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Research paper

Development, characterization and *in vivo* evaluation of benzocaine-loaded liposomes

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Abstract

This study reports the development and *in vivo* evaluation of a liposomal formulation of the local anaesthetic benzocaine. Multilamellar (MLV) and small uni-lamellar (SUV) vesicles entrapping benzocaine were prepared using 50:50 w/w phosphatidylcholine–cholesterol as lipophilic phase and 50:50 v/v ethanol–water as hydrophilic phase. Liposome size, Zeta-potential, encapsulation efficiency and skin penetration properties were determined. Drug permeation from liposomal dispersions, as such or formulated in Carbopol gel, was evaluated through artificial lipophilic membranes and excised abdominal rat skin, whereas *in vivo* anaesthetic effect was tested on rabbits. Interestingly, addition of the drug into the hydrophilic phase, rather than into the lipophilic one, during liposome preparation enabled an improvement of the MLV's entrapment efficiency from 29.7% to 82.3%. On the other hand, sonication conditions to obtain SUV influenced size and polydispersity index of the vesicles and reduced the entrapment efficiency by about 30%. All liposomal-benzocaine formulations showed sustained release properties and a more intense anaesthetic effect than plain drug. Permeation experiments from drug solutions in gel containing the same amount of ethanol as in the liposomal formulations made it possible to exclude a possible enhancer effect of this solvent, at least when not used in liposomal formulations. MLV with the drug added into the hydrophilic phase gave the most effective formulation, showing a permeability coefficient value 2.5 times higher than that of the plain drug and allowing a significant improvement ($P < 0.01$) not only of intensity but also of duration of anaesthetic effect of benzocaine. These results suggest that a suitably developed liposomal formulation of benzocaine can be of actual value for improving its clinical effectiveness in topical anaesthesia.

Keywords: Benzocaine; Liposome; Skin permeation; *In vivo* anaesthetic effect

1. Introduction

Local anaesthetics are a class of drugs able to induce pain relief by virtue of their ability to bind to the sodium channel of excitable membranes, thus blocking the influx of sodium ions and the propagation of the nervous impulse [1]. Among these, benzocaine is an ester-type local anaesthetic mainly used in topical, dermal and mucosal formulations. Its anaesthetic action is characterized by a rapid but short effect, compared with the potential duration of pain. Moreover, toxic effects of ester-type local anaesthetics, due to their systemic absorption, have been reported in the literature [1,2]. Therefore, the development of a new effective topical drug delivery system intended to suitably modulate the benzocaine release rate, thus prolonging its anaesthetic effect, and to enhance its localization in the skin, thus reducing its systemic toxicity, could be particularly useful.

Liposomes are colloidal phospholipidic vesicles extensively investigated as safe and effective drug carrier systems. Among their several possible pharmaceutical

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applications, they have been widely applied in topical drug delivery [3–6]. In particular, an increase in clinical efficacy has been demonstrated for liposomal formulations of some local anaesthetics such as tetracaine and lidocaine with respect to administration of the plain drugs [7–13].

A study was thus undertaken to develop a new liposomal formulation of benzocaine, intended to improve its clinical effectiveness in topical anaesthesia in terms of both enhanced intensity and prolonged duration of action. With this aim, in the present work we investigated in depth the role of the structure (uni- or multi-lamellar) of the vesicle membrane as well as the effect of drug encapsulation into the lipophilic or the hydrophilic liposomal phase on the properties of the final product in order to find the most suitable operative conditions for improving both liposome encapsulation efficiency, and benzocaine permeation properties and *in vivo* activity. On the basis of preliminary experiments, a 50:50 w/w phosphatidylcholine–cholesterol mixture was selected as lipid phase, due to the well-recognised beneficial role of cholesterol on the stability of the vesicles. In fact, cholesterol molecules insert into the liposomal bilayers with the hydroxyl group oriented towards the aqueous phase and the aliphatic chain parallel to the acyl chains of phosphatidylcholine, thus increasing phospholipid packing densities and, at the same time, reducing possible electrostatic and hydrogen bonding interactions between choline head groups [14,15]. On the other hand, considering the lipophilic nature of the drug ($\log P$ 1.9 [16]), a 50:50 v/v water–ethanol mixture was used as hydrophilic phase, in order to obtain adequate drug solubility in the medium (14 g/L). A series of benzocaine-entrapped liposomal systems were then prepared by keeping the composition of both lipid and hydrophilic phases constant and varying the drug concentration and the experimental preparation conditions. The formulations were characterized for encapsulation efficiency, particle size, Zeta-potential, morphology, and skin-uptake properties by using, respectively, dialysis, light scattering and Confocal Laser Scanning Microscopy techniques. Permeation properties of the drug from these systems were evaluated using both artificial lipophilic membranes simulating the skin behaviour [17] and excised abdominal rat skin, whereas *in vivo* anaesthetic effect and duration of activity were tested on rabbits.

2. Materials and methods

2.1. Materials

Benzocaine base (BZC) (ethyl-4-aminobenzoate) ($pK_a = 2.8$, $\log P = 1.9$ [16]) was from Fluka–Sigma–Aldrich (Italy). Cholesterol (CH), l-α-phosphatidylcholine (PC), and rhodamine 6G were provided by Sigma–Aldrich (Italy). Carbopol® 940 (polyacrylic acid) was kindly supplied by Noveon, Inc. (Cleveland, OH, USA). All other reagents were of analytical grade.

