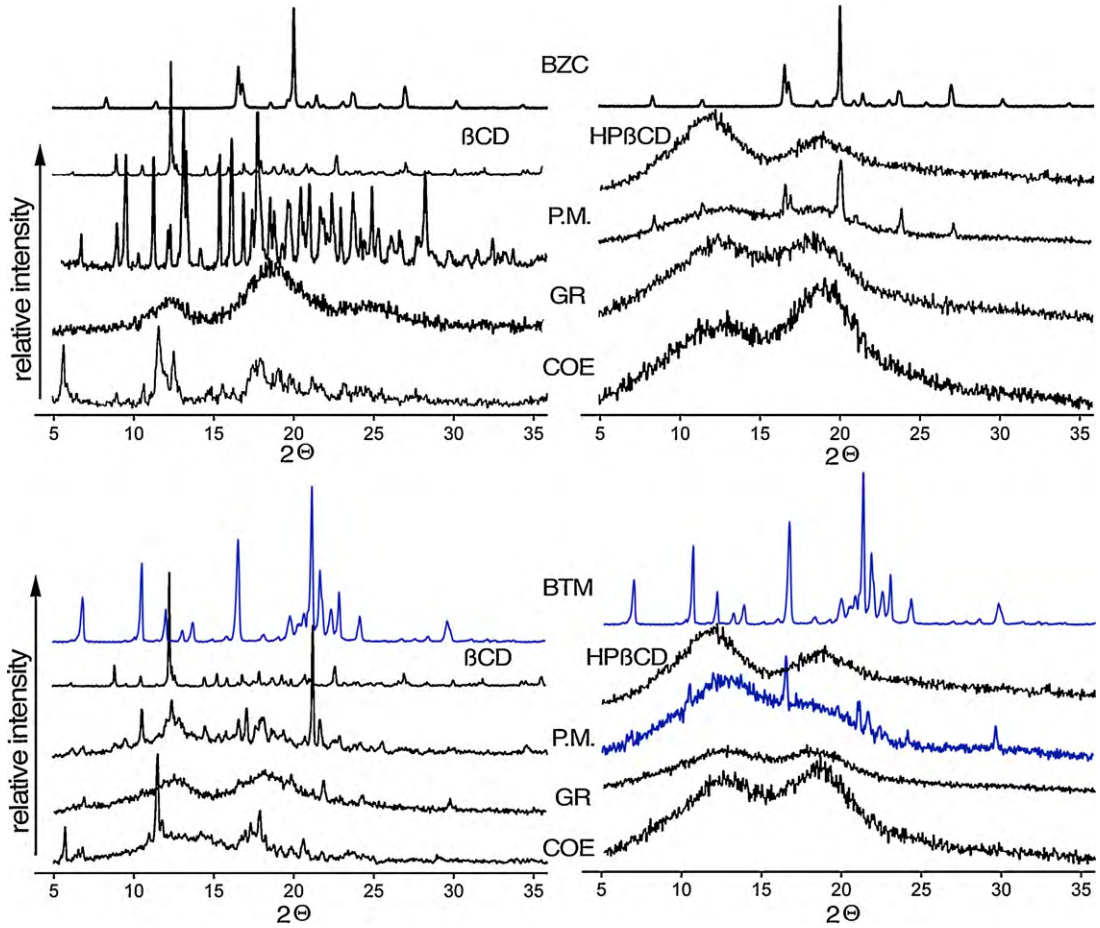

Fig. 3. X-ray powder diffraction spectra of pure benzocaine (BZC), butamben (BTM), β-CD and HPβCD, and of equimolar drug–CD systems obtained by physical mixing (P.M.), co-grinding (GR) or coevaporation (COE).

lial cells (Trotta et al., 2004; Bragagni et al., 2010), thus resulting in better drug permeability and prolonged pharmacological action (Piemi et al., 1999). Therefore, we evaluated the effect of adding in the liposomal formulation of different amounts of stearylamine.

