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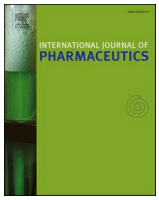

Cyclodextrin complexation as a fruitful strategy for improving the performance of nebivolol delivery from solid lipid nanoparticles

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ABSTRACT

Oral bioavailability of nebivolol (NEB), a highly-selective β1-adrenergic receptor antagonist specially used in hypertension treatment, is limited by its low aqueous solubility. In this work we investigated the possibility of developing a new effective oral formulation of NEB by exploiting a combined strategy based on NEB complexation with hydroxypropyl-βCyclodextrin (HPβCD) and complex incorporation into solid lipid nanoparticles (SLNs). Solubility studies enabled to choose Imwitor 491 and 988 as solid lipids for SLN preparation. The effect of their separated or combined use, at different amounts, and of different surfactants on nanoparticles dimensions, homogeneity and surface charge was examined. The best formulations were selected for drug loading, as such or as complex with HPβCD, and evaluated for physicochemical properties, morphology, entrapment efficiency and drug release. A comparison of the two kinds of formulations revealed that the presence of HPβCD improved SLNs quality in terms of reduced dimensions, higher homogeneity and greater physicochemical stability, avoiding the sharp Zeta Potential reduction observed when loading the plain drug; moreover, it allowed a marked increase in entrapment efficiency and better control of drug release. Furthermore, the use of HPβCD gave the opportunity of doubling drug loading without noticeable variations in SLNs physicochemical properties and maintaining excellent entrapment efficiency.

1. Introduction

Nebivolol Hydrochloride (NEB) is a third generation β_1-selective beta-blocker with vasodilating properties and good tolerability, widely used in the control and management of hypertension and hearth failure (Fongemie and Felix-Getzik, 2015; Borghi et al., 2017; Olawi et al., 2019; Ferri, 2021). According to the Biopharmaceutical Classification System (BCS), NEB belongs to the Class II drugs, being characterized by high membrane permeability and very poor aqueous solubility and dissolution rate. For such a class of therapeutic agents, dissolution in the gastrointestinal tract represents the rate-controlling step in the oral absorption process. In fact, the low and variable oral bioavailability of NEB has been mainly ascribed to its low water solubility (Gielen et al., 2006; Moen and Wagstaff, 2006). Therefore, several strategies aimed at improving NEB solubility and dissolution rate, and then, hopefully, its bioavailability, have been investigated during last years, such as the development of solid dispersions with hydro-soluble carriers (Shah et al., 2015; Raj and Kumar, 2018; Mude et al., 2021), nanosuspension tablets (Thadkala et al., 2015), self-emulsifying systems (Rao et al., 2016; Siriah and Puranik, 2018), cocrystals (Nikam and Patil, 2020; Devi et al., 2023), microemulsions (Kaur et al., 2021), liquid self-nanoemulsions (Sabri and Hussein, 2021), liquisolid compacts (Sabri and Hussein, 2020; Sura et al., 2022) or micellar systems (Paul Raj et al., 2024).

Complexation with cyclodextrins (CDs) is another approach that has been widely and successfully exploited for improving the solubility and bioavailability of several kinds of poorly water-soluble drugs (Saravana et al., 2013; Vikas et al., 2018). CDs are cyclic oligosaccharides formed by (α-1,4)-linked D-glucopyranose units. Their peculiar three-dimensional structure, presenting an internal non-polar cavity and an outer hydrophilic surface, makes them able to encapsulate a variety of non-polar molecules, leading to the formation of inclusion complexes (Del Valle, 2004). Both natural and derivative CDs have found large applications in the pharmaceutical field, mainly in virtue of their ability of enhancing aqueous solubility, stability and bioavailability of hydrophobic drug molecules as a consequence of inclusion complexes formation (Duchêne and Bochot, 2016; Sarabia-Vallejo et al., 2023). We recently proved the usefulness of CD complexation in improving NEB

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solubility and dissolution rate from conventional tablets (Maestrelli et al., 2024).

A further interesting and profitable strategy proposed for enhancing the oral bioavailability of poorly-water soluble drugs is the use of lipid-based nanoparticulate carriers, such as the solid lipid nanoparticles (SLNs) (Ekambaram et al., 2012; Geszke-Moritz and Moritz, 2016; Scioli Montoto et al., 2020). SLNs formulations are usually obtained as a dispersion in an aqueous phase of solid, lipid matrices composed of biocompatible and biodegradable excipients. They have demonstrated to represent a valid alternative to liposomes, showing comparable safety in use but greater stability as well as more protection of the incorporated drug, in virtue of the presence of a solid matrix, in addition to the lower cost of their raw materials, compared to the phospholipids (Mehnert and Mäder, 2012; Mishra et al., 2018). It has been reported that NEB-loaded PEG-modified SLNs exhibited improved in vitro permeability compared to conventional tablets (Üstündağ-Okur et al., 2016; Kaynak et al., 2018).

Combined strategies based on the combined use of cyclodextrins (CDs) and nanocarriers, including SLNs, proved to provide numerous advantages, allowing to overcome most of the issues associated with such carriers when used separately, while joining their respective benefits (Mura, 2020; Real et al., 2021). In particular, the effectiveness of simultaneously exploiting the CD complexing ability, to improve the solubility of poorly water-soluble drugs, together with the carrier function of solid lipid nano-carriers, has been shown by different authors (Chirio et al., 2009; Carlotti et al., 2012; Spada et al., 2012; Cirri et al., 2017, 2018; Pires et al., 2019; Dudhipala et al., 2021).

Therefore, based on these premises, the aim of the present work was to develop a new effective oral formulation of NEB in SLN carriers and to investigate the usefulness of loading the drug as CD complex rather than as pure drug, in order to assess the actual advantages of the above described joined approach. Different solid lipids and surfactants were tested for SLN preparation. Selected formulations were loaded with the drug alone or as CD complex and suitably characterized and compared in terms of particle size, polydispersity index, Zeta-potential, encapsulation efficiency, release properties, cytotoxicity and stability.