2.2. Methods

2.2.1. Liposome preparation

Liposomes consisting of mixtures of 50% w/w PC and 50% w/w CH were prepared by thin layer evaporation (obtaining multi-lamellar vesicles (MLV)) or by sonication of MLV (obtaining small uni-lamellar vesicles (SUV)).

According to the thin layer evaporation (TLE) technique [18], the lipid phase, consisting of a mixture of 50 mg PC and 50 mg CH, was dissolved in chloroform which was then removed under reduced pressure in a rotary evaporator at 58 °C, thus obtaining a thin film of dry lipid on the flask wall. Evaporation was continued for 1 h after the dry residue appeared, to completely remove all the traces of the solvent. The film was then hydrated by adding 10 mL of a water–ethanol (50:50 v/v) mixture under vigorous mechanical shaking with a vortex mixer until vesicle formation.

According to the sonication method, MLV suspensions (obtained as described above) were submitted to ultrasonication (Eurosonic 44 ultrasonic bath, Wilten Woltil, de Meern, The Netherlands) under different conditions of temperature (25 or 58 °C) and energy (335–120 W) and different input times (1–5 min) and number of cycle iterations (from 2 to 10), with an idle time between cycles of 1 min.

BZC was dissolved at different concentrations (0.1–0.5–1.0% w/v) in the lipophilic (L) or hydrophilic (H) phase during liposome preparation. All products were sealed in glass containers and stored in darkness at 4 °C.

2.2.2. Determination of liposome encapsulation efficiency

Liposome encapsulation efficiency was determined using the dialysis technique for separating the non-entrapped drug from liposomes [17,19,20]. The suitability of this method (which gave results comparable to those obtained by the ultracentrifugation technique) has been previously demonstrated [21]. Preliminary experiments, performed by both ultracentrifugation and dialysis techniques, showed also in the present case similar results in terms of encapsulation efficiency values, thus further confirming the reliability of the dialysis technique. Then, according to this method, 3 mL of drug-loaded liposomal dispersion was dropped into a cellulose acetate dialysis bag (Spectra/Por®, MW cut-off 12,000, Spectrum, Canada) immersed in 150 mL of a 50:50 v/v water–ethanol solution and magnetically stirred at 30 rpm. Samples, taken at time intervals from the receiver solution, were replaced with equal volumes of fresh solvent. BZC was spectrometrically assayed at 282 nm (UV-1601 Shimadzu). The experiment was stopped when constant drug concentration values 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 then calculated according to the following equation:

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

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

2.2.3. Determination of liposomal particle size and Zeta-potential

The average diameter of the vesicles and their Zeta-potential were determined using a Zetamaster apparatus (Malvern Instruments, Malvern, UK) at a temperature of 25 ± 0.1 °C. Samples were analyzed 24 h after their preparation.

For the particle size measurements, the liposome suspensions were suitably diluted with distilled water in order to avoid multiscattering phenomena. Microscopic observations allowed exclusion of drug crystallization phenomena as a consequence of water dilution of the samples. The intensity of the laser light scattered by the samples was detected at an angle of 90° with a photomultiplier. For each kind of liposomal suspension, five independent samples were taken, each of which was measured at least twice, up to four times. For each specimen, 10 autocorrelation functions were analysed using a cumulative analysis. From this analysis, the z-average value was obtained, which is an approximation of the diameter of the liposomes. The polydispersity index was used as a measurement of the width of the size distribution.

For liposome surface charge determinations, about 2 mL of each liposomal suspension, suitably diluted with distilled water, was dropped into the Zetamaster electrophoretic cell and the Zeta-potential was determined by Electrophoretic Mobility (μ) measurements. The mobility μ was converted into a Zeta-potential by the Smoluchowski equation $Z = \mu\eta/\varepsilon$, where η is the viscosity and ε is the permittivity of the solution.

2.2.4. Gel preparation

A 0.5% w/v 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 by mixing (50:50 w/w) Carbopol gel with BZC aqueous or water–ethanol (50:50 v/v) solution or liposomal suspension obtaining the final drug concentrations of 0.05–0.25–0.5% w/w.

2.2.5. In vitro permeation studies through artificial membrane

Permeation studies of BZC from the different formulations through artificial membranes were performed for 24 h at 37 ± 1 °C with Franz diffusion cells (Vidrafoc, Barcelona, Spain), in a six-unit assembly (effective permeation area 2.54 cm^2). A cellulose nitrate membrane impregnated with lauryl alcohol (membrane weight increase 90–110%) as lipid phase was used as artificial lipophilic membrane simulating the epidermal barrier [22]. The receiver compartment (14.5 mL) consisted of a degassed pH 7.4 phosphate buffer solution. Permeation experiments were performed in non-occlusive mode. The donor compartment was filled with 0.15 g of the liposome suspension or 0.3 g of the corresponding Carbopol gel formulations. The solubility of the drug in the receiver medium at 37 °C was 1.6 mg/mL, thus enabling 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. At predetermined intervals, 0.55 mL samples were withdrawn from the receptor compartment and spectrometrically assayed for drug content at 285 nm.No interference was found for other components. The samples were replaced with equal volumes of fresh receptor medium and the correction for the cumulative dilution was calculated. Experiments were performed in sextuplicate. The cumulative amount of drug transferred into the receptor side was calculated and the results were averaged (C.V. $< 1.5\%$).

