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Development of liposomal and microemulsion formulations for transdermal delivery of clonazepam: Effect of randomly methylated β-cyclodextrin

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ABSTRACT

Transdermal administration of clonazepam, a poorly water-soluble benzodiazepine, is an interesting strategy for overcoming the drawbacks of its oral administration. With this aim, two nano-carrier formulations, based on ultra-deformable liposomes and microemulsions, have been developed to favour clonazepam transdermal delivery. Considering the solubilizing power of methyl-βcyclodextrin (Me-βCD) toward clonazepam and its potential positive influence on transdermal drug delivery, the effect of its addition to these formulations was investigated. Artificial lipophilic membranes simulating the skin allowed a rapid evaluation of the drug permeation properties from the systems, compared with those from an aqueous drug suspension, with or without Me-βCD. The best formulations were further characterized by permeation through excised rabbit ear skin. All the formulations increased drug permeability, ranging from 2-fold (liposomes without Me-βCD), up to over 4-fold (microemulsions containing Me-βCD). The different formulations allowed for pointing out different possible permeation enhancing mechanisms of Me-βCD: increase in drug solubility and thermodynamic activity in the vehicle, when added to the drug aqueous suspension; interactions with the vesicle bilayer, in case of liposomal formulations; interactions with the skin membrane lipids, as evidenced in experiments with excised rabbit ear for microemulsions containing Me-βCD, that were then selected for further *in vivo* studies.

1. Introduction

Transdermal drug delivery has been attracting increasing attention over the last years, due to the number of advantages offered over oral or intravenous administration, such as reduced systemic toxicity, absence of hepatic first-pass metabolism and better control of blood levels. Unfortunately, only a limited number of active compounds appear to be able to penetrate the skin at a rate sufficiently high to exert systemic effects and have therapeutic efficacy (Hadgraft, 2001; Notman and Anwar, 2013). Therefore, several strategies have been proposed and investigated in the attempt of overcoming this drawback and improving drug permeability through the skin.

Liposomal formulations constitute a promising approach for the development of effective dermal and transdermal drug delivery systems (Neubert, 2011). In particular, the potential of liposomes as carrier systems for transdermal drug delivery can be improved by adding suitable pharmaceutically-acceptable "edge-activators" in the vesicle bi-layer, able to increase its elasticity, thus obtaining ultra-deformable liposomes, also named *transfersomes* (Cevc, 2004; El Zaafarany et al., 2010).

Another interesting formulative strategy, which can be exploited for promoting transdermal drug delivery, is represented by microemulsions, thermodynamically stable, isotropic liquid mixtures of oil and water stabilized by an interfacial film of a suitable surfactant-cosurfactant mixture (Moulik and Paul, 1998). Microemulsions present ease of preparation, high solubilization capacity both for hydrophilic and lipophilic drugs and good penetration enhancing effects when administered on the skin (Peltola et al., 2000; Rhee et al., 2001; Kogan and Garti, 2006; Chen et al., 2006; Heuschkel et al., 2008). The effectiveness of both such kinds of formulations in promoting transdermal drug delivery can be further improved by the addition of a suitable "skin penetration enhancer" (Pahri et al., 2012). Depending on the type of enhancer and on the nature of the drug, different mechanisms could be involved, such as increase of the effective concentration of the drug in the vehicle, improvement of drug partitioning from the formulation to the skin, increase of the drug diffusion coefficient,

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decrease of the skin barrier properties (Moser et al., 2001). Cyclodextrins are reported among the different possible skin penetration enhancers, even though their mechanism of action is still under debate (Loftsson and Másson, 2001; Loftsson et al., 2007). The combination in a same formulation of different penetration enhancers, including cyclodextrins, can give rise sometimes to a synergistic effect (Loftsson and Màsson, 2001; Loftsson et al., 2007; Karande and Mitragotri, 2009; Pahri et al., 2010).

Different authors evidenced the potential advantages over conventional dosage forms of the transdermal administration of different kinds of benzodiazepines (Nokhodchi et al., 2003; Kravchenko et al., 2003; Balaguer-Fernandez et al., 2010; Soler et al., 2012). Among these, clonazepam (CLZ), a potent benzodiazepine derivative mainly employed for its anxiolytic, hypnotic and antiepileptic properties, is considered a very interesting candidate for transdermal administration, due to its pharmacological characteristics, such as high first pass metabolism, wide blood levels oscillations, low dose size, need for long-term treatment (Ogiso et al., 1989; Mura et al., 1996, 2000; Corti et al., 1998; Puglia et al., 2001). On the other hand, the very low water solubility of CLZ gives rise to a dissolution rate-limited absorption, generally recognized as directly related to poor and/or erratic absorption and bioavailability (Amidon et al., 1995). For such a reason, in a recent work we investigated and compared the complexing and solubilizing efficacy of different native and chemically-modified cyclodextrins toward CLZ, as a first step for the future development of innovative transdermal delivery systems of the drug (Mennini et al., 2014). Among the examined carriers, the randomly methylated-beta-cyclodextrin (Me-βCD) provided the best results in terms of increase in drug solubility and dissolution rate (Mennini et al., 2014).