With this aim, a series of MLV formulations were prepared, whose lipophilic phase consisted of a mixture of phosphatidylcholine (PC), cholesterol (CH), and stearylamine (SA) in different molar ratios (1:1:0; 6:1:1; 5.5:1:1.5). All formulations contained 1% of BZC as model drug dissolved in the hydrophilic phase, consisting of a 60:40 (v/v) water:ethanol mixture, to allow drug solubilization.

The mean particle size, polydispersity index, *Z* potential, encapsulation efficiency of these liposomal dispersions are summarised in Table 1. As expected the presence of stearylamine caused a change of the *Z* potential sign from negative (−43.84 mV), in the absence of the cationic additive, to positive (+33.27 mV). Moreover, a reduction of vesicles particle size was observed, whose mean diameter passed from 627 nm to about 390 nm for liposomes without and with stearylamine. This finding seems to be attributable to a rearrangement or the liposomal bilayer as a consequence of the insertion of stearylamine molecules. In fact, simple changes in cholesterol:phosphatidylcholine ratios generally did not give rise to significant variations in the liposome size (Mohammed et al., 2004).

Table 1
Effect of variation of the lipophilic phase composition of MLV vesicles, containing 1% benzocaine in the hydrophilic core, on encapsulation efficiency (*EE*%), particle size, polydispersity index (P.I.) and Zeta potential.

PC:CH:SA molar ratio	*EE*% ± s.d.	Particle size (nm ± s.d.)	PI	Z pot. (mV ± s.d.)
1:1:0	35.7 ± 1.5	627.0 ± 20	0.2	−43.84 ± 3.02
6:1:1	40.6 ± 1.7	392.8 ± 18	0.3	+30.38 ± 1.04
5.5:1:1.5	50.5 ± 1.6	390.2 ± 12	0.1	+33.27 ± 1.32

Liposomal suspensions were then dispersed (50:50, w/w) in an aqueous Carbopol gel for performing *in vivo* studies, so that to obtain a more reproducible deposition of a constant drug amount in the conjunctival sac of the eye's rabbit, and therefore to reduce the variability of the experimental conditions. Results of *in vivo* studies, summarised in Table 2, indicated a favourable effect of the presence of stearylamine in the liposomal bilayer in improving drug anaesthetic effect. In particular, the liposomal formulation with the PC/CH/SA 5.5/1/6.5 molar ratio showed the best performance, allowing a significant ($P < 0.05$) enhancement in intensity of the drug anaesthetic effect and also a significant extension of its duration. Such findings can be attributed to the higher penetration ability through the tissue of deformable liposomes. An additional role may be played by the use of a positively charged delivery system, which may contribute to assure a better interaction with the tissue cells, which are negatively charged, thus resulting in better drug contact and a prolonged pharmacological action (Piemi et al., 1999).

Therefore this formulation was chosen for preparing the series of differently drug-loaded liposomes.

3.5. Development of drug-loaded liposomal formulations

In order to investigate in depth the actual effectiveness and advantages of the proposed "double-loading" technique of lipo-