2.2.6. In vitro permeation studies through rat skin

Franz diffusion cells were used in the permeation studies of BZC through rat skin. The studies were carried out for 24 h at 37 ± 1 °C as described above, on the same formulations. Rat skin was obtained from the abdominal skin of Wistar rats stemming from the Laboratory Animal Service of University of Seville (aged 17–22 weeks, weight 250–300 g). 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. 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 of 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 horny layer facing the donor compartment and the dermal side toward the receptor fluid, which was stirred with a magnetic bar at 50 rpm. The receiver compartment was sampled at appropriate interval times and the drug concentration was determined by a HPLC technique (Hitachi D-7000 HPLC Multi System Managersystem), using a LiChrospher RP-C18 column, a mobile phase of pH 2.5 phosphate buffer:acetonitrile (60:40 v/v) at a flux rate of 1 mL/min. The UV detection wavelength was 285 nm. The HPLC technique was used to avoid possible interferences due to release of some skin components. The samples were replaced with equal volumes of fresh receptor medium and the correction for the cumulative dilution was calculated. Experiments were performed in sextuplicate. The cumulative amount of drug transferred into the receptor side was calculated and the results were averaged (C.V. $< 5.5\%$).

2.2.7. Data analysis and statistics of permeation experiments

In vitro steady-state drug fluxes (mg/h cm^2) were calculated by least square linear regression analysis from the linear portion of the cumulative amount of drug diffused versus time plots. The permeability coefficients (K_p, cm/h) of the drug were determined by dividing the drug fluxes by the initial concentration of the drug in the donor phase. Results are expressed as means ± standard deviation ($n = 6$ independent samples). 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 $P < 0.01$.

2.2.8. Confocal laser scanning microscopy (CLSM)

CLSM studies were performed in order to investigate the skin penetration ability of liposomes through the skin layers. Towards this aim, some liposomal dispersions were prepared by adding a hydrophobic fluorescent probe, i.e. rhodamine 6G (10^{-5} M), in the lipid or in the water–ethanol phase, according to the two different techniques used to entrap BZC into the vesicles. Blank preparations containing the plain marker at the same concentration as in the liposomal dispersions were also evaluated for comparison purpose. Rhodamine 6G was selected as an equivalent marker for BZC due to their comparable lipophilic properties, as indicated by their respective oil/water partition coefficients (log *P* 2.0 for rhodamine 6G [23] and 1.9 for BZC [16]). Therefore, a similar partitioning behaviour from the lipid layers of the liposomal vesicles into the skin can be reasonably expected. After a 24-h incubation period at 37 °C in the Franz diffusion cell in the presence of these preparations (as such, or dispersed in the Carbopol gel), rat skin was rinsed with pH 7.4 phosphate buffer solution, rapidly frozen by liquid nitrogen and then stoked at −4 °C; 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 [24]. Analysis was carried out using a Leica TCS SP II Laser scanning Confocal Imaging System (Leica, Heidelberg, Germany) equipped with a Kr–Ar–He–Ne ion 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. Each experiment was performed in sextuplicate.

2.2.9. In vivo studies

The anaesthetic activity of BZC formulated in aqueous Carbopol gel, as such or entrapped in liposomes, was assayed *in vivo* in albino rabbits according to the conjunctival reflex test [25]. Male albino rabbits (2.5–3.0 kg body weight) 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 Guide 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 four to six rabbits) as the number of formulations to test. A fixed amount of each sample was instilled in the conjunctival sac of the right eye of the rabbit, whereas a corresponding blank formulation (without drug) was simultaneously instilled in the left eye as control. The external sides of rabbit eyes were then stimulated at interval times with a cat whisker, and the number of stimuli to induce the conjunctival reflex and, consequently, the palpebral closure was determined. For each tested formulation, separate experiments were performed within each group of rabbits and the results were averaged. The local anaesthetic activity of the drug is evidenced by the necessity of a higher number of stimuli to provoke the reflex.

3. Results and discussion

3.1. Preparation and characterization of MLV liposomes

The physicochemical properties of the drug, especially solubility and partition coefficient, can be important determinants for the extent of its liposomal incorporation. Therefore, considering the lipophilic nature of the drug (log *P* 1.9) and its high solubility in chloroform [16], a first series of liposomal formulations was prepared by dissolving BZC in this organic solvent, together with CH and PC, and the influence of varying the drug concentration (0.1%, 0.5% and 1.0% w/v) was evaluated. The obtained liposomal dispersions were characterized for encapsulation efficiency (EE%), particle size, polydispersity index and Zeta-potential (Table 1). The results indicate that, as