2. Materials and Methods

2.1. Materials

Nebivolol.HCl (NEB) (2,2′-Azanediylbis(1-(6-fluorochroman-2-yl) ethanol) hydrochloride) was a kind gift of Menarini S.p.a. (L'Aquila, Italy). Hydroxypropyl-βCD (HPβCD), loss on drying 10 % max, total impurities (on D.S.) 1 % max, Molar substitution (MS) 0.81–0.99, was kindly supplied by Roquette (Lestrem, France) (Roquette, 2022). Glyceryl palmitostearate (Precirol® ATO5), Glyceryl behenate (Compritol® 888 ATO), Glycerol monostearate (Geleol®) and Polyoxyl-32 Stearate (Gelucire® 48/16) were kindly gifted by Gattefossé (Saint-Priest, Cedex, France). Cetyl palmitate and glyceryl tripalmitate were from Sigma Aldrich (St. Louis, MO, USA). Glyceryl monostearate (Imwitor® 491) and glycerol monocaprylate Type 1 (Imwitor® 988) were kindly provided by IOI Oleo GmbH (Witten, Germany). Polyoxyethylene sorbitan monooleate (Tween® 80) was from Merck (Hohenbrunn, Germany) and Poloxamer 188 (Pluronic® F68) from BASF (Ludwigshafen, Germany). All other chemicals and solvents were of analytical reagent grade.

2.2. Screening of solid lipids

An initial screening of various solid lipids as possible components for the preparation of SLNs was performed by evaluating their solubilizing ability towards the drug. Briefly, the experiments were carried out by adding 1 mg at a time of NEB to 250 mg of each lipid; the obtained mixture was then heated under stirring up to about 80 °C to melt the lipid. The drug solubility was then visually assessed verifying the attainment of a clear solution and the absence of drug crystals (Kasongo et al., 2011; Cirri et al., 2017). The procedure was repeated until drug saturation was achieved.

2.3. Preparation of the solution containing the NEB-HPβCD inclusion complex

According to the results of previous studies (Maestrelli et al., 2024), the NEB-HPβCD inclusion complex was formed by dissolving 25 mM HPβCD in water, adding NEB (2.5 mM) and stirring over 72 h. The obtained solution contained 1 mg/mL NEB as HPβCD complex.

2.4. Preparation of solid lipid nanoparticles (SLNs)

SLNs were prepared using the Hot High-Shear Homogenization (HSH) technique as described by Lopes et al. (Lopes et al., 2012) and Gonçalves et al. (Gonçalves et al., 2016), slightly modified. Briefly, the selected solid lipids (singularly or in mixture and in different amounts, depending on the formulation) were heated in a water bath at 80 °C, up to melting. In the meantime, the aqueous phase, containing the surfactant (Tween® 80 or Pluronic® F68 at 1.0 or 1.5 % w/v) was heated at the same temperature, and then 15 mL were added to the fused lipid. An emulsion was then obtained by homogenization at 10,000 rpm (high shear homogenizer Silverson L5M, Chesham, UK). Finally, the obtained dispersion was cooled down to 4 °C. In the case of SLNs incorporating NEB (5 mg), when it was used as such, it was dissolved into the melted lipid phase, while, when it was used as HPβCD complex, the hot aqueous phase to be added to the melted lipid contained also the suitable amount of the drug-HPβCD solution (Carlotti et al., 2012; Spada et al., 2012).

2.5. Determination of particle size, polydispersity index (PDI) and Zeta Potential of SLNs

Measurements of SLN mean diameter and polydispersity index (PDI) were performed at 25 °C by dynamic light scattering using a Malvern Zetasizer NanoS (Malvern Instruments Ltd, Malvern, UK). The surface charge (Zeta Potential, ZP) was determined by laser doppler microelectrophoresis at 25 °C by Malvern Zetasizer NanoZ (Malvern Instruments Ltd, Malvern, UK). All measurements were carried out after suitable dilution of the formulations with distilled water.

2.6. Transmission electron microscopy (TEM)

The samples were analyzed using a Gaia 3 (Tescan s.r.o, Brno, Czech Republic) FIB-SEM (Focused Ion Beam-Scanning Electron Microscope) operating in high-vacuum mode with electron beam voltage of 10 kV and bright-field transmission electron microscope detector.

To perform the analysis a drop (10 μL) of sample dispersion is applied on a carbon film-covered copper grid. Most of the dispersion is blotted from the grid with filter paper to form a thin film specimen, which is stained with a 1 % w/v phosphotungstic acid solution (PTA) in distilled water, and then the samples are dried for 3 min before the characterization. The digital images were taken with a magnification appropriate to display the morphology of the formulations.

2.7. Determination of drug entrapment efficiency (EE%)

Entrapment efficiency of SLNs was assessed by using the indirect method (Cirri et al., 2012; Gonçalves et al., 2016). The amount of the unentrapped drug present in the aqueous dispersion was estimated after separation from SLNs by ultrafiltration–centrifugation (Amicon Ultra-4 centrifugal filters, 100 kDa MWCO, Merck Millipore, Darmstadt, Germany) at 4500 rpm for 20 min at 4 °C. The concentration of free drug in the aqueous phase, collected in the outer chamber of the ultrafiltration device, was then determined by UV assay at 281 nm (UV/Vis 1601 Shimadzu Spectrophotometer, Tokyo, Japan). The entrapment efficiency (EE%) was finally obtained using the following equation:

$$EE\% = \frac{Wtotal\ drug - Wfree\ drug}{Wtotal\ drug} x100 \quad (1)$$

where $W_{total\ drug}$ is the drug amount initially added and $W_{free\ drug}$ the amount of drug in the aqueous phase after ultrafiltration-centrifugation.

2.8. In vitro drug release studies

Release studies from SLNs were carried out by a dialysis membrane method (Silva et al., 2017). Each sample (1 mL) was put into the semi-permeable dialysis bag (SpectraPor® Biotech Grade Cellulose Ester membranes, MWCO 100 kDa, Repligen, Waltham, MA, USA), and then immersed 2 h in 15 mL of pH 1.2 simulated gastric fluid (HCl 0.1 N and NaCl 2.0 g/L) and after 4 h in 15 mL of pH 6.8 simulated intestinal fluid (6.8 g/L KH_2PO_4 and NaOH 0.89 g/L) both thermostated at 37 °C and stirred at 300 rpm. Samples (200 μL) were taken at given intervals up to 360 min, and immediately replaced by an equal volume of fresh medium kept at the same temperature. A correction was made for the cumulative dilution. The amount of released drug was assayed by UV-spectrophotometry at 281 nm (UV/Vis 1601 Shimadzu Spectrophotometer, Tokyo, Japan). Each test was repeated at least three times. Drug release data were analyzed according to the zero-order, first-order Higuchi, and Korsmeyer–Peppas kinetic models. The model better fitting experimental data was determined on the basis of the highest correlation coefficient (r^2) value. The principal mechanism of drug release was also deduced by the value of the exponent n of the Korsmeyer–Peppas equation:

$$\frac{M_t}{M_\infty} = K_{KP} t^n \quad (2)$$

where M_t is the cumulative amount of drug released at time t, M_∞ the initial total NEB amount, K_{KP} the release constant and n the diffusional exponent. Values of $n < 0.5$ indicate Fickian diffusion, n between 0.5 and 0.9 non-Fickian (anomalous) transport and $n > 0.9$ type-II transport.

