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Liposomes containing cyclodextrins or meglumine to solubilize and improve the bioavailability of poorly soluble drugs

Carolina Aloisio [a], Sophia G. Antimisiaris [b], Marcela R. Longhi [a,*]

[a] Unidad de Investigación y Desarrollo en Ciencia y Tecnología Farmacéutica (UNITEFA-CONICET). Departamento de Farmacia, Facultad de Ciencias Químicas-Universidad Nacional de Córdoba, Ciudad Universitaria, X5000HUA Córdoba, Argentina
[b] Laboratory of Pharmaceutical Technology, Department of Pharmacy, University of Patras, Rio 26500, Greece

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ABSTRACT

Poorly soluble drug-loaded liposomes are well known for their ability to solubilize and improve the bioavailability of the carried molecules, and may provide benefits as oral drug delivery systems. In this work, we aim to evaluate the effect of the incorporation of β-cyclodextrin (βCD), methyl-βCD (MβCD), hydroxypropil-βCD (HPβCD) and meglumine (MEG) in liposomes for the oral delivery of the poorly water-soluble drugs, sulfamerazine (SMR) and indomethacin (INM). Liposomes with egg phosphatidylcholine (PC) and cholesterol (CHO), incorporating SMR or INM as plain drug or inclusion complexes, were prepared using the thin film hydration method or dehydration-rehydration method, respectively. The systems were characterized by particle size, polydispersity and zeta potential measurements, and drug-component interaction studies were performed by ^{1}H NMR. Liposome stability in presence of SMR, INM, CD and MEG was determined by the retention of vesicle encapsulated calcein after incubation in solutions of pH 7.4, at 37 °C for up to 48 h. Drug entrapment, as well as drug release, were estimated for all liposome types prepared. The ^{1}H NMR studies revealed that the drugs presented interaction with lipids of the liposomes, suggesting the location of the drugs in the lipid bilayer. The liposomes presented high stability in the presence of the drugs, βCD, HPβCD or MEG. The highest entrapment values were achieved for SMR and INM with PC:CHO 3:1 liposomes when MEG and HPβCD were used, respectively (5636.28 and 439.54 mmol/mol), meaning that 18 and 43 times higher incorporation of SMR and INM were achieved in comparison with the ligand-free formulation. The in-vitro release studies showed a strong influence of the ligands on the delivery of the drugs from the liposomes.

1. Introduction

Several approaches have been investigated to develop nanosized drug delivery systems in recent years. Lately, a great deal of interest has been focused on lipid-based carriers due to the lack of suitable large-scale production methods for polymeric nanoparticle based products and to the toxicity of polymers [1]. Liposomes are colloidal vesicles ranging from few nanometers to several micrometers in diameter with one or more lipid bilayers surrounding aqueous compartments [2]. It is well known that liposomes offer many advantages for the delivery and/or targeting of drugs [3–6], since they are biodegradable, biocompatible, non-toxic and non-immunogenic [2]. Liposomal drug formulations can be used to overcome a drug's non-ideal properties, by loading the hydrophilic drugs into the inner aqueous phase, whereas hydrophobic drugs can be inserted into the hydrophobic lipid bilayers [7,8].

Although drug-loaded liposomes have been mainly used for the parenteral administration, their potential application as oral drug delivery systems has also been studied [8–10]. These systems are well known for their ability to solubilize and deliver poorly soluble drugs with significant improvements in bioavailability, observed following liposomal encapsulation [8,11]. Besides, incorporation of poorly permeable small molecule drugs into liposomes also yielded improved oral absorption [5,12–17]. Oral liposomes may also provide protection from the hostile environment in the gastrointestinal tract [8,12] and enable sustained release of the carried drugs [18–22]. Moreover, these systems offer the advantage of reducing toxicity [21,23–25], another side effect that can be observed using similar or lower concentrations compared to the required dose for therapeutic activity [25–27]. Furthermore, they may improve biodistribution [24,28,29] and produce specific site delivery [27,28,30].

Abbreviations: βCD, β-cyclodextrin; CD, cyclodextrins; CHO, cholesterol; Δ, chemical shifts in ^{1}H NMR; Δδ, induced chances in the ^{1}H NMR chemical shifts; D/L, molar ratio of drug over the total lipid concentration; DCL, drug-in- cyclodextrin-in-liposome; DRV, dried-reconstituted vesicles; F_{AT}, calcein fluorescence value measured after the addition of Triton X-100; F_{BT}, calcein fluorescence value measured before the addition of Triton X-100; HPβCD, hydroxypropil-βCD; INM, indomethacin; MEG, meglumine; MLV, multilamellar vesicles; MβCD, methyl-βCD; PBS, phosphate buffer solution; PC, egg phosphatidylcholine; SMR, sulfamerazine; SUV, small unilamellar vesicles; TFH, thin film hydration method.

* Corresponding author.
E-mail addresses: caloisio@fcq.unc.edu.ar (C. Aloisio), mrlcor@fcq.unc.edu.ar (M.R. Longhi).