On the basis of all the above considerations, and in continuation of our previous studies on transdermal delivery of CLZ (Mura et al., 1990, 1992, 1996, 2000; Corti et al., 1998), the present work was aimed at the development of a new innovative and effective transdermal delivery system of CLZ. With this purpose, we developed different ultra-deformable liposomal and microemulsion formulations containing potential skin penetration enhancers and investigated their performance in improving the drug permeation properties. Moreover, considering the potential co-enhancer effect of cyclodextrins in transdermal drug delivery (Loftsson et al., 1998, 2007), and also based on the high solubilizing efficacy of Me-βCD toward CLZ (Mennini et al., 2014), the effect of the addition of such CD to both liposomal and microemulsion formulations was also evaluated. The permeation properties of CLZ from these delivery systems through both skin-simulating lipophilic artificial membranes and rabbit ear excised skin, used as a percutaneous absorption model, were evaluated and compared with those of a simple drug aqueous suspension (Mura et al., 1996, 2007).

2. Materials and methods

2.1. Materials

Cholesterol (CHL), Clonazepam (5-(2-chlorophenyl)-7-nitro-3H-1,4-benzodiazepin-2(1H)-one) (CLZ), lauryl alcohol, L-α-phosphatidylcholine from egg yolk (PC), octadecylamine (OCT), oleic acid, polyoxyethylene sorbitan monolaurate (Tween 20), sodium cholate hydrate (SC), sorbitan monoleate (Span 80), sorbitan trioleate (Span 85), triethanolamine (TEA) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Randomly methylated-beta-cyclodextrin (Me-βCD) was kindly provided by Wacker Chemie Gmbh (Munich, Germany). Caprylic/capric triglycerides (Labrafac CC), caprylocaproyl macrogol-8 glycerides (Labrasol), highly purified diethylene glycol monoethyl ether (Transcutol HP), linoleoyl macrogol-6 glycerides (Labrafil M2125CS), medium-chain triglycerides (Labrafac Lipophile WL1349), octyldodecylmyristate, polyglyceryl-3 dioleate (Plurol Oleique CC 497), polyglycolized glycerides (Labrafac Hydro WL 1219), propylene glycol dicaprylocaprate (Labrafac PG), and propylene glycol monolaurate (Lauroglycol 90) were kindly donated by Gattefossé Italia s.r.l. (Milan, Italy). Isopropyl myristate and polyethylene glycol 400 (PEG 400) were purchased from Merck Schuchardt OHG (Hohenbrunn, Germany). Carbopol 940 was obtained from Lubrizol (Cleveland, OH, USA). Water was obtained from a Milli-Q water purification system (Millipore, Billerica, MA, USA). All other chemicals were of analytical grade.

2.2. Screening of oils for microemulsions

The solubility of CLZ in the different oil phases (Labrafil M2125CS, Labrafac CC, Lauroglycol 90, Labrafac PG, Labrafac Hydro WL 1219, Labrafac lipophile WL1349, oleic acid, isopropyl myristate) was determined, in order to select the oil with the highest solubilizing power to use as the oil phase in the microemulsions. An excess amount of drug was added to 5 mL of each oil; each sample was sealed, initially shaken using a vortex mixer and then kept under magnetic stirring at 25 ± 1.0 °C for 48 h to reach equilibrium. The suspensions were then centrifuged at 3000 rpm for 15 min mantaining constant the temperature. The supernatant was filtered through a 0.45 μm cellulose acetate membrane filter and the concentration of CLZ in the filtrate was determined by HPLC, as described below. The experiments were performed in triplicate.

2.3. High-performance liquid chromatography (HPLC) assay of clonazepam

Quantitative assay of CLZ was carried out by HPLC (Merck Hitachi, Darmstadt, Germany) equipped with an Elite Lachrom UV–vis detector (Merck Hitachi). A Hypersil RP C18 column (Thermo Electron Co., Waltham, MA, USA), 2.4 μm particle size, 100 mm × 4.6 mm, was used as stationary phase. The mobile phase was a 30:70 v/v mixture of acetonitrile:water; the flow rate was 0.9 mL/min. UV detection was carried out at 310 nm. The injection volume was 20 μL. The temperature was maintained at 40 ± 1.0 °C. The retention time of CLZ under these experimental conditions was about 8 min. A calibration curve in the 5–20 mg/L concentration range was prepared. The method was validated performing repeated analyses of decreasing analyte concentrations (Ermer, 2001). The lower limit of quantification and the limit of detection were 0.6 mg/L and 0.25 mg/L, respectively.

2.4. Construction of phase diagrams and preparation of microemulsions

Pseudo-ternary phase diagrams were constructed by progressive titration with water of the component mixtures. Each surfactant (Labrasol, Span 80, Span 85, Plurol Oleique CC 497, Tween 20) was mixed in a 1:1 v/v ratio with Transcutol, selected as co-surfactant. Each surfactant/co-surfactant (S/CoS) mixture was then mixed with Labrafac Hydro WL 1219, the oil phase selected on the base of previous solubility studies, in different oil:S/CoS v/v ratios. Each mixture was then titrated by adding water drop by drop up to clouding. The surfactant which allowed the obtainment of the largest existence area of the microemulsion (Tween 20) was selected for the subsequent step where the experiments were repeated at different S/CoS v/v ratios.

Analysis of the obtained pseudo-ternary phase diagrams allowed the choice of the best components and selection, within

Table 1
Solubility of clonazepam (CLZ) in different oils at 25 °C.