2.9. Cytotoxicity assays

CaCo-2 cells (human epithelial colorectal adenocarcinoma cell line) provided by the American Type Cell Culture Collection, ATCC® HTB-37™, Rockville, MD, USA) were used for cytotoxicity studies. Cytotoxicity was tested using propidium iodide (PI) exclusion assay, following a previously set procedure (Gaspar et al., 2016; Nerli et al., 2023).The day before the experiment, the cells were seeded on sterile 96-well plates (Greiner Bio-One, Kremsmünster, Austria) at a cell density of $2x10^5$ cells/mL (100 μL per well), and incubated at 37 °C and 5 % CO_2 in RPMI 1640 culture medium supplemented with 10 % foetal serum bovine, 100 units/mL of penicillin G (sodium salt), 100 μg/mL of streptomycin sulphate and 2 mM L-glutamine (all from Thermo Fisher Scientific, Waltham, MA, USA). After 24 h, the medium was replaced by fresh medium containing the different empty and NEB-loaded (0.33 mg/mL 0.75 mM) SLN samples (each analyzed in 4 wells per plate in 3 independent plates). The culture medium was used as negative control and sodium dodecyl sulphate (SDS) at 1 mg/mL as positive control. After further 24 h, the medium was replaced with 0.3 μM propidium iodide (PI) solution (stock solution 1.5 mM in DMSO) diluted with culture medium (1:5). PI is a red fluorescent probe that only penetrates through cells whose membrane integrity is compromised; when PI reaches and interacts with nucleic acids, its fluorescence strongly increases and therefore it is used to verify the cellular membrane integrity. Fluorescence (excitation wavelength 485 nm; emission wavelength 590 nm) was measured by a Microplate Reader (FLUOstar Omega, BMGLabtech GmbH, Ortenberg, Germany). The data were expressed as PI uptake by using the following equation:

$$PI\ uptake = \frac{Fluorescence_{sample}}{Fluorescence_{control}} \quad (3)$$

Table 1
Solubility of NEB in melted solid lipids.

Melted solid lipid (250 mg)	drug added(mg)	System appearance
Precirol® ATO5	1	Turbid
Compritol® 888ATO	1	Turbid
Geleol®	1	Turbid
Gelucire®	1	Turbid
Cetyl palmitate	1	Turbid
Tripalmitin	1	Turbid
Imwitor® 988	2	Transparent
Imwitor® 988	3	Turbid
Imwitor®491	3	Transparent
Imwitor®491	4	Turbid

where $Fluorescence_{sample}$ and $Fluorescence_{control}$ are the fluorescence intensity values obtained for cells treated with samples and for cells incubated with culture medium, respectively.

2.10. Stability studies of the selected SLN formulations

Drug-loaded selected SLN formulations were stored for 2 months at 4 ± 1°C and checked every 15 days for mean diameter, PDI, and Zeta potential by DLS as described in Section 2.5. Samples were also visually inspected to point out possible formation of mold or appearance of aggregation or crystallization/precipitation phenomena. At the end of the storage period, SLN formulations were also examined for drug EE%. All the measurements were carried out in triplicate and the results were averaged.

2.11. Statistical data analysis

Statistical evaluation of data was carried out by one-way analysis of variance (ANOVA) and the Student-Neman-Keuls comparison post hoc test (GraphPad Prism software, version 6.0, San Diego, CA, USA) was used to assess the significance of the differences. P values < 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Screening of solid lipids for SLNs preparation

An adequate solubility of the drug in the lipid carrier is an important factor to enable a good loading ability into SLNs (Müller et al., 2000). Therefore, as a first step in developing the SLNs formulations, the solubility of NEB in various melted solid lipids has been evaluated, in order to select the most effective ones.

A turbid dispersion was obtained after the addition of only 1 mg drug to most of the melted solid lipids, indicating the presence of undissolved drug (Table 1). A clear solution was obtained only in the case of Imwitor®491 and 988, that enabled the complete NEB dissolution. Further drug additions indicated that 250 mg of melted Imwitor® 988 and Imwitor®491 allowed to dissolve 2 mg and 3 mg drug, respectively.

3.2. Preparation and characterization of SLNs

Based on the solubility screening with various types of solid lipids, Imwitor® 491 and Imwitor® 988 were selected in virtue of their greater solubilizing power towards the drug. Pluronic® F68 and Tween® 80 were instead chosen as non-ionic hydrophilic surfactants/stabilizers, due to their common use in SLN preparation (Müller et al., 2011; Cirri et al., 2017). In fact, surfactants act by decreasing the interfacial tension between hydrophobic surface of the lipid nanoparticles and surrounding aqueous medium, thus contributing to stabilize the whole SLN structure (Müller et al., 2011). A preliminary series of empty SLN formulations was prepared using as solid lipid Imwitor® 491, alone or in mixture with Imwitor® 988, dispersed in 15 mL of Pluronic® F68 (1.5 %) or Tween®

Table 2
Composition of the first series of empty SLNs formulations.

Formulation code	Imwitor® 491 (mg)	Imwitor® 988 (mg)	Stirring time (min)	Surfactant solution (mL)	
				Pluronic®F68 1.5 %	Tween® 80 1.5 %
SLN1 a	300	–	8	15	–
SLN1 b	300	–	5	15	–
SLN1 c	300	–	3	15	–
SLN2 a	300	–	8	–	15
SLN2 b	300	–	5	–	15
SLN2 c	300	–	3	–	15
SLN3	250	250	5	–	15
SLN4	250	250	5	15	–

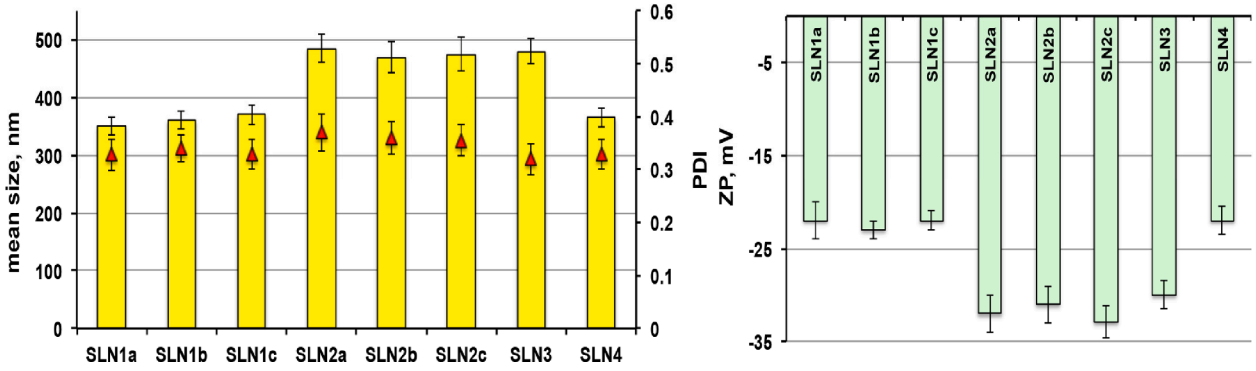

Fig. 1. Mean size, polydispersity index (PDI) and Zeta Potential (ZP) of the first series of empty SLNs. See Table 1 for their composition.