Table 1
Effect of benzocaine (BZC) concentration, added in the lipophilic solution, on the entrapped drug amount, encapsulation efficiency (EE%), particle size, polydispersity index (P.I.) and Zeta-potential (ζ) of MLV

c(BZC) (%w/w)	Entrapped BZC (mg/mL)	EE%	Particle size (nm ± SD)	P.I.	ζ (mV)
0.1	0.11 ± 0.01	29.3 ± 1.9	372 ± 4	0.029	−44.9
0.5	0.40 ± 0.02	26.5 ± 1.5	395 ± 10	0.052	−42.3
1.0	0.90 ± 0.05	29.7 ± 1.8	560 ± 28	0.180	−43.1

expected, the total amount of drug entrapped in the vesicles progressively increased with increasing BZC concentration initially dissolved in the lipophilic phase during vesicle preparation. However, the EE% was scarcely influenced, and did not in any case exceed 30%. Furthermore, light scattering analysis showed an increase in vesicle size and a reduction in homogeneity with increasing the amount of entrapped drug, as indicated by the progressive increase of the polydispersity index. These findings could be explained by considering that, when the drug is dissolved together with the PC–CH mixture, it will be located within the liposomal bilayer, where the acyl chains of phospholipids provide a favourable environment for the lipophilic BZC molecules. However, the intercalating incorporation of the drug into the bilayer could alter the microstructure of the vesicular membrane, thus giving rise to a re-arrangement of the membrane structure and a decrease in ordering and stability of the system, and hindering an effective drug entrapment. This hypothesis is supported by the reduction of the liposomal membrane organization order, as has been reported for other local anaesthetics [26,27].

Then, considering that other factors such as the liposome preparation method have also been shown to contribute to determining drug liposomal incorporation [28], the influence of dissolving BZC (at the highest investigated concentration, i.e. 1.0% w/v) in the hydrophilic phase, rather than in the lipophilic one, was investigated. Interestingly, this variation in the experimental conditions allowed a noticeable improvement in the amount in entrapped drug. In fact, it was almost threefold when the same drug amount was dissolved in the lipophilic phase, passing from 0.90 to 2.5 mg per mL of liposomal dispersion, with a corresponding EE% value of 82.3% compared to 29.7%. Both the good solubility of BZC in the selected hydro-alcoholic hydration phase (14 g/L) and the higher stability of the membrane bilayer surrounding the aqueous core could be considered responsible for this unexpected result. Moreover, the vesicles were characterized by a higher homogeneity, as demonstrated by the polydispersity index that dropped from 0.18 to 0.09.

On the other hand, measurements of Zeta-potential values indicated that it was scarcely influenced by both the presence and concentration of drug, and the modality of its addition during the MLV preparation. Therefore, a contribution of the drug to the liposomal charge can be excluded. In this regard, BZC was almost completely in its undissociated form in the liposomal dispersion (pH about 6.5). Therefore, the negative charge of the vesicles, which in all cases was in the range between −39.5 and −45 mV, could be attributable to the ionisation of the phosphate groups of PC molecules. Similar Zeta-potential values have been reported for 50:50 mol/mol PC:CH liposomes containing or not Amphotericin B [29].

3.2. Preparation and characterization of SUV liposomes

SUV were realized by sonication of MLV prepared according to the experimental conditions selected as the most effective i.e. by adding BZC at 1% w/v in the hydrophilic phase. Since the modalities of performing the sonication process can influence the vesicle particle size and homogeneity [30], a series of studies were performed by suitably varying temperature, energy, input time, and number of cycle iterations, in order to select the best conditions to obtain the smallest vesicles with the narrowest particle size distribution.

The results are summarized in Table 2. As can be seen, sonication at room temperature (25 °C) gave rise to larger and highly heterogeneous vesicles. In accordance with Muller et al. [30], higher temperature was more effective in particle size reduction. As for the other examined parameters, no significant variations were observed by varying the sonication energy from 120 to 355 W, whereas a reduction of both particle size and polydispersity index was obtained by reducing the input time and increasing the number of cycle iterations.

The selected parameters (58 °C, 355 W energy, input time 60 s, idle time 60 s, with a cycle number of 10 reiterations) allowed the obtainment of SUV with small particle size (251 ± 10 nm) and the lowest polydispersity index (0.15). However in all cases the sonication process gave rise to a reduction, with respect to the original MLV, of EE% values. In fact, independent of the sonication process conditions, they were in all cases ranged between 28% and 30%. The reduced volume of the aqueous compartment, due to the smaller dimensions of SUV, in comparison with the MLV, can be considered the main factor responsible for this result [31]. On the contrary, as expected, no important variations of Zeta-potential were observed and in all cases it ranged between −41 and −43 mV.