OIL	CLZ solubility (mg/L)
Labrafac Hydro WL 1219	71.4
Isopropyl myristate	54.5
Labrafac CC	23.0
Oleic acid	16.7
Labrafac PG	16.2
Lauroglycol 90	13.3
Labrafac Lipophile WL1349	7.9
Labrafil M2125CS	<0.6
Octyldodecylmyristate	<0.6

the zone of microemulsion formation, of their most suitable relative amounts to prepare stable microemulsions. For preparation of CLZ-loaded microemulsions, the drug (1% w/v) was previously dissolved under magnetic stirring in the oil phase. In the case of microemulsions containing also MeβCD, it was previously dissolved in the aqueous phase (50 mg/mL).

2.5. Preparation of ultradeformable liposomes

Large multi-lamellar vesicles were prepared by the thin layer evaporation (TLE) technique. According to this method, the components of the lipid phase were dissolved in chloroform in a 250 mL round bottom flask (Mura et al., 2007). The solvent was then removed under reduced pressure in a rotary evaporator, obtaining a thin layer of dry lipid on the flask walls. Evaporation was continued for 2 h for a complete removal of solvent residues. The film was then hydrated with 10 mL of water. To obtain loaded liposomes, the drug was dissolved in the lipophilic phase. Me-βCD, when present in the formulation, was instead dissolved in the aqueous phase (50 mg/mL). The CD was added at the maximum compatibile with liposomes formation (data not shown) in light excess amount respect to the 1:1 molar ratio with the drug.

The hydrated film was submitted to a water bath heating at 60 ± 1.0 °C for 10 min and vortexing for 2 min; the treatment was repeated, performing 5 cycles in total. The suspension was left cooling down and probe sonicated for 5 min in a ice bath using a Sonopuls HD2070 (Bandelin GmbH, Berlin, Germany) equipped with a titanium probe (DS73, Bandelin), setting the instrument at 50% of the power. The suspension was then hermetically sealed, protected from light and stored at 4 °C.

2.6. Characterization of microemulsions and liposomes

The average size of the microemulsions droplets and the mean diameter and Zeta potential of the vesicles of liposomal suspensions were investigated by dynamic light scattering, using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK). Samples were analysed 24 h after their preparation. Before analysis, the liposomal suspensions were properly diluted with deionized water to avoid multi-scattering phenomena. Six independent samples were taken from each dispersion and measured at 25 ± 0.1 °C, both for particle size and Zeta potential determination.

Liposome encapsulation efficiency was indirectly determined by using the dialysis method. The suitability of this technique (which gave results comparable to those obtained by the ultracentrifugation technique) has been previously demonstrated (Maestrelli et al., 2005; Mura et al., 2007). Briefly, 3 mL of liposomal suspension were dropped into a cellulose acetate dialysis bag (Spectra/Pore®, MW cut-off 12,000, Spectrum, Canada), which was sealed, immersed into 200 mL of deionized water and magnetically stirred at 30 rpm. At predetermined time intervals,

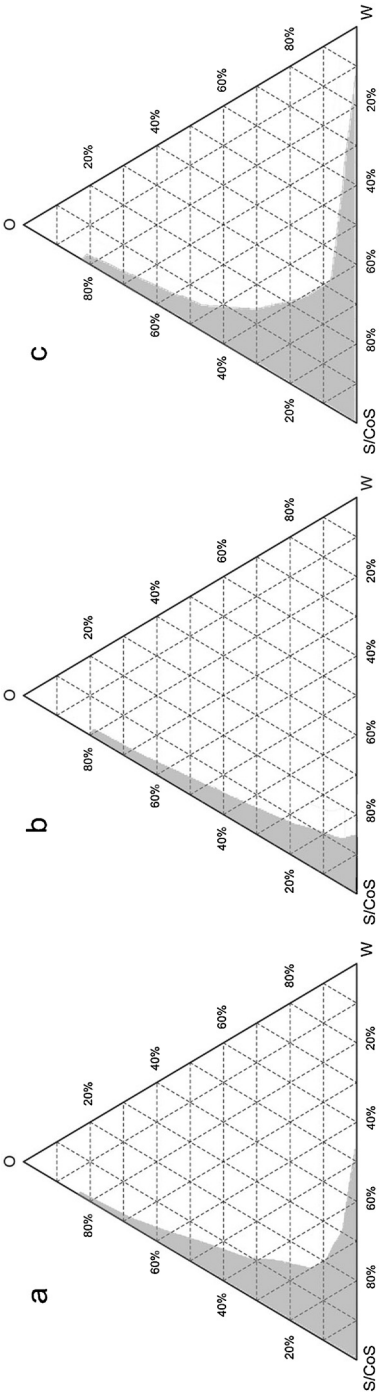

Fig. 1. Pseudo-ternary phase diagrams obtained by the aqueous titration method using Labrafac Hydro WL 1219 as oily phase (O) and different 1:1 v:v Surfactant/CoSurfactant (S/CoS) mixtures: (a): Labrasol/Transcutol; (b) Span 80/Transcutol; (c) Tween 20/Transcutol. The shaded areas represent the region of existence of the microemulsions.

a 0.5 mL sample of receiving medium was withdrawn and immediately replaced with an identical volume of fresh water. The amount of diffused drug was determined by HPLC analysis as described above. The percent of encapsulation efficiency (EE%) was calculated according to the following equation:

$$EE\% = \frac{[\text{totaldrug}] - [\text{diffuseddrug}]}{[\text{totaldrug}]} \times 100$$

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

The liposome deformability was evaluated by determining the mean size of the liposomes before and after 11 times of extrusion through a 100 nm nitrocellulose membrane filter (Isopore, Millipore, Bedford, MA, USA) utilizing a LiposoFast-Basic membrane extruder (Avestin GmbH, Manneheim, Germany) joined to a 3 atm pressure source. Each result is the mean of five separate experiments.