80 (1.5 %) aqueous solution, representing the aqueous phase. Formulations containing Imwitor® 491 were prepared using different homogenization time (8, 5 or 3 min), to evaluate the influence of such a variable on the SLNs properties. The composition of these preparations is shown in Table 2.

The obtained SLNs were characterized for particle size, polydispersity index (PDI) and Zeta Potential (ZP), that are recognized as the most crucial parameters affecting their biological performance and physical stability. Generally, a small particle size, around or below 300 nm, is preferred, since it seems that it should provide a more rapid action of the carried drug and an improvement in cellular uptake rate (Andrysek, 2003). On the other hand, low PDI values are desirable (possibly < 0.3), as indicative of highly homogeneous systems; on the contrary high ZP values (i.e. possibly around $\pm$ 30 mV or even higher), are important to assure the physical stability of the nanoparticles dispersion, avoiding potential agglomeration, aggregation and/or flocculation phenomena, due to electrostatic repulsion of the nanoparticles (Mehnert and Mäder, 2012).

As can be seen in Fig. 1, the dimensions of SLNs prepared using Imwitor® 491 and Pluronic® F68 (series SLN1) were significantly smaller compared to the corresponding ones prepared with Tween® 80 (series SLN2). Analogous results have been found by other authors (Bhupinder and Newton, 2016; Cirri et al., 2017) and explained with a different mode of incorporation of the molecules of the two surfactants into the nanoparticles solid shells (Radomska-Soukharev, 2007). Moreover, Tween® 80-containing SLNs exhibited slightly higher PDI values than those containing Pluronic® F68, indicative of a little less homogeneous dispersion. On the other hand, Tween® 80 allowed to obtain more highly negative ZP values than Pluronic® F68. Moreover, no significant differences ($P > 0.05$) in terms of mean dimensions, PDI or particle surface charge were observed when comparing the results obtained for SLN1 and SLN2 formulations when the high-shear homogenization time was changed (3, 5 or 8 min, series a, b, c, respectively). For this reason, all the following formulations were prepared using a constant homogenization time of 5 min.

As for the SLNs containing the mixture of solid lipids, they showed similar particle size and slightly lower PDI values than the previous corresponding ones containing only Imwitor® 491. Moreover, also in this case, the formulation with Tween® 80 (SLN3) showed a more negative ZP value than the corresponding with Pluronic®F68 (SLN4). Thus, despite the greater mean dimensions of the nanoparticles, SLN3 formulation was chosen for drug loading. Based on the previous solubility study (Table 1), 5 mg of drug were added to the solid lipid mixture. Actually, the drug-loaded formulation (SLN3DL) showed a rather satisfying entrapment efficiency (60 $\pm$ 2 %). However, the presence of NEB gave rise to a strong decrease of the negative ZP, that was reduced to -9 ± 2 mV, value considered not sufficient to effectively prevent potential aggregation phenomena and thus provide an adequate physical stability of the colloidal dispersion.

It was then decided to prepare a new series of SLNs formulations by varying the amount of Imwitor® 491 or using it in combination with Imwitor® 988, while Pluronic®F68 was selected as surfactant, considering its ability to give rise to nanoparticles of smaller dimensions (Fig. 1). The composition of the new formulations is presented in Table 3, while their physicochemical properties are shown in Fig. 2.

As expected, SLNs particle size slight increased with increasing the amount of solid lipid in the formulation. Anyway, their dimensions did not exceed in any case 350 nm. PDI values were always lower than 0.35,

Table 3
Composition of the second series of empty SLN formulations.

Formulation code	Imwitor® 491 (mg)	Imwitor® 988 (mg)	Pluronic®F68 solution (mL)	
			1.5 %	1.0 %
SLN5	100	–	–	15
SLN6	250	–	–	15
SLN7	100	–	15	–
SLN8	250	–	15	–
SLN9	100	100	15	–
SLN10	250	250	15	–
SLN11	100	100	–	15
SLN12	250	250	–	15

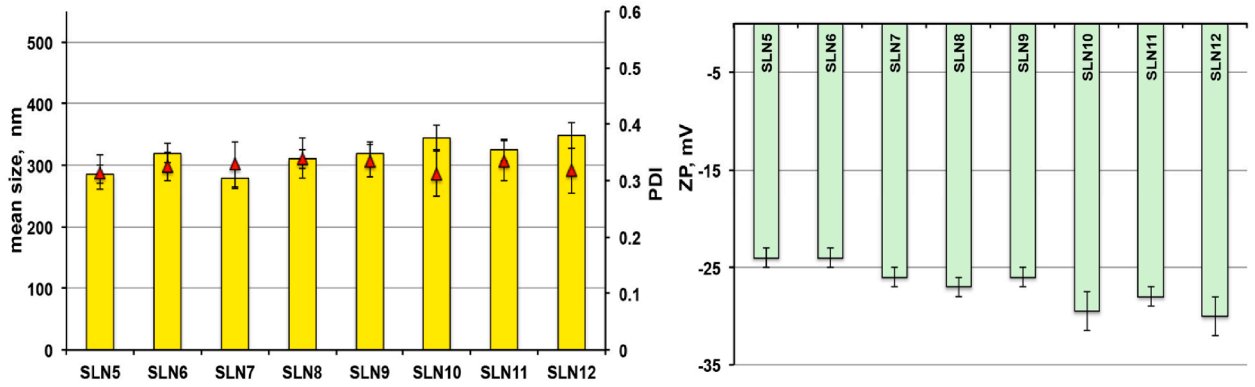

Fig. 2. Mean size, polydispersity index (PDI) and Zeta Potential (ZP) of the second series empty SLNs. See Table 3 for their composition.

Table 4
Composition of the series of SLN formulations loaded with the NEB-HPβCD complex.