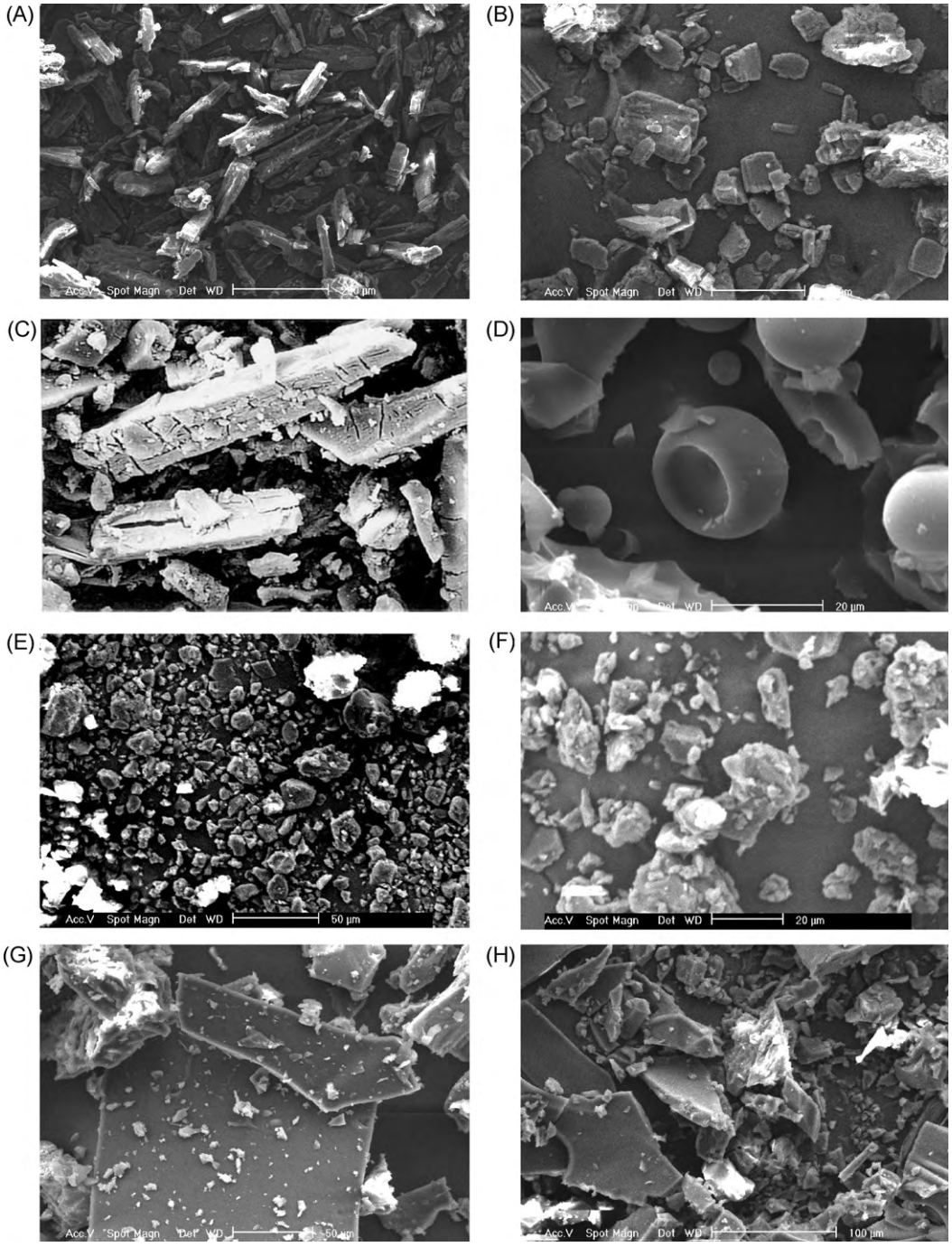

Fig. 4. SEM micrographs of pure butamben (BTM) (A), benzocaine (BZC) (B), βCD (C), HPβCD (D), and BTM-HPβCD and BZC-HPβCD co-ground (E and F) and coevaporated (G and H) products.

somes, in comparison with the traditional "single-loading" one, we developed three different kinds of liposomal formulations, all containing a total drug amount of 1% (w/v): (a) liposomes double-loaded with BZC or BTM as hydrophilic complex with HPβCD in the aqueous phase at their saturation solubility (respectively, 0.5%, w/v and 0.3%, w/v) and the remaining amount up to 1% (w/v) as free drug in the lipophilic one; (b) liposomes loaded with free BZC or BTM in the lipophilic phase; (c) liposomes loaded with free BZC in a 60:40 (v/v) water:ethanol mixture as aqueous phase. In the case of BTM, due to its lower hydrosolubility, it was not possible to prepare this third kind of formulation at the required drug concentration.