Table 2
Effect of sonication conditions on the encapsulation efficiency (EE%), particle size, and polydispersity index (P.I.) of the SUV

Energy (W)	Input time (s)	Idle time (s)	Temperature (°C)	*n* Cycles	EE%	Particle size (nm ± SD)	P.I.
355	60	60	58	10	28.8 ± 1.6	251 ± 10	0.15
355	300	60	58	2	29.3 ± 1.8	334 ± 9	0.20
355	60	60	25	10	30.0 ± 1.9	682 ± 154	0.49
355	300	60	25	2	29.7 ± 1.9	607 ± 157	0.68
120	120	60	58	10	29.0 ± 1.7	302 ± 3	0.16
120	60	60	58	10	28.0 ± 1.5	256 ± 99	0.19

3.3. Permeation studies through artificial membranes

The results of permeation studies across artificial membranes of BZC from the different MLV and SUV liposomal dispersions as such or from the corresponding Carbopol gel formulations are summarized in Table 3 in terms of drug permeability coefficient (K_p), while the corresponding drug permeation profiles are shown in Fig. 1(a and b) together with the release curve obtained from the simple drug solution in the gel. It can be observed that all liposomal formulations (Fig. 1a) showed an initial fast release phase followed, after 6–8 h, by a plateau phase. MLV liposomes containing the drug in the lipophilic phase (MLV-L) showed the lowest drug release values ($P < 0.001$). On the contrary, the highest values were obtained with the MLV containing the drug in the hydrophilic phase (MLV-H), and they were significantly better ($P < 0.01$) than those given by the SUV. The not thermodynamically favourable partitioning of BZC from the lipid bilayer into the phosphate buffer solution can be considered in all cases as the principal factor controlling the drug release rate from the vesicles. The lesser effectiveness of MLV-L can be mainly attributed to their lower EE% (29.7%) with respect to the MLV-H ones (82.3%). On the other hand, the vesicle structure (multi- or unilamellar) and dimensions can play a role in the performance of the final formulation. The satisfying behaviour of SUV could be explained by their reduced dimensions and the uni-lamellar structure, which allow faster drug diffusion [32], thus in part counterbalancing their low EE% (28.8%). An analogous behaviour was observed for the corresponding formulations in gel (Fig. 1b), with the difference that the presence of the polymeric network gave rise, as expected, to a general reduction of the drug permeation rate ($P < 0.001$) and allowed obtainment of a more regular release profile as a function of time, with respect to the simple liposomal dispersions. Interestingly, the gel formulation containing the drug solution showed a faster drug release ($P < 0.01$) in comparison to the gel containing MLV with the drug in the lipophilic phase, while it was slower ($P < 0.001$) than from gels incorporating SUV or MLV with the drug in the aqueous phase. In particular, gels with MLV gave opposite results, with respect to the solution formulation, depending on the modality of drug addition during their preparation, since it strongly affected the vesicle entrapment efficiency

Table 3
Permeability coefficient (K_p) values of benzocaine (BZC) from the different formulations through the artificial lipophilic membrane (mean ± SD, $n = 6$)

Formulation	K_p (cm/h)
MLV-H dispersion	0.1230 ± 0.0018
MLV-L dispersion	0.0618 ± 0.0009
SUV dispersion	0.1068 ± 0.0016
MLV-H gel	0.0360 ± 0.0005
MLV-L gel	0.0174 ± 0.0003
SUV gel	0.0324 ± 0.0005
Solution in gel	0.0240 ± 0.0004

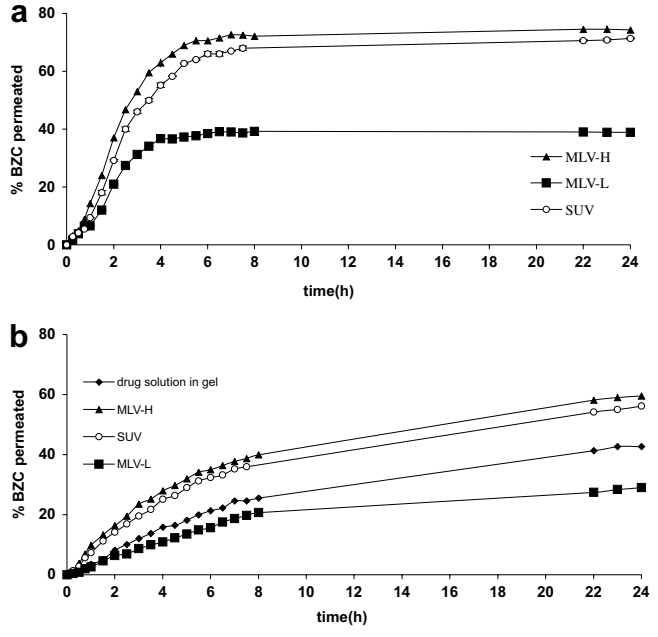

Fig. 1. Permeation profiles through artificial lipophilic membrane of benzocaine (BZC) from 1% w/v drug-loaded liposomal dispersions as such (a) or dispersed (50:50 w/w) in an aqueous Carbopol gel (b).