2.7. Preparation of gels

A 2% w/v Carbopol gel was initially prepared, by dispersing 2 g of Carbopol 940 in water under stirring, and then adding TEA up to pH 7.0 for gelification. Gels loaded with the drug were then prepared by mixing (50:50 w/w) the Carbopol gel with an aqueous suspension (1% w/w) of CLZ (alone or in the presence of Me-βCD), or with the liposomal or the microemulsion formulations, containing or not Me-βCD (50 mg/mL). For all the gels, the final

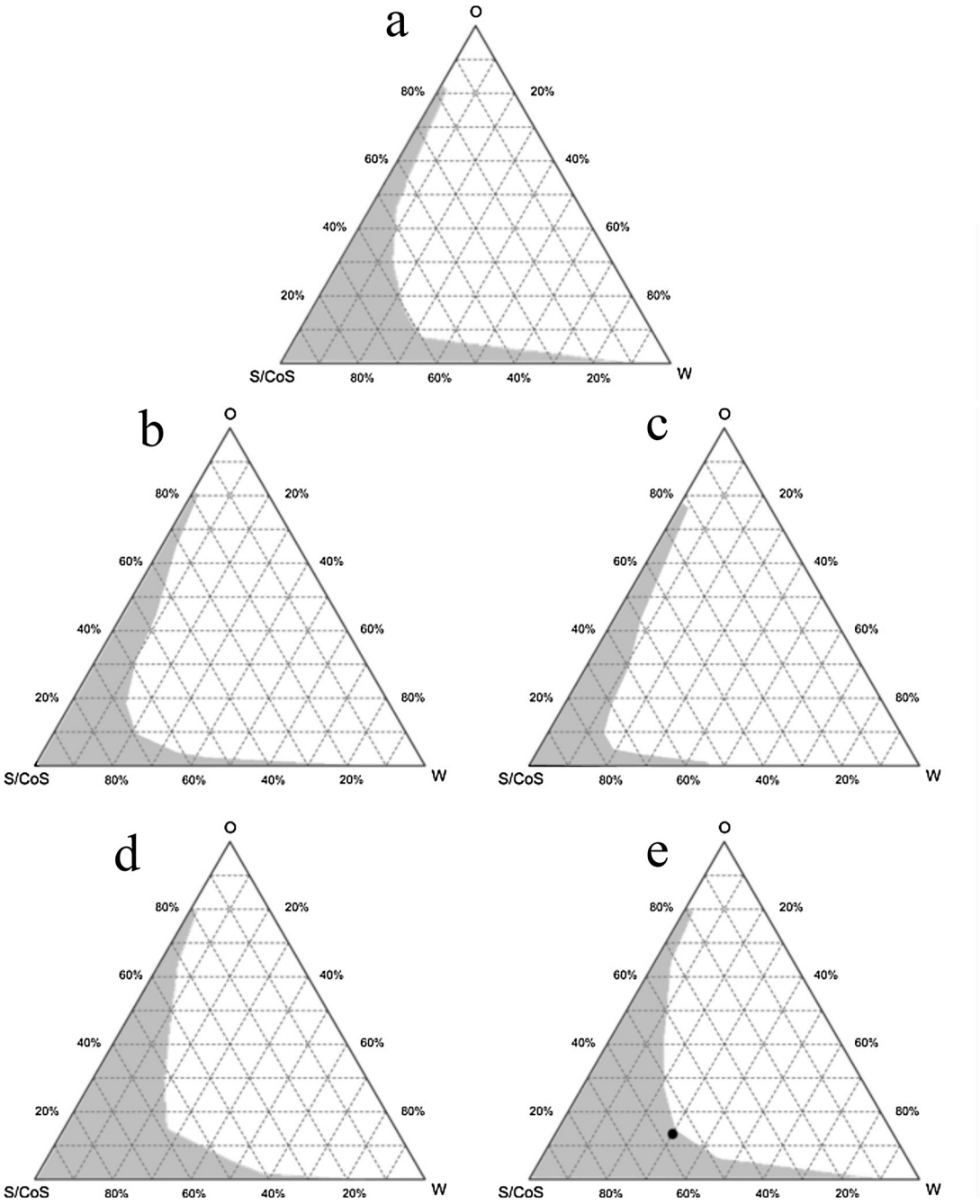

Fig. 2. Pseudo-ternary phase diagrams obtained by the aqueous titration method using Labrafac Hydro WL 1219 as oily phase (O) and Tween 20/Transcutol (S/CoS) mixtures at different v/v ratios: (a) 1:1; (b) 1:2; (c) 1:4; (d) 2:1; (e) 4:1. The shaded areas represent the region of existence of the microemulsions, and the black dot (●) corresponds to the composition selected for the microemulsion formulation.

Table 2
Mean diameter and polydispersity index (PDI) of the droplets of the selected microemulsion formulation (13% Labrafac Hydro WL1219, 30% water and 57% Tween20/Transcutol 4:1 v/v) in the presence or absence of clonazepam (CLZ) (1% w/v) and MeβCD (50 mg/mL).