Loaded liposomes were in all cases successfully obtained and neither the double-loading technique, or the cyclodextrin presence did not influence the liposomal morphology, with respect to the traditional drug single-loading in the vesicle lipophilic phase, as shown by CSLM studies (Fig. 6). The obtained liposomal formulations were characterized and compared in terms of mean particle size, polydispersity index, *Z* potential and encapsulation efficiency (Table 3). No important differences in size were observed between the double-loaded liposomes with respect to the corresponding single-loaded ones with the plain drug in the lipophilic phase. A marked increase in size was instead observed for both these liposomes in comparison with those containing the drug

Table 2
Effect induced on the rabbit conjunctival reflex test by benzocaine liposomal formulations in a Carbopol gel. The lipid phase of liposomes consisted of a mixture of phosphatidylcholine (PC), cholesterol (CH), and stearylamine (SA) in different molar ratios.

Ocular treatment	Eye	Number of stimuli to induce conjunctival reflex time after treatment							
PC:CH:SA molar ratio		5 min	10 min	15 min	20 min	25 min	30 min	40 min	60 min
1:1:0	Left	32.6 ± 2.1^	30.1 ± 2.2^	28.2 ± 3.1^	26.3 ± 2.4^	20.1 ± 3.0^	14.2 ± 2.3^	6.6 ± 3.1^	2.2 ± 1.0
Control[a]	Right	1.0 ± 0.0	1.5 ± 0.3	1.2 ± 0.2	1.0 ± 0.0	1.2 ± 0.2	1.1 ± 0.2	1.4 ± 0.2	1.0 ± 0.0
6:1:1	Left	36.7 ± 2.0^,*	33.9 ± 2.1^,*	30.5 ± 2.2^	28.6 ± 2.3^	22.4 ± 3.1^	16.4 ± 2.0^	6.4 ± 1.2^	1.5 ± 0.0
Control[a]	Right	1.4 ± 0.2	1.0 ± 0.0	1.2 ± 0.3	1.1 ± 0.2	1.0 ± 0.0	1.2 ± 0.3	1.0 ± 0.0	1.1 ± 0.2
5.5:1:1.5	Left	1.2 ± 0.3	1.5 ± 0.3	1.3 ± 0.5	1.2 ± 0.2	1.4 ± 0.2	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Control[a]	Right	1.2 ± 0.3	1.5 ± 0.3	1.3 ± 0.5	1.2 ± 0.2	1.4 ± 0.2	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0

There were 6 rabbits per group. Each value represents the mean of six separate experiments.
[a] The control consisted in a Carbopol gel containing the corresponding liposomal formulation without drug.
^ $P < 0.01$ in comparison with control.
* $P < 0.05$ in comparison with the other formulations.

loaded in the internal core. This result can be attributed to the presence of the drug in the bilayer, which can give rise to a rearrangement of the liposomal structure, and, as a consequence, to an increase in the vesicle dimensions. The lower *EE*% values of double-loaded liposomes with respect to those containing all the drug in the lipid compartment could be ascribed to both the high affinity of the lipophilic free drug for the lipid phase of the vesicles, and to the smaller volume of the aqueous phase of MLV with respect to the lipidic one. In confirmation of this hypothesis, the highest *EE*% value was obtained for vesicles loaded with the most lipophilic drug, i.e. BTM, in the lipophilic phase; in the same way, the most marked *EE*% reduction was instead observed for BZC-double-loaded vesicles, which contained a higher amount of drug in the aqueous phase as CD complex (0.5% vs 0.3% in the case of BTM).