3.4. Permeation studies through rat skin

Permeation studies across rat skin were performed on the same formulations examined in the previous studies with artificial lipophilic membranes, under the same experimental conditions. The results are summarized in Table 4 in terms of drug permeability coefficient (K_p), and the corresponding permeation profiles are presented in Fig. 2(a and b). As a general observation, a reduced drug permeation rate was noted, due to the more complex permeation process through the rat skin than through the artificial membrane. Also, the difference between the use of the liposomal dispersion as such (Fig. 2a) or formulated in the Carbopol gel (Fig. 2b) was less evident, probably due to the major controlling effect exerted by the skin on the drug

Table 4
Permeability coefficient (K_p) and lag time values of benzocaine (BZC) from the different formulations through rat skin (mean ± SD, $n = 6$)

Formulation	K_p (cm/h)	Lag time (min)
MLV-H dispersion	0.0222 ± 0.0040	44
MLV-L dispersion	0.0162 ± 0.0015	52
SUV dispersion	0.0161 ± 0.0013	69
MLV-H gel	0.0180 ± 0.0021	55
MLV-L gel	0.0102 ± 0.0010	42
SUV gel	0.0096 ± 0.0008	74
Solution in gel	0.0072 ± 0.0006	96

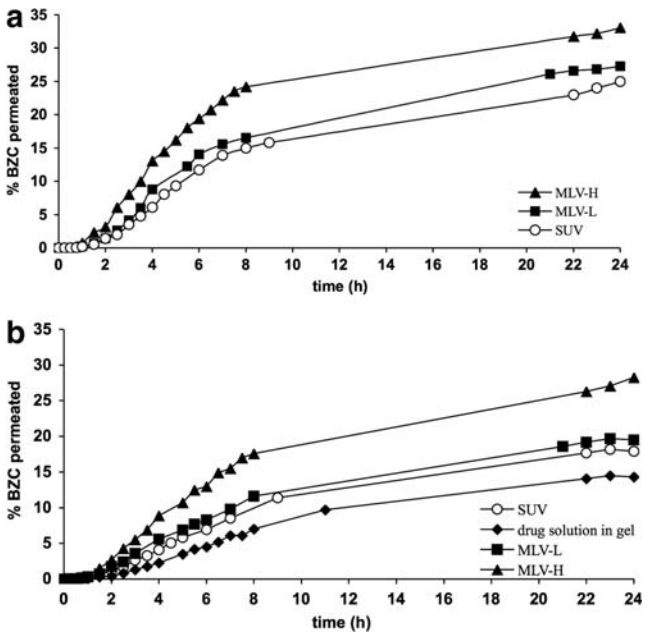

Fig. 2. Permeation profiles through rat skin of benzocaine (BZC) from 1% w/v drug-loaded liposomal dispersions as such (a) or dispersed (50:50 w/w) in an aqueous Carbopol gel (b).

permeation rate. Moreover, an initial lag-phase was present, which, on the contrary, was not detectable in the previous series of experiments. The observed lag-times (Table 4) were attributed to the longer time necessary to saturate the skin membrane and reach a pseudo steady-state flux condition between donor and receiver compartments.

However, other more interesting and important differences can be observed when comparing the results given by the different formulations in the two series of experiments. In particular, while MLV-H still showed the best performance ($P < 0.001$), as in the experiments with the artificial membrane, there was unexpectedly a clear worsening of the SUV behaviour, which was not significantly different ($P > 0.05$) from that of the MLV-L. Therefore, the reduced particle size of the small uni-lamellar vesicles did not play a major role in affecting the drug permeation rate across the skin, contrary to prior observations in the experiments with artificial membranes. Previous studies showed that the employed artificial membranes can give reliable results, comparable to the skin behaviour, only when passive diffusion is the main driving force of the drug permeation process and no permeation enhancer effects are present [17]. Evidently, in the present case, the drug permeation rate was affected by specific interactions of liposomes with the skin components, whose effect can be appreciated by using an animal membrane, such as rat skin, but however cannot be evaluated with the artificial membranes.

Moreover, permeation experiments through rat skin revealed a possible correlation between liposome entrapment efficiency and drug delivery. In fact, the BZC permeability coefficient values from SUV (EE%: 28.8) and MLV-L (EE%: 29.7) were very similar ($P > 0.05$), independent of whether the entrapped drug is in the aqueous (SUV) or in the lipophilic (MLV-L) liposomal phase, and of their different dimensions and structure (uni-lamellar or multi-lamellar bilayer).

Finally, the lowest drug permeation ($P < 0.001$) observed from simple gel solution in comparison with all the gel liposomal formulations (differently from that found in previous experiments across artificial membranes) seems to further confirm the hypothesized permeation enhancing effects of the liposomal vesicles on drug delivery, which cannot be efficiently estimated by using the artificial membrane. A possible co-solvent effect due to the ethanol released from the liposomal formulation, which could change the properties of the receiving medium, can be excluded, considering that the total amount of this solvent present in 0.15 g of the liposomal dispersion used as donor phase was about 0.075 mL, which can be considered negligible with respect to the 14.5 mL of phosphate buffer receiving solution. However, a possible enhancer effect due to the ethanol present in the liposomal formulation could be assumed. To investigate this possibility, permeation studies across rat skin were also performed on BZC formulated in a hydro-alcoholic Carbopol gel. A slight, even though not significant ($P > 0.05$), reduction in the drug flux was observed with respect to the corresponding drug formulation in the aqueous gel, and it was explained by considering the higher affinity of BZC for the water–ethanol rather than for the aqueous solvent, which lowered its escaping tendency from the polymeric network. Therefore, this result excluded the enhancer effect of ethanol, at least when it was not used in liposomal formulations. In fact, it could be reasonably hypothesized that, as already observed by other authors [33], the presence of ethanol within the liposomes could favourably affect their performance by loosening the structure of PC bilayers, thus improving their interaction and penetration abilities through the skin.