	Mean diameter (nm)	PDI
Unloaded microemulsion	76.4 ± 14.4	0.466 ± 0.004
CLZ-loaded microemulsion	68.4 ± 12.4	0.436 ± 0.011
CLZ-loaded microemulsion with MeβCD	61.5 ± 13.7	0.445 ± 0.013

concentration of CLZ was held constant and equal to 0.5% w/w, and the Me-βCD concentration, when present, was 25 mg/mL.

2.8. In vitro *permeation studies through artificial membranes*

In vitro permeation studies were carried out by using vertical Franz diffusion cells (Rofarma, Gaggiano, Italy). Artificial membranes of cellulose nitrate with pore size 0.1 μm (Millipore VCWP, Merck, Darmstadt, Germany) impregnated with lauryl alcohol as lipid phase simulating the epidermal barrier were employed for the study (Mura et al., 1996, 2007). Briefly, each membrane was accurately weighed, immersed for 15 min into lauryl alcohol at 50 °C, dried by a filter paper, weighed again to check that its weight increase was always between 90 and 110%, and immediately mounted on the cell. The acceptor medium, thermostated at 37 °C and kept under gentle agitation, consisted of 7 mL of pH 7.4 phosphate buffer containing 25% v/v of PEG 400 to increase CLZ solubility in order to maintain sink conditions during the experiments (Mura et al., 1990, 1992, 1996). An appropriate amount of each gel, containing 0.5 mg of CLZ, was placed in the donor compartments of the cells. At predetermined time intervals, 0.5 mL of medium were withdrawn from the receiving chamber and the drug concentration was assayed by HPLC as described above. A correction for the cumulative dilution due to the sample replacement with an equal volume of fresh medium was calculated. The experiments were performed in triplicate for every formulation and the results were averaged.

The apparent permeability coefficient of the drug was calculated according to the formula:

$$P_{app} = \frac{dQ}{\mathrm{d}tAC_0}$$

where $dQ/\mathrm{d}t$ is the cumulative amount of drug permeated per unit of area vs. time, A the effective surface area exposed to the medium and C_0 the initial concentration of CLZ in the donor compartment.

2.9. In vitro *permeation studies through excised animal membranes*

This second series of permeation studies was performed to evaluate more in depth the performance of the hydrogel formulations which provided the best results in previous *in vitro* experiments with artificial membranes. The experiments were carried out with the same apparatus and under the same experimental conditions of previous *in vitro* studies, but using rabbit ear skin as a percutaneous absorption model (Mura et al., 1996). The skin was obtained from the ears of freshly killed rabbits destined to be slaughtered for human alimentation. When not used immediately, the skin was kept at 2–5 °C and used within 24 h. After depilation and washing, the skin was excised from the connective tissue by a lancet, washed with a pH 7.4 buffer solution, carefully dried with filter paper and immediately mounted in the Franz diffusion cell with the stratum corneum side facing toward the donor compartment, and the dermal side toward the receptor medium, 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 HPLC. The samples were replaced with equal volumes of fresh receptor medium and the correction for the cumulative dilution was calculated. Experiments were performed in sextuple. The cumulative amount of drug transferred into the receptor side was calculated and the results were averaged. The drug apparent permeability coefficient was calculated as described for previous *in vitro* experiments.

2.10. Statistical analysis

All data were statistically analysed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparison post-4 software Inc.-San Diego, CA, USA). The differences were considered statistically significant when $P < 0.05$.

3. Results and discussion

3.1. Screening of oils for microemulsion formulation

Solubility studies of CLZ in the different oils chosen as possible candidates as component of the oily phase of the microemulsion formulation were carried out, in order to individuate the oil endowed with the highest solubilizing power towards the drug. The CLZ solubility values obtained with all the examined oils are presented in Table 1. The drug solubility in both Labrafil M2125CS

Table 3
Composition of the liposomal formulations and corresponding values of deformability, mean diameter, polydispersity index (PDI), Z-potential and encapsulation efficiency (EE%).

	Lipid phase	Aqueous phase	Deform.	Mean diameter (nm)	PDI	Z-potential (mV)	EE%
#1	PC 220 mg OCT 20 mg CHL 20 mg SC 20 mg CLZ 100 mg	Water	1.05 ± 0.04	216.4 ± 13.4	0.296 ± 0.004	+56.0 ± 1.92	88.6 ± 2.3
#2	PC 220 mg OCT 20 mg CHL 20 mg SC 20 mg CLZ 100 mg	Water + Me-βCD	1.08 ± 0.03	212.8 ± 32.3	0.413 ± 0.057	+54.3 ± 1.10	89.6 ± 2.6

Table 4
Amount of clonazepam (CLZ) permeated per unit area through the artificial lipophilic membrane and apparent permeability coefficient from the different gel formulations at 0.5% drug, containing or not Me-βCD (25 mg/mL).

CLZ formulation	μg/cm^2 CLZ permeated	*P* app (cm/s × 10^{-6})
Aqueous suspension	3.22 ± 0.15	1.10 ± 0.05
Aq. suspension + Me-βCD	5.52 ± 0.28	2.21 ± 0.09
Liposomes	6.90 ± 0.32	2.52 ± 0.10
Liposomes + Me-βCD	10.24 ± 0.49	3.77 ± 0.12
Microemulsion	13.24 ± 0.61	4.09 ± 0.13
Microemulsion + Me-βCD	13.93 ± 0.63	4.25 ± 0.15

and octyldodecylmyristate was very low and it was under the limit of quantification of the HPLC assay method. Labrafac Hydro WL 1219 was the best solvent for CLZ, allowing to reach a drug solubility of 71.4 mg/L, over 5 folds the value of its solubility in water (<15 mg/L) and therefore it was selected as the oily phase of the microemulsion.