Formulation code	Imwitor® 491 (mg)	NEB (mg)*	surfactant solution (mL)		
			Pluronic®F68 1.5 %	Pluronic®F68 1.0 %	Tween®80 1.5 %
SLN13	300	–	15	–	–
SLN14	300	–	–	15	–
SLN15	300	–	–	–	15
SLN13DL	300	5	15	–	–
SLN14DL	300	5	–	15	–
SLN15DL	300	5	–	–	15
SLN16DL	300	7.5	15	–	–
SLN17DL	300	10	15	–	–

*mg NEB as NEB-HPβCD complex.

indicating the satisfying homogeneity of all the colloidal systems. Finally, as for ZP values, they ranged from −24 to −30 mV, suggesting good physical stability of the nanoparticles. Based on the obtained results, SLN10 and SLN12 were selected for NEB loading (5 mg) considering not only their highest ZP values, but also their greater solubilizing power towards the drug (due to the higher content in lipids).Unfortunately, as previously observed in the case of formulation SLN3, the drug addition to both such formulations (SLN10DL and SLN12DL) gave rise to a sharp reduction of the ZP, that decreased up to −10 ± 2 mV, independent on their different Pluronic ®F68 concentration (1.5 or 1.0 %, respectively). Moreover, an entrapment efficiency of 35 ± 1 % and 28 ± 1 % was obtained for SLN10DL and SLN12DL respectively, clearly lower than that obtained for SLN3DL (60 ± 2 %).

3.3. Preparation and characterization of SLNs containing the drug as CD complex

Due to the problems encountered in the phase of drug loading, both in terms of unsatisfying entrapment efficiency and of loss of physico-chemical stability of the colloidal dispersion, owing to the sharp decrease of the nanoparticles surface charge, as a continuation of the study, we investigated the possibility of improving the SLN formulation performance by loading NEB as CD complex, considering the successful results achieved by applying such a combined strategy in previous SLN formulations (Cirri et al., 2017).

Among the different CDs previously tested for their complexing and solubilizing properties towards NEB (Maestrelli et al., 2024), hydroxypropyl-β-CD (HPβCD) was selected, considering both its absence of toxicity, being it admitted also for parenteral use, and the appropriate value of the stability constant of its complex with NEB (654 M^{-1}); on the

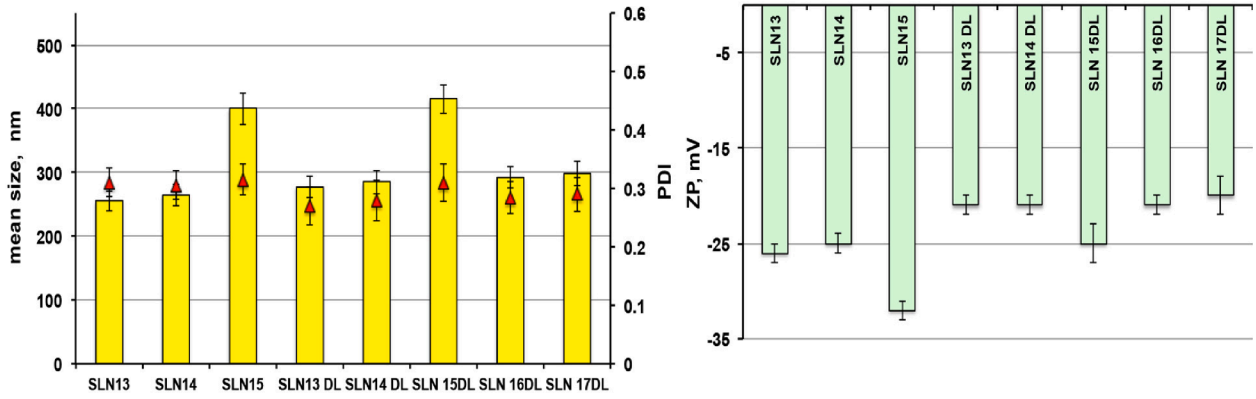

Fig. 3. Mean size, polydispersity index (PDI) and Zeta Potential (ZP) of the new series of SLNs empty or drug-loaded (DL) with NEB as HPβCD complex. See Table 4 for their composition.

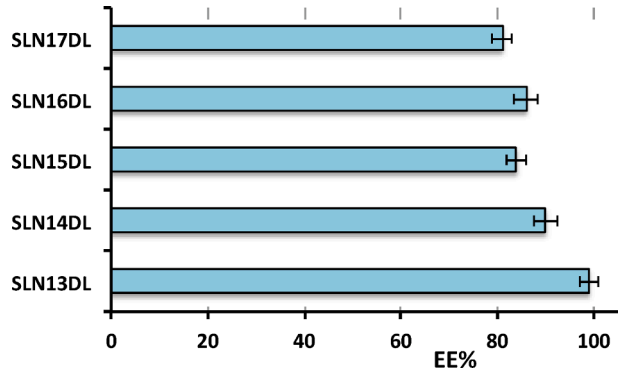

Fig. 4. Entrapment efficiency (EE%) values of the new series of SLNs loaded with NEB as HPβCD complex. See Table 4 for their composition.

contrary, the use of sulfobutylether-β-CD (SBEβCD) was discarded, despite its higher solubilizing power towards NEB, since the stability constant of the NEB-SBEβCD complex (2306 mM^{-1}) (Maestrelli et al., 2024) was considered too high, and thus able to excessively slow down the drug release (Cavalli et al., 1999). Moreover, it has been previously found that the SBEβCD presence gave rise to problems in preparing both SLN and NLC formulations (Cirri et al., 2018, 2017), that were ascribed to possible interactions between this anionic CD and the surfactants present in this kind of formulations. On the other hand, HPβCD, is nonionic and its interaction with drug and SLN components can be ascribed only to hydrophobic interactions with the internal cavity and the surfactant properties of the CD.

For obtaining drug-loaded SLNs, the hot aqueous phase of the formulation to be added to the melted solid lipid contained also the required volume of the prepared solution with the NEB-HPβCD complex. The composition of these formulations and their physicochemical properties are shown in Table 4 and in Fig. 3, respectively.

As we can see, the presence of HPβCD in the aqueous phase of the empty formulations (SLN13-15), did not interfere with the formation of SLNs which showed comparable values of ZP with respect to those of previous analogous empty formulations (SLN1 and SLN2) not containing the CD. On the other hand, some reduction in the particle size and PDI values was observed and attributed to both the lower amount of lipid present in these last formulations, as well as to the CD presence (Cavalli et al., 1999). Moreover, as already observed above, SLNs containing Tween®80 presented larger dimensions and higher negative ZP values than the corresponding containing Pluronic®F68, while no significant variations were observed by varying the Pluronic®F68 content (1.0 or 1.5 %).