3.5. Confocal laser scanning microscopy (CLSM)

In order to evaluate the actual skin accumulation and penetration properties of the examined liposomal formulations, sections of the rat skin were analysed by CLSM at the end of permeation experiments. For these studies, a fluorescent hydrophobic marker such as rhodamine 6G, with an oil/water partition coefficient [23] similar to that of BZC [16], was added, like the drug, in the hydrophilic or lipophilic phase during liposome preparation. CLSM images revealed that all the examined liposome formulations penetrated deeply into the stratum corneum, and diffused into the whole skin thickness. The only difference observed was a more marked concentration in the most external skin layer for formulations based on MLV-L; on the contrary, a more homogeneous distribution in all the skin layers was observed for liposomal formulations containing the hydrophobic probe in the hydrophilic phase (MLV-H) (Fig. 3b and c). The greater ability to penetration through the skin due to the liposomal vesicles was

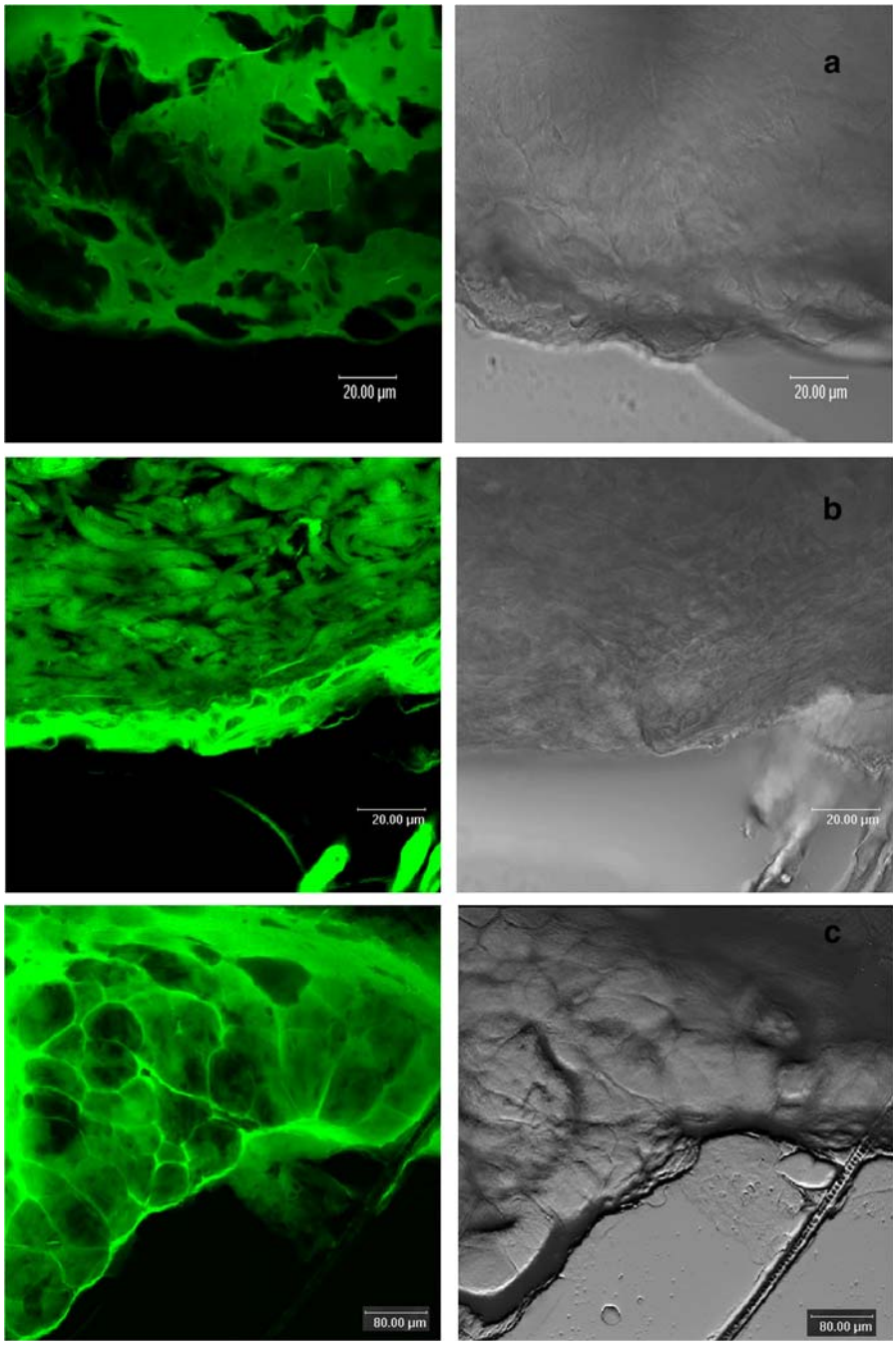

Fig. 3. CLSM images of cross sections of abdominal rat skin after 24-h incubation in Franz diffusion cell with rhodamine 6G solution (a) or with MLV liposomes containing rhodamine 6G in the lipophilic (b) or hydrophilic (c) phase.

confirmed by control experiments performed using the marker directly dispersed into the Carbopol gel formulation, where remarkably less intense skin depositions and penetration effects were observed (Fig. 3a).