3.2. Construction of phase diagrams

Pseudo-ternary phase diagrams were constructed, as described in the Methods section, by titration with water of mixtures of the selected oily phase (i.e. Labrafac Hydro WL 1219), with 1:1 v/v combinations of Transcutol, selected "a priori" as co-surfactant, in virtue of its skin permeation enhancer properties, with each surfactant examined (Labrasol, Span 80, Span 85, Plurol Oleique CC 497, Tween 20), in order to determine the surfactant able to give rise to the largest existence area of stable microemulsions (Mura et al., 2000; Gannu et al., 2008; Shokri et al., 2012). In the presence of the surfactants Span 85 and Plurol Oleique CC497, the formation of stable microemulsions was not possible at any ratio of the components, and therefore they were discarded. The pseudo-ternary phase-diagrams obtained by using the other surfactants are shown in Fig. 1. The shaded areas in these diagrams represent the existence field of stable, clear and transparent O/W microemulsions. The diagram relative to the use of Tween 20 (Fig. 1c) presents a wider existence area of the microemulsions, in comparison with the diagrams relative to the use of Labrasol (Fig. 1a) and Span 80 (Fig. 1b). On the basis of these results, Tween 20 was selected as the surfactant of the microemulsion formulation.

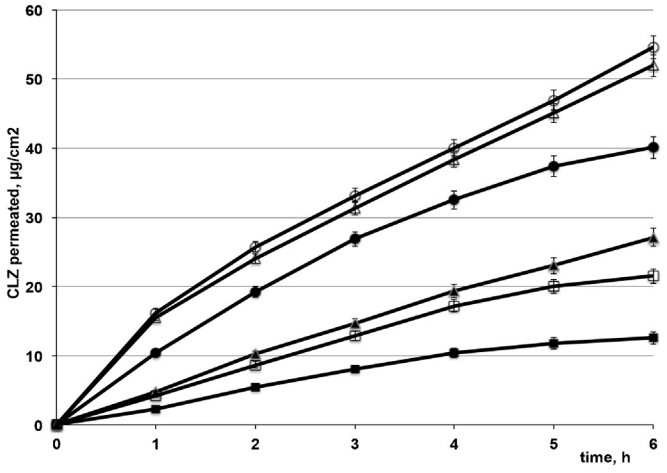

Fig. 3. Permeation studies through artificial membranes of clonazepam (CLZ) from aqueous suspensions or liposomal dispersions or microemulsions incorporated in a Carbopol gel (0.5% w/w): ■ aqueous suspension, □ aqueous suspension with Me-βCD, ▲ liposomal suspension, ● liposomal suspension with Me-βCD (25 mg/mL), △ microemulsion, ○ microemulsion with Me-βCD (25 mg/mL).

A further series of pseudo-ternary phase-diagrams were then constructed, where the effect of varying the Tween20/Transcutol v/v ratio (1:1, 1:2, 1:4, 2:1 and 4:1) was evaluated. As shown in Fig. 2, the existence field of the microemulsions increased whit increasing the S/CoS ratio. Among the tested combinations, the Tween20/Transcutol 4:1 v/v ratio (Fig. 2e) allowed the obtainment of the largest area of existence of the microemulsions, and was then selected for the preparation of drug-loaded O/W microemulsions.

3.3. Preparation and characterization of microemulsion formulations

The composition of the final formulation was selected inside the microemulsion region defined by the Labrafac Hydro WL 1219-Tween20/Transcutol 4:1 v/v-water pseudo-ternary phase diagram (Fig. 2e). Different contrasting aspects, which require a suitable compromise, have to be taken into account. On one hand, being the O/W microemulsion destined to be incorporated into a hydrophilic gel, and having to dissolve in its aqueous phase a proper amount of the hydrophilic CD, a high water content is desirable. On the same time, the amount of the oily phase should be enough to allow the complete dissolution of the lipophilic drug CLZ at the required dose. Finally, a content of Transcutol not less than 10% should be desirable, in order to exploit its enhancer properties. On the basis of all these considerations, the composition corresponding to 13% Labrafac Hydro WL1219, 30% water and 57% Tween20/Transcutol 4:1 v/v mixture, was selected as the best compromise for the preparation of the drug loaded microemulsion. CLZ was previously dissolved in Labrafac Hydro WL 1219, while Me-βCD, when present, was previously dissolved in the aqueous phase. The addition of both the drug (1% w/v) and the CD (50 mg/mL) did not affect the formation of the microemulsion, which appeared as a clear transparent solution even six months after its preparation.

For each tested combination of components, light-scattering analyses were carried out within the microemulsion existence field defined by the respective pseudo-ternary phase-diagram. The mean size of the droplets of the dispersed phase was in all cases below 100 nm, and the PDI always less than 0.5, thus confirming the actual formation of homogeneous microemulsions. In particular, in Table 2 the data of the final selected formulation, containing or not the drug and the CD are presented. The addition to the formulation of both CLZ and Me-βCD did not significantly influence ($P > 0.05$) the droplets dimensions and the homogeneity of the microemulsion.