However, it is important to observe that in this case, unlike the previous ones, the drug loading caused only a limited reduction of the ZP which decreased from –32 to −25 mV and from −25 to −20 mV for SLNs containing Tween®80 or Pluronic®F68, respectively. This result can be explained considering that in such last formulations the drug was added not as such but as complex with HPβCD, and then we are in the presence of a combined drug-in CD-in SLN system. The ZP values obtained for these drug-loaded SLN formulations can be considered enough to assure a good nanoparticles protection against agglomeration phenomena, considering also the stabilizing properties of the emulsifiers Tween®80 and, particularly, Pluronic®F68 (Zimmermann and Müller, 2001). Moreover, no further reduction of ZP values was observed by increasing the drug loading amount up to 10 mg (formulations SLN16DL and SLN17DL). Finally, according to the literature (Cavalli et al., 1999; Chirio et al., 2009), a favorable effect of the CD presence on the mean size and homogeneity of the SLN colloidal dispersion was also observed, as indicated by the smaller nanoparticle dimensions and lower PDI values (all < 0.3) than the previous formulations.

The results of Entrapment Efficiency (EE%) determination are shown in Fig. 4.

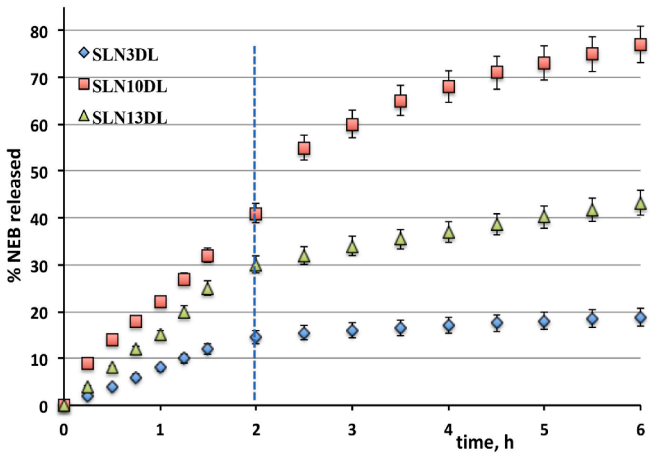

Fig. 5. Release curves in simulated gastric fluid (first 2 h) and in simulated intestinal fluid of NEB from SLN3DL, SLN10DL and SLN13DL formulations (see Tables 2, 3 and 4, respectively, for their composition).

As we can see, a beneficial effect of CD complexation emerged for all formulations, that showed EE% values > 80 %, clearly higher than those obtained with all the previous SLNs loaded with the drug as such. In particular the highest EE%, around 100 %, was obtained with the formulation SLN13DL, containing Pluronic®F68 at 1.5 %; on the contrary, being the same the drug loaded amount (5 mg), the lowest EE% (around 84 %) was found for the formulation SLN15DL, containing Tween®80 at 1.5 %. On the other hand, being the Pluronic®F68 content the same (1.5 %), a progressive slight decrease of EE % was found with increasing the drug loading amount; however, the total amount of drug actually entrapped into the nanoparticles continued to increase, since SLN16DL and SLN17DL have been formulated with a higher amount of NEB as NEB-HPβCD complex (Table 4).

Comparable positive effects on EE% improvement when loading the drug in combination with a suitable CD have been reported in case of SLNs loaded with hydrocortisone as βCD complex, (Cavalli et al., 1999), or of curcumin as complex with γ-CD derivatives (Chirio et al., 2009) as well as of NLCs loaded with ketoprofen as complex with polymeric βCD (Cirri et al., 2012), obtaining in this last case an EE% increase of 70 and 77 %, respectively, compared to the corresponding formulations loaded with the plain drug. A similar positive finding was also observed for SLN and NLC formulations loaded with hydrochlorothiazide in the presence of HPβCD (Cirri et al., 2017, 2018).

3.4. Release assay

The *in vitro* release profiles of NEB from the three kinds of drug-loaded SLN formulations are shown in Fig. 5.

As can be observed, very different results were obtained from SLN3DL and SLN10DL formulations, despite the only difference in their composition was the use as surfactant of Tween® 80 (SLN3DL) or Pluronic®F68 (SLN10DL), respectively. It seemed that the use of Tween® 80 gave rise to a too slow NEB release, achieving only about 20 % after 6 h; on the contrary, a higher release rate was observed for the formulation containing Pluronic®F68 (SLN10DL), reaching around 80 % of released drug after 6 h. However, it has to be considered that SLN3DL contained a higher % of entrapped drug than SLN10DL (60 vs 30 %), and this contributed to provide a slower, more prolonged release.

An intermediate release rate was obtained from the formulation SLN13DL, containing 1.5 % Pluronic®F68 as surfactant, as formulation SLN10DL, but loaded with the drug as HPβCD complex, which reached around 45 % of released drug after 6 h. The slower release rate can be attributed to the almost 100 % entrapment of the drug into the SLNs, compared to only 30 % obtained with SLN10DL, as well as to the presence of the drug as CD complex, that can further control the drug release

Table 5
Correlation coefficients (R^2) and release exponent (n) values obtained from the different kinetic models for the selected SLNs formulations.

Kinetic Model	SLN3DL	SLN10DL	SLN13DL
Zero-order	0.8220	0.9316	0.8765
First-order	0.6106	0.6105	0.5903
Higuchi	0.9246	0.9765	0.9592
Korsmeyer-Peppas	0.9422	0.9942	0.9764
Korsmeyer-Peppas (n)	0.56	0.75	0.68

rate (Cavalli et al., 1999; Pires et al., 2019; Spada et al., 2012). In fact, it has been found that the release rate of drug-CD complexes from SLNs was inversely related to the stability of the complexes (Cavalli et al., 1999).

In order to gain some information about the mechanism of drug release form the developed SLNs formulations, and the possible influence exerted by the HPβCD presence, a kinetic evaluation of the release profiles was carried out by fitting the data into the zero-order, first-order, Higuchi and Korsmeyer–Peppas kinetic models. The goodness-of-fit of the different models to the release curves was evaluated on the basis of the obtained correlation coefficients (R^2), reported in Table 5. The release exponent n obtained by the Korsmeyer–Peppas equation has been also presented.

As we can see, the NEB release profile from all the examined formulations showed the best fit to the Korsmeyer-Peppas model, followed by the Higuchi one. Moreover, in all cases, n values > 0.5 were obtained, considered indicative of a non-Fickian anomalous diffusion transport, which has been recognized as common in many kinds of porous media (Yang and Wang, 2019).