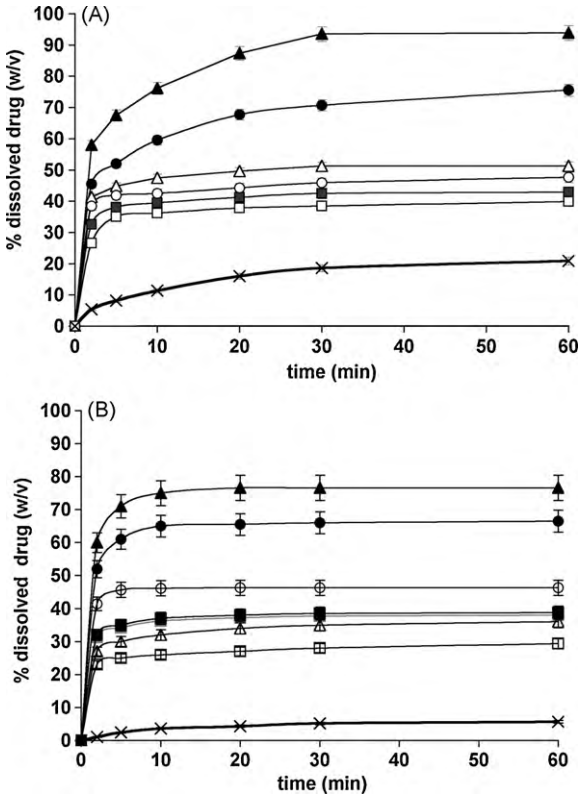

Fig. 5. Dissolution curves of benzocaine (A) and butamben (B), alone (×) and from equimolar physical mixtures (□, ■), co-ground (○, ●) and coevaporated (△, ▲) products with βCD (open symbols) and HPβCD (filled symbols).

As for the *Z* potential values, no important variations were observed among the differently drug-loaded vesicles. In fact, for the series of BZC-loaded vesicles, only a progressive slight reduction was observed when passing from those containing all the free drug in the aqueous phase (+33.27 ± 1.32) to the double-loaded ones (+31.18 ± 2.84) up to those loaded with all the free drug in the bilayer (+26.72 ± 2.26), indicating a good stability of all the formulations.

The different liposomal suspensions, dispersed (50:50, w/w) in an aqueous Carbopol gel, were then subjected to *in vivo* studies, in comparison with gel formulations containing the same drug amount added as simple hydroalcoholic solution. Optical microscopy analysis allowed to verify that mixing in the Carbopol gel did not modify morphology and dimensions of liposomal vesicles (data not shown). The results of *in vivo* studies (Table 4) demonstrated that all liposomal gel formulations allowed a significant improvement in terms of intensity and duration of action with respect to the gel formulation containing the simple drug solution. Moreover, the type of drug loading played an important role in determining the performance of the liposomal formulation. In fact, MLV loaded with the drug in the internal aqueous core rather in the external lipid phase showed a significant increase ($P < 0.05$) in the duration of drug action, in virtue of the better control of drug release. However, the best results for both BZC and BTM were obtained with the double-loading technique with free and complexed drug, which not only assured a fast release, due to the presence of free drug in the external bilayer, but also a prolonged effect, due to the presence of the complexed drug in the internal core. Thus, a further significant improvement ($P < 0.05$) of both intensity and duration of the drug therapeutic effect with respect to MLV containing only the plain drug was observed. An enhancer effect of cyclodextrin on epithelial cells, able to improve *in vivo* permeation and anaesthetic effect, together with the controlled release due to the drug complexation, can be also hypothesized to explain this result (Matsuda and Arima, 1999; Schoch et al., 2007).

Table 3
Effect of benzocaine (BZC) or butamben (BTM) loading technique on encapsulation efficiency (*EE*%), particle size, polydispersity index (P.I.) and Zeta potential of liposomes.

sample	*EE*% ± s.d.	Particle size (nm ± s.d.)	IP	*Z* pot. (mV ± s.d.)
BZC in lipidic phase	83.9 ± 3.4	720.2 ± 0.2	0.2	+26.72 ± 2.26
BZC in aqueous phase	50.5 ± 1.6	390.2 ± 12	0.1	+33.27 ± 1.32
BZC double-loaded	59.16 ± 2.8	661.2 ± 0.3	0.3	+31.18 ± 2.84
BTM in lipidic phase	94.40 ± 3.6	703.6 ± 0.2	0.2	+22.47 ± 4.14
BTM double-loaded	82.23 ± 3.0	680.8 ± 0.2	0.2	+27.33 ± 2.53