3.4. Preparation and characterization of the liposomal formulations

It has been reported that classic liposomes are not able to deeply penetrate trough the skin, thus presenting only a limited capacity to promote an effective drug skin delivery (Touitou et al., 2000). The liposome skin penetration ability can be improved by addition in the lipid phase of the vesicles of a charged surfactant, which enhance the bilayer fluidity (Fang et al., 2006; Mura et al., 2008; Bragagni et al., 2010). Furthermore, it has been proved that the presence in the vesicle bilayer of an "edge-activator" can give rise to the formation of very flexible vesicles, able to penetrate the skin membrane intact and carry the drug in the deepest layers of the skin (Cevc and Blume, 2001; Maestrelli et al., 2010; Bragagni et al., 2012). Therefore, an ultra-deformable liposomal formulation has been developed, whose lipid phase contained octadecylamine, selected as cationic surfactant, and sodium cholate, selected as edge activator (El Zaafarany et al., 2010). Two different drug-loaded ultra-deformable liposomes have been prepared, where the drug was always dissolved in the lipid phase, while the aqueous phase contained or not Me-βCD.

Table 5
Amount permeated per unit area through the excised rabbit ear skin and apparent permeability of clonazepam (CLZ) from its 0.5% w/v microemulsion in gel formulations, containing or not Me-βCD (25 mg/mL).

CLZ formulation	μg/cm^2 CLZ permeated	P app (cm/s $\times 10^{-7}$)
Microemulsion	0.285 ± 0.026	1.571 ± 0.149
Microemulsion + Me-βCD	0.667 ± 0.063	3.149 ± 0.295

The composition of the liposomal formulations and their main properties in terms of deformability (expressed as the ratio of the vesicle diameter before and after extrusion), mean diameter, polydispersity index (PDI), Z-potential and drug entrapment efficiency (EE%) are reported in Table 3. Both liposomal dispersions showed good deformability properties, being the ratio of the vesicles diameter before and after extrusion near to the unit, independent of the presence or not of the CD. The addition of the CD to the formulation did not significantly influence ($P > 0.05$) the dimensions of the vesicles, while it gave rise to some loss of homogeneity of the liposomal suspension, as indicated by the significant increase ($P < 0.05$) in the PDI value. The markedly positive values of Z-potential, due to the presence of octadecylamine in the preparation, should concur to improve the physical stability of the dispersions over time, reducing aggregation and flocculation phenomena. As expected, considering the neutral character of Me-βCD, its addition did not change the Z-potential of the vesicles ($P > 0.05$). The drug was successfully encapsulated in both liposomal formulations, with very similar EE% values near to 90% ($P > 0.05$).

3.5. In vitro *permeation studies through artificial membranes*

Previous studies about the percutaneous absorption of CLZ from different topical formulations proved the reliability of the use of a suitable artificial lipophilic membrane, purposely developed, which allowed a good simulation of the skin permeation behavior, giving results well correlable to those obtained using animal membranes such as rabbit ear skin (Corti et al., 1998; Mura et al., 2000; Puglia et al., 2001). Moreover, artificial membranes present the advantage of a greater simplicity in use and of a higher reproducibility with respect to animal membranes, thus requiring a lower number of experiments to obtain trustworthy results.

Therefore, *in vitro* permeation studies through the skin-simulating lipophilic artificial membrane were performed to carry out a rapid screening and compare the effectiveness of the developed formulations (gels containing CLZ microemulsions or liposomal dispersions) in improving the drug permeation rate with respect to a gel loaded with a simple drug aqueous suspension, and to evaluate the influence of the presence or not of Me-βCD in such formulations. The results of these studies are summarized in Table 4 in terms of amount of permeated drug and apparent permeability coefficient values, while the corresponding permeation profiles are shown in Fig. 3.

The drug permeation rate from the gel loaded with its simple aqueous suspension was very low, and only 2.5% of CLZ diffused through the artificial membrane after 6 h. The addition of Me-βCD (50 mg/mL) to the drug aqueous suspension gave rise to a significant improvement ($P < 0.05$) in the drug permeation rate, with an about 1.7 times increase of the amount of permeated drug at 6 h. This result can be be mainly attributed to the high solubilizing power of Me-βCD toward CLZ, which enhanced the drug thermodynamic activity in the vehicle and, consequently, the amount of drug available to diffuse through the lipophilic absorption barrier (Mennini et al., 2014).

As for the gels containing the liposomal formulations, it was expected that incorporation of the drug into ultra-deformable liposomes should improve its concentration at the level of the skin-simulating artificial membrane, and thus facilitate its penetration into and across the barrier membrane. However, the liposomal formulation not containing Me-βCD allowed a significant ($P < 0.05$) but rather limited (about 2.3 times) increase of CLZ apparent permeability with respect to the simple aqueous suspension of the drug. This result was probably due to the high affinity of the lipophilic CLZ for the vesicle components, which reduced its escaping tendency from the vehicle. On the contrary, the liposomal dispersion containing Me-βCD enabled a more marked increase of CLZ permeation rate, significantly higher ($P < 0.05$) also than that obtained with the aqueous suspension of the drug containing the CD. In order to explain this result, it has been hypothesized that, in this case, Me-βCD could interact with the vesicle bilayer components, in virtue of its complexing ability, further improving the vesicle deformability and malleability, and thus favouring the drug release.