3.6. In vivo studies of anaesthetic activity

Only gel formulations were studied in the rabbit model, since they allowed deposition of a more constant and reproducible amount of the drug with respect to liquid formulations, and therefore reduced the variability of the experimental conditions.

Results of *in vivo* studies, summarized in Table 5, indicated that the entrapment of BZC in liposomes allowed the achievement of both the prefixed goals, i.e. a statistically significant extension ($P < 0.01$) of its duration of activity and also a significantly stronger anaesthetic effect with respect to the corresponding BZC solution at the same drug concentration. Such findings can be attributed to the favourable effect of the liposomal carriers that, in virtue of their high affinity for the skin, undergo a preferential

Table 5
Effect induced by some benzocaine (BZC) liposomal formulations on the rabbit conjunctival reflex test

Ocular treatment	Eye	Time after treatment			
		10 min	20 min	30 min	40 min
		Number of stimuli to induce conjunctival reflex			
Control (empty gel)	Left	1.0 ± 0.0	1.5 ± 0.3	1.2 ± 0.2	1.0 ± 0.0
Control (empty gel)	Right	1.0 ± 0.0	1.2 ± 0.2	1.0 ± 0.0	1.0 ± 0.0
Gel + empty liposomes	Left	1.2 ± 0.3	1.5 ± 0.3	1.0 ± 0.0	1.0 ± 0.0
Gel + empty liposomes	Right	1.4 ± 0.2	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Gel + aqueous solution	Left	1.4 ± 0.3	1.2 ± 0.2	1.0 ± 0.0	1.0 ± 0.0
Gel + 0.5%BZC aq. sol.	Right	21.4 ± 3.3*	17.8 ± 2.5*	14.1 ± 1.6*	2.3 ± 0.2
Gel + MLV-L	Left	1.0 ± 0.0	1.4 ± 0.2	1.4 ± 0.3	1.0 ± 0.0
Gel + MLV-L 0.5%BZC	Right	29.3 ± 3.7*,†	24.1 ± 2.3*	18.4 ± 1.5*	1.6 ± 0.4
Gel + MLV-H	Left	1.3 ± 0.5	1.6 ± 0.2	1.0 ± 0.0	1.0 ± 0.0
Gel + MLV-H 0.5%BZC	Right	35.2 ± 4.6*,†	28.8 ± 4.5*,†	23.5 ± 5.2*,†	11.7 ± 4.9*,†
Gel + SUV	Left	1.8 ± 0.4	1.5 ± 0.3	1.9 ± 0.5	1.0 ± 0.0
Gel + SUV 0.5%BZC	Right	26.3 ± 3.9*,†	23.2 ± 4.2*	17.7 ± 4.0*	10.5 ± 3.3*

There were 4–6 rabbits per group. Each value represents the mean of 4–6 separate experiments.
* $P < 0.01$ in comparison with control.
† $P < 0.01$ in comparison with GEL + BZC solution 0.5% w/w.

uptake and enable a better skin penetration of the entrapped drug than the simple solution, according to the results of CLSM studies. In particular, the best results were obtained with MLV-H, containing the BZC added in the hydrophilic phase, in accordance with the more efficacious drug release obtained with this kind of liposome in the previous permeation experiments with both artificial membranes and rat skin.

On the other hand, in spite of the reduced particle size and thus of their greater diffusion properties, as emerged from permeation studies across artificial membranes, and the expected better skin permeation properties [32], SUV (obtained by sonication of MLV-H) showed a behaviour comparable to that of MLV-L, but clearly inferior to that of the original MLV-H. These results differed from those of permeation experiments across artificial membranes, while they were in complete agreement with those of permeation experiments across rat skin. This finding confirmed the previous supposition that the dominant role in determining liposomal formulation effectiveness was not played by the liposomal dimension or structure, but by its EE%, which was about 82% for MLV-H and about 30% for MLV-L and SUV. Therefore the importance of adding the drug in the aqueous or lipophilic phase during the liposome preparation is only indirect, arising from its influence in determining the liposome EE%.

4. Conclusions

Liposomal formulations of BZC allowed a significant improvement of its therapeutic effectiveness in terms of both intensity of the anaesthetic effect and duration of action.

These findings can be mainly ascribed to the powerful liposomal carrier functions, that not only enabled a more intense localization of the drug in the layers of the skin (as indicated by Confocal Laser Microscopy studies), thus improving its therapeutic effectiveness, but also acted as an effective drug reservoir, thus prolonging the anaesthetic effect of BZC.

The technique used for the vesicle preparation significantly influenced the performance of the final product. In particular the modality of addition of the drug during the vesicle preparation was notably important, since it had a direct and strong influence on the EE%, which was the actual dominant factor in determining the effectiveness of the final formulation. On the contrary, the reduced dimensions of the vesicles did not have a significant effect on either the permeation across rat skin or the *in vivo* drug therapeutic effectiveness, in contrast to that reported by Verma et al. [32].

Finally, the critical comparison between the results obtained in permeation experiments with artificial membranes and those with rat skin was useful to provide more insight into the factors determining the drug skin permeability and to point out the essential role of the liposomal carriers.

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