Interestingly the different way of drug entrapment, i.e. as pure drug in the lipid phase or as HPβCD complex in the aqueous phase of the SLNs, as well as the presence or not of HPβCD strongly influenced entrapment efficiency and drug release rate but did not substantially affect the drug release mechanism.

3.5. Morphological studies

The three selected drug-loaded formulations (SLN3DL, SLN10DL and SLN13DL) were also subjected to TEM investigations, to confirm the nanoparticle formation and point out eventual differences in morphology due to their different composition as well as to the presence or no of HPβCD. As can be seen in Fig. 6, where some selected photomicrographs are shown, the TEM study showed that nanoparticles were actually obtained in all cases, all exhibiting an almost uniform, round shape. Moreover, no agglomeration or cluster-formation phenomena were detected, indicating the obtainment in all cases of homogeneous colloidal dispersions. Finally, no clear differences in morphology were observed between SLNs containing the drug as such or as HPβCD inclusion complex.

3.6. Cytotoxicity assays

A cytotoxicity evaluation of these formulations was finally performed according to the Propidium Iodide (PI) test (Fig. 7).

Values of PI uptake close to 1 (as obtained for the negative control) are indicative of the membrane cellular integrity and then of absence of cytotoxicity. As can be seen, both the empty and the drug-loaded SLN formulations showed higher PI uptake values than the control, even though clearly lower than the positive control, indicating a slight cytotoxicity. However, after a 1:1 dilution, PI values around 1, similar to those of the negative control ($P > 0.1$), were obtained for SLN10and SLN13 formulations, both empty or drug-loaded, denoting total absence of cytotoxicity. On the contrary, in the case of formulation SLN3, both in the absence and in the presence of NEB, some cytotoxicity was ever observed also after the 1:1 dilution; moreover, it is noteworthy to point out that the blank formulation showed a PI uptake value slightly higher (even if not statistically significant ($P > 0.05$)) with respect to the corresponding one containing the drug. This indicated that the cytoxicity issue was due to some formulation component, and not to the drug addition. A 1:4 dilution was necessary to achieve a full cytotoxicity absence. Interestingly, formulation SLN3 was the only one of the three formulations that contained Tween® 80 as surfactant. Therefore this finding indicated that Tween® 80, despite having shown the lowest cytotoxic effect compared to other five different surfactants (Arechabala et al., 1999), resulted more cytotoxic than Pluronic®F68.

3.7. Storage stability studies

A first short-term storage stability study was finally performed on the selected drug-loaded SLN formulations, that were kept two months at 4 ± 1°C. The colloidal dispersions were monitored in terms of particle size, PDI and Zeta potential variations considered as an index of their physicochemical stability (Fig. 8).

As can be observed in Fig. 8, a slight but progressive increase in particle size and, more evident, in PDI, with a concomitant slight, even though not significant ($P > 0.05$), reduction of the surface charge of the nanoparticles was detected in the case of SLN3DL and SLN10DL formulations, that could be a sign indicative of a potential tendency to agglomeration phenomena. On the contrary, such variations were not pointed out in the case of SLN13DL formulation, containing the drug as inclusion complex with HPβCD, whose parameters remained almost constant during the considered storage period, thus confirming the expected greater physical stability of such a colloidal dispersion, based on its higher Zeta Potential. At storage study termination, the entrapment efficiency (EE%) of the formulations was checked, to evaluate the extent of possible drug leakage occurrence. In all cases a very small and not significant ($P > 0.05$) EE% reduction was found, never exceeding 3 %, indicating a low tendency of all the formulations to an early drug expulsion. However, SLN13DL emerged also in this case as the best formulation, showing to be able to maintain EE% values around 100 %,

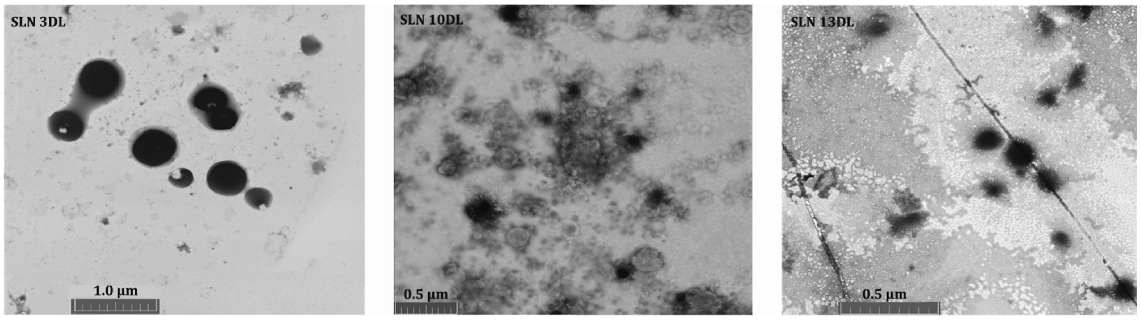

Fig. 6. TEM photomicrographs of the selected drug-loaded SLNs. The images were taken with different appropriate magnifications, to appreciate the morphology of the different kinds of nanoparticles.