The gels containing the microemulsions gave the best results. In fact, also in the absence of Me-βCD, a more than four-folds increase of the amount of drug permeated after 6 h was observed in comparison with the simple aqueous suspension of the drug. The highly homogeneous and extremely fine dispersion of the drug, completely dissolved into the nano-sized droplets of the O/W microemulsion, probably allowed to maximize the drug concentration gradient at level of the barrier membrane. The solubilizing and carrier effect of Transcutol toward CLZ is also be taken into account (Mura et al., 2000). Interestingly, in the case of microemulsions, the addition of Me-βCD to the formulation did not cause an appreciable increase in the drug permeation rate. In fact, in this case, the solubilizing effect of CD was not useful, being the drug already completely dissolved in the oily phase of the microemulsion; moreover, differently from what observed for liposomal formulations, Me-βCD did not improve the CLZ escaping tendency from the vehicle.

3.6. In vitro *permeation studies through excised animal membranes*

Microemulsion formulations were then selected for further *in vitro* permeation studies, using excised rabbit ear skin as model of percutaneous absorption, in order to investigate more in depth their mechanism of action in promoting drug permeability (Mura et al., 1996). The results of these studies are summarized in Table 5 in terms of amount of permeated drug and apparent

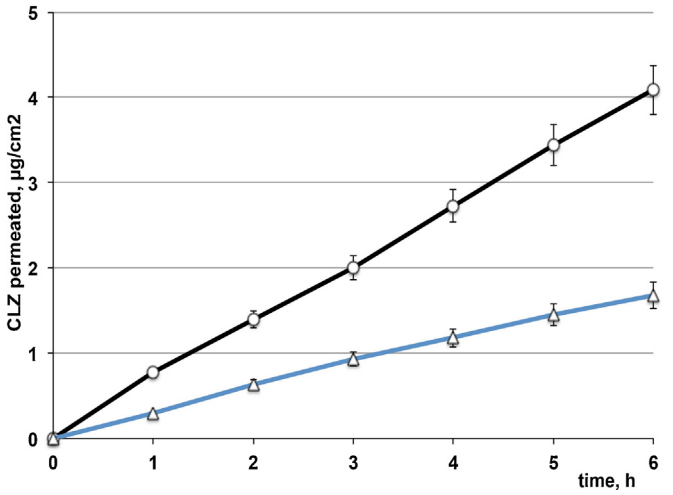

Fig. 4. Permeation studies through excised rabbit ear skin of clonazepam (CLZ) from microemulsions incorporated in a Carbopol gel (0.5% w/w): △ microemulsion, ○ microemulsion with Me-βCD (25 mg/mL).

permeability coefficient, while the corresponding permeation profiles are shown in Fig. 4.

As expected, the drug penetration rate through excised rabbit ear skin was clearly slower than that through the artificial membrane, due to the major barrier effect exerted by the biological membrane. Interestingly, differently from that observed in previous experiments with the artificial membrane, the presence of Me-βCD in the microemulsion brought about a significant ($P < 0.05$) and very marked improvement in drug permeation properties. In fact, both the amount of permeated drug and its apparent permeability coefficient were almost doubled with respect to the values obtained for the corresponding formulation without CD. Evidently, the permeation experiments through the biological membrane allowed to highlight a true skin penetration enhancer effect of Me-βCD, in addition to its solubilizing ability toward the drug. In particular, the obtained result can be attributed to the capacity of Me-βCD to extract lipids, such as cholesterol and triglycerides, from the stratum corneum and to complex them, thus temporaneously decreasing the skin barrier properties (Másson et al., 1999; Lopez et al., 2000; Babu and Pandit, 2004). A synergistic effect with Transcutol can be also hypothesized, considering the ability to this last enhancer to interact with the lipid components of the skin (Gannu et al., 2008; Shokri et al., 2012).

4. Conclusions

Gels containing liposomal or microemulsion formulations for transdermal delivery of CLZ have been successfully developed and characterized and the possible enhancing effects provided by the addition of Me-βCD to the different preparations have been investigated.

Permeation studies through artificial membranes evidenced a positive effect of liposomal formulations on drug permeability with respect to the simple aqueous suspension of the drug, and this effect became significantly more evident ($P < 0.05$) in the presence of Me-βCD, reasonably in virtue of its interaction with the bilayer of the liposomal vesicles. However, gels containing O/W microemulsions, were significantly more effective than liposomal formulations ($P < 0.05$) in improving the drug permeation rate, allowing a more than 4-fold increase in CLZ apparent permeability with respect to the simple aqueous suspension of the drug, and no significant changes were observed in the presence of Me-βCD.

On the contrary, permeation studies through excised rabbit ear as skin membrane model performed on microemulsion-based formulations, showed a more than 100% increase in the drug apparent permeability as a consequence of Me-βCD addition to the formulation, putting in evidence a true skin penetration enhancer effect of Me-βCD related, in this case, to its complexing power toward the skin lipids, probably facilitated by the presence of Transcutol, in virtue of its known ability to interact with the components of the skin barrier.

In conclusion, the microemulsion formulation containing Me-βCD was the most effective in improving CLZ skin permeation. Further *in vivo* studies will be performed to evaluate the actual therapeutic effectiveness of the developed transdermal formulation of CLZ, as an alternative to its oral administration.

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