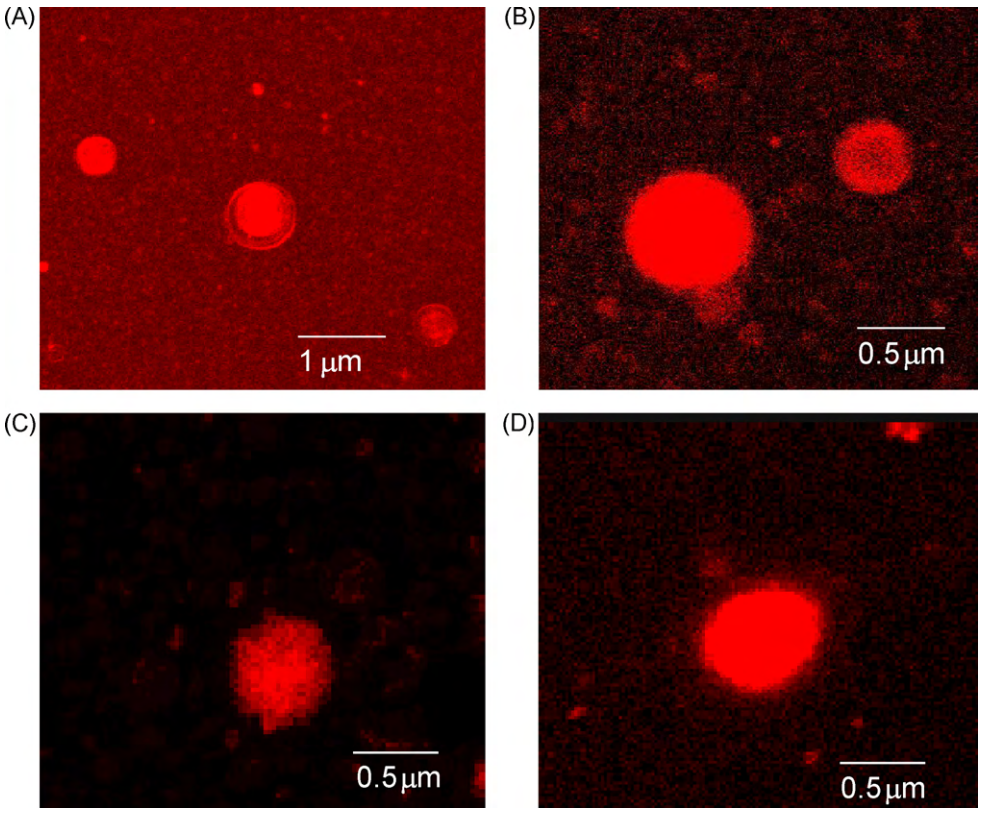

Fig. 6. Confocal laser scanner micrographs of liposomes single-loaded in the external bilayer (A and B) or double-loaded (C and D) with benzocaine or butamben, respectively.

Table 4
Effect induced on the rabbit conjunctival reflex test by different Carbopol gel formulations containing benzocaine (BZC) and butamben (BTM) as hydroalcoholic solution or MLV liposomal dispersion.