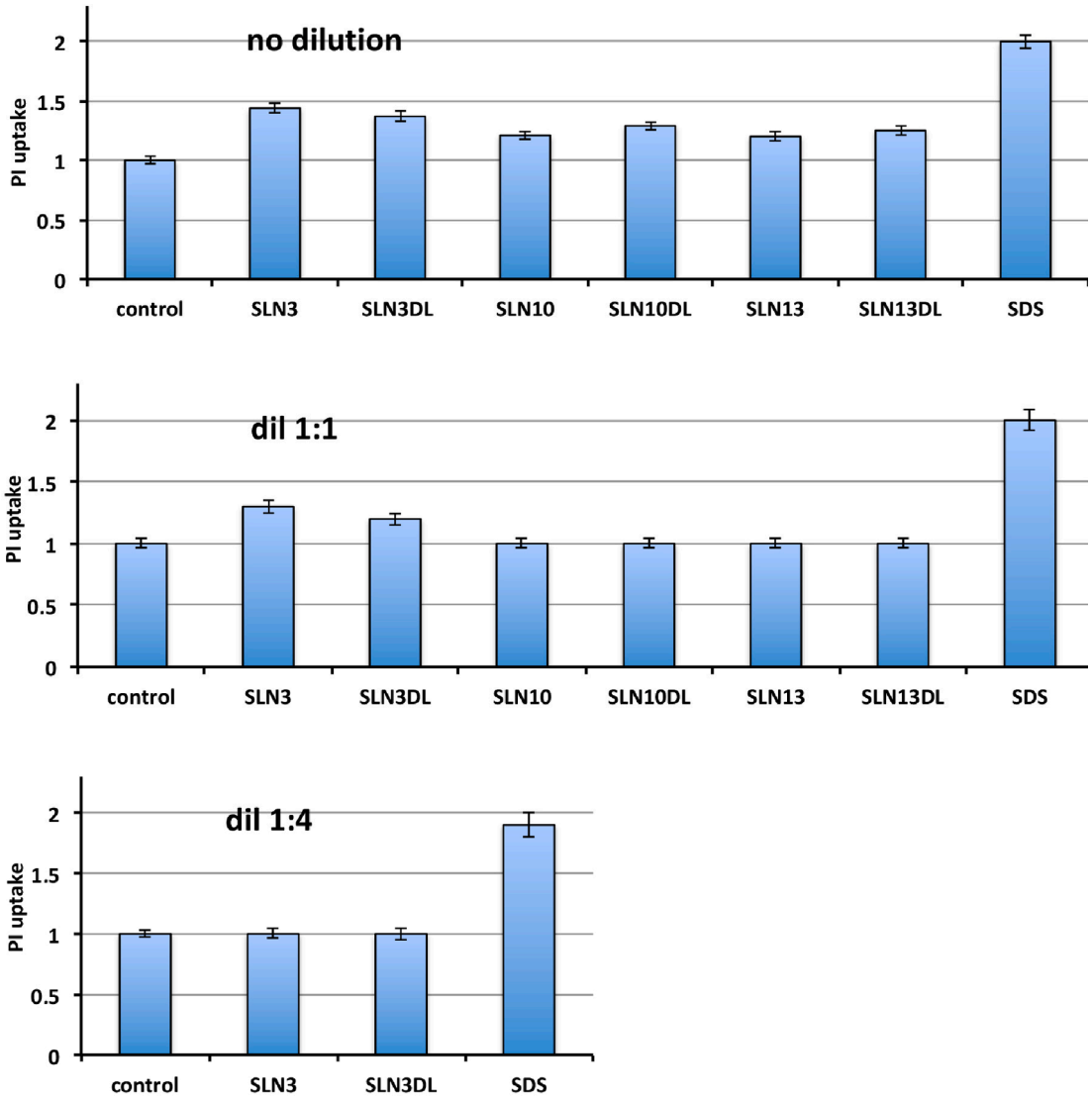

Fig. 7. Propidium iodide (PI) uptake by Caco-2 cell line after 24 h incubation with empty and NEB-loaded SLNs (see Tables 1, 2 and 3 for their composition). Fresh culture medium and SDS (1 mg/mL) were used as negative and positive control. Results are expressed as relative fluorescence intensity (RFI) with respect to cells incubated with culture medium (mean ± SD, 4 replicates per plate in 3 independent plates).

with respect to the around 60 % and 35 % values obtained with SLN3DL and SLN10DL formulations, respectively.

4. Conclusions

The possibility of developing a new effective oral formulation of NEB in SLN carriers has been investigated, as well as the usefulness of loading the drug as HPβCD complex rather than as pure drug. Based on the obtained data we can conclude that the use of HPβCD actually allowed to significantly improve the performance of the SLN formulation. In fact, it not only did not interfere in anyway with the preparation of SLNs formulation, but, on the contrary, gave rise to a series of important beneficial effects.

First of all, the presence of HPβCD improved the SLNs quality in terms of smaller dimensions, higher homogeneity (PDI < 0.3) and greater physicochemical stability of the colloidal dispersion, allowing to avoid the sharp reduction of ZP values caused by loading the drug as such.

Furthermore, loading of the drug as HPβCD complex made it possible to clearly increase its entrapment efficiency (from 60 to about 100 %) and to better control and sustain its release rate.

Finally, it was also proved that the use of HPβCD offered the possibility of increasing up to doubling the drug loading without appreciable changes in the physicochemical properties of the SLNs and maintaining a very high entrapment efficiency.

CRediT authorship contribution statement

P. Mura: Writing – original draft, Visualization, Resources, Data curation. **F. Maestrelli:** Writing – review & editing, Supervision, Methodology, Conceptualization. **L.M.D. Gonçalves:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis. **M. Cirri:** Writing – review & editing, Validation, Funding acquisition. **N. Mennini:** Writing – review & editing, Software, Project administration. **A.J. Almeida:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

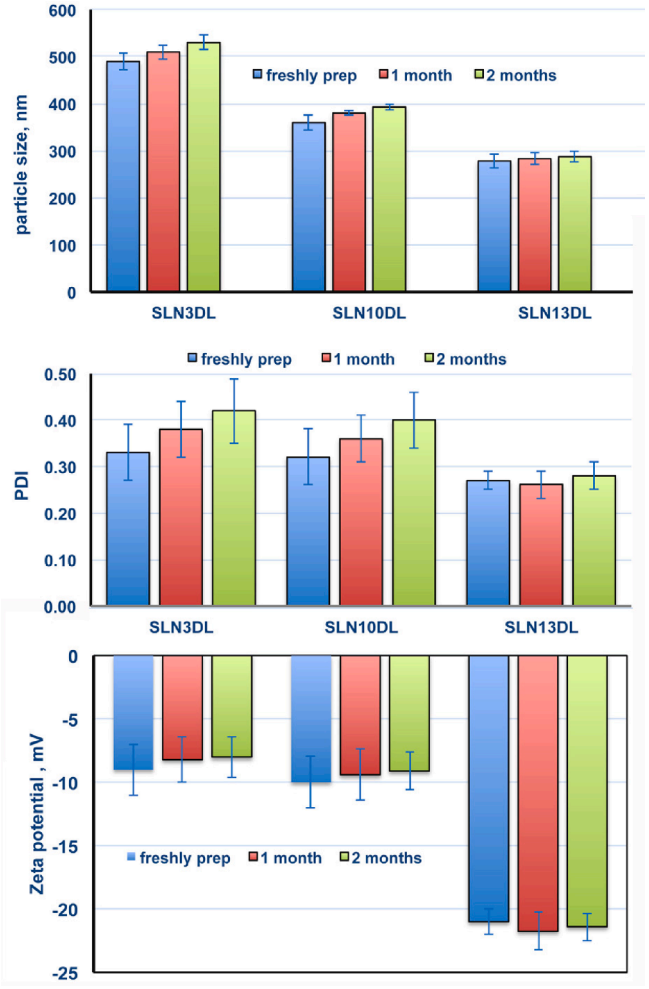

Fig. 8. Effects of storage at 4 ± 1°C on particle size, polydispersity index (PDI) and Zeta Potential of the selected drug-loaded SLN formulations.

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Data availability

Data will be made available on request.

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