Sample	Eye	Number of stimuli to induce conjunctival reflex time after treatment							
		5 min	10 min	15 min	20 min	25 min	30 min	40 min	60 min
BZC in solution	Left	28.6 ± 2.1	21.4 ± 3.3	28.2 ± 3.1	17.8 ± 2.5	15.1 ± 3.0	10.1 ± 1.6	2.3 ± 0.9	1.2 ± 1.0
Control[a]	Right	1.0 ± 0.0	1.3 ± 0.3	1.1 ± 0.2	1.2 ± 0.0	1.0 ± 0.2	1.1 ± 0.2	1.3 ± 0.2	1.0 ± 0.0
BZC in MLV lipid phase	Left	37.2 ± 3.0[^]	38.4 ± 2.1[^]	34.1 ± 2.5[^]	24.6 ± 3.8[^]	22.2 ± 2.2[^]	14.4 ± 2.0[^]	5.4 ± 1.2[^]	1.0 ± 0.2
Control[a]	Right	1.3 ± 0.2	1.0 ± 0.0	1.0 ± 0.3	1.1 ± 0.2	1.2 ± 0.0	1.1 ± 0.3	1.0 ± 0.0	1.0 ± 0.2
BZC in MLV aqueous phase	Left	38.8 ± 2.1[^]	36.4 ± 2.6[^]	34.6 ± 2.3[^]	32.9 ± 2.1[^,*]	27.7 ± 2.0[^,*]	23.6 ± 2.5[^,*]	18.9 ± 2.9[^,*]	11.5 ± 2.8[^,*]
Control[a]	Right	1.2 ± 0.3	1.5 ± 0.3	1.3 ± 0.5	1.2 ± 0.2	1.4 ± 0.2	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
BZC in MLV double-loaded	Left	49.2 ± 3.1[^,*]	47.5 ± 3.7[^,*]	41.8 ± 2.3[^,*]	37.9 ± 2.1[^,*]	33.7 ± 2.0[^,*]	28.6 ± 2.5[^,*]	20.9 ± 2.9[^,*]	14.5 ± 2.8[^,*]
Control[a]	Right	1.1 ± 0.3	1.3 ± 0.3	1.4 ± 0.5	1.1 ± 0.2	1.2 ± 0.2	1.0 ± 0.0	1.1 ± 0.0	1.1 ± 0.0
BTM in solution	Left	27.4 ± 3.4	19.7 ± 2.4	14.2 ± 2.8	10.7 ± 3.5	5.8 ± 2.2	2.3 ± 0.3	1.2 ± 0.2	1.1 ± 0.9
Control[a]	Right	1.3 ± 0.3	1.2 ± 0.3	1.4 ± 0.5	1.0 ± 0.2	1.3 ± 0.2	1.1 ± 0.0	1.0 ± 0.0	1.3 ± 0.0
BTM in MLV lipid phase	Left	40.6 ± 2.5[^]	35.8 ± 4.0[^]	31.5 ± 3.5[^]	25.3 ± 3.0[^]	21.3 ± 3.1[^]	17.6 ± 2.5[^]	8.3 ± 2.6[^]	1.0 ± 0.5
Control[a]	Right	1.1 ± 0.3	1.3 ± 0.3	1.4 ± 0.5	1.1 ± 0.2	1.2 ± 0.2	1.0 ± 0.0	1.1 ± 0.0	1.1 ± 0.0
BTM in MLV double-loaded	Left	49.3 ± 3.1[^,*]	46.1 ± 2.9[^,*]	40.2 ± 3.5[^,*]	33.6 ± 2.7[^,*]	29.3 ± 2.0[^,*]	24.6 ± 2.5[^,*]	19.5 ± 2.3[^,*]	10.5 ± 2.3[^,*]
Control[a]	Right	1.0 ± 0.3	1.2 ± 0.3	1.4 ± 0.5	1.3 ± 0.2	1.0 ± 0.2	1.1 ± 0.0	1.2 ± 0.0	1.3 ± 0.0

There were 6 rabbits per group. Each value represents the mean of six separate experiments.
[a] The control consisted in a Carbopol gel containing the corresponding formulation without drug.
[^] $P < 0.01$ in comparison with drug solution.
[*] $P < 0.05$ in comparison with the other MLV formulations.

4. Conclusion

The study demonstrated the critical importance of the vesicle composition and of the drug loading method in determining the therapeutic efficacy of the developed liposomal formulation.

In particular, as for the vesicle composition, investigation on the influence of stearylamine addition to the liposomal lipid phase evidenced its important role in improving the deformability and skin penetration ability of liposomes. In fact, vesicles containing the cationic surfactant allowed a significant ($P < 0.05$) improvement of the drug anaesthetic effect in terms of intensity and duration of action with respect to the corresponding formulation containing only cholesterol and phosphatidylcholine

As for the loading method, significant differences were observed when the plain drug was single-loaded in the aqueous or lipophilic phase of the vesicles, or double-loaded as CD complex in the aqueous phase and as plain drug in the lipophilic one. The double-loading technique, which exploits the favourable effects of drug–CD

complexation, was the most effective preparation method, giving rise to a significant enhancement of both strength and length of the drug therapeutic effect with respect to both the single-loaded formulations.

Finally, the analogous results observed with both BZC and BTM, in spite of their different lipophilicity, indicated the essential influence of the liposomal carrier function and cyclodextrin complexation on the formulation performance, irrespective of the drug physicochemical properties.

Acknowledgements

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