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0167-7322/

Encapsulating a sufficient amount of the active ingredient is one of the most desirable properties for the usage of liposomes. Factors affecting the encapsulation efficiency of the drug in the liposomes are various and come from the properties of both the liposomes (as the preparation method) and encapsulated drugs (as the hydrophilic or lipophilic properties or tendency to interact with the membrane bilayer) [11,31]. Accommodation of a poorly water-soluble drug in the lipid bilayer of liposomes is often limited in terms of drug to lipid mass ratio [11]. Cyclodextrins (CD) are well known for their ability to form inclusion complexes with a variety of guest molecules providing solubility-enhancing properties for lipophilic poorly-soluble drugs [32–35]. The strategy of combining liposomes and cyclodextrin complexes of lipophilic drugs, by forming drug-in- cyclodextrin-in-liposome (DCL) formulations, provided a novel system in drug delivery for the entrapment of water-soluble cyclodextrin–drug inclusion complexes in the aqueous interior of liposomes [11,20,36]. This could potentially increase the drug to lipid mass ratio to levels above those attained by conventional drug incorporation into the lipid phase [11].

N-acetyl glutamine, also known as meglumine (MEG), is a polyhydroxy organic amine that has been demonstrated to raise solubility [37,38], drug release rate [38–41] and stabilization [41] of weakly acidic molecules. In a previous work developed in our laboratory, MEG showed a significant solubilization enhancement of sulfamerazine (SMR), which is a very slightly water-soluble (0.22 mg/ml) [38] sulfonamide, compared with the free drug and the SMR-CD complexes, and proved to be responsible for a solubility improvement via multiple factors rather than just providing a favorable pH. Moreover, it was demonstrated that the complexation of this active ingredient with βCD, MβCD, HPβCD and MEG resulted in a decrease in the release rate of the drug through cellulose acetate membrane, thereby enabling sustained drug delivery systems [38].

Following on from these studies, we now aimed to examine various liposome-based formulation approaches, from biodegradable components, and to evaluate the effect of the incorporation of βCD, MβCD, HPβCD and MEG on the oral delivery of poorly water-soluble drugs, using SMR and indomethacin (INM) as model drugs. Experimental studies applied to these molecules can also provide information for other poorly water-soluble drugs with similar physicochemical properties. The characterization of the systems was carried out by particle size and zeta potential determinations, and drug-component interaction studies were performed by ^{1}H NMR. In addition, the integrity of the liposomes was measured in order to evaluate the vesicle stability after the incorporation of the drugs and ligands. Moreover, the effect of encapsulation on the solubility and release rate of the drugs, which affects the bioavailability, was also tested.

2. Materials and methods

2.1. Materials

Phosphatidyl-choline (PC), cholesterol (CHO), calcein, βCD, MβCD KLEPTOSE® CRYSMEB (DS = 0.5), HPβCD (DS = 0.45–0.95) and Meglumine were purchased from Sigma Aldrich® Hellas, Greece. Sulfamerazine and indomethacin were obtained from Parafarm®, Argentina. All the other materials and solvents were of analytical grade or better. Purified water was obtained from Millipore Milli-Q Water Purification System.

2.2. Preparation of liposomes

2.2.1. Thin film hydration method (TFH)

Multilamellar vesicles (MLV) were prepared by the TFH method. For this, PC (20 mg/ml) and CHO (10 mg/ml) were dissolved in chloroform/methanol (2:1 v/v) and mixed using different PC:CHO ratios (3:1, 2:1, 1:1, 1:0) that were subsequently evaporated under vacuum (in a round bottomed flask connected to a rotor evaporator) until a thin lipid film was formed and the traces of the solvent were removed under a stream of nitrogen. The lipid film was hydrated with the appropriate volume of pH 7.4 Phosphate Buffer solution (PBS). Small unilamellar vesicles (SUV) were prepared by probe sonication. The large liposome suspension initially produced was sonicated for at least two 15-min cycles using a vibra cell sonicator (Sonics and Materials, UK), equipped with a tapered microtip. In all cases, the initially turbid liposomal suspension was well clarified after sonication. The Ti-fragments that leaked from the probe during sonication, as well as any Multilamellar vesicles or liposomal aggregates present in the samples, were removed by centrifugation at 14.000 *g* for 10 min at 201C (Spectrafuge 16 M, Labnet, Germany). For the preparation of drug-loaded liposomes, the same procedure as for empty liposomes was applied, and SMR or INM were incorporated in the chloroform/methanol (2:1 v/v) dispersion in a 1 mg/ml concentration. For measurement of drug entrapment in liposomes, liposomal drug was separated from the non-solubilized drug by filtration thought 1 μm pore size membrane.

2.2.2. Dehydration- rehydration method

Dried-reconstituted vesicles (DRV) were prepared by the procedure of Kirby and Gregoriadis [42], due to the demonstrated high entrapment efficiency of drugs in the liposomes. The 3:1 lipid ratio was selected since it showed high encapsulation of the drugs when the THF method was applied (Section 2.2.1). SMR or INM were incorporated in the vesicles in absence and presence of βCD, MβCD, HPβCD and in the presence of MEG for SMR, in a 1:1 stoichiometric relation, chosen from previous studies. The INM-loaded liposome formulation containing MEG was dismissed because a negative effect on INM stability, caused by the ligand, was observed in previous studies.

2.3. Particle size, polidispersity and zeta potential

The droplet size, polidispersity and zeta potential of the liposomes were determined at 25 °C using a Malvern Zetasizer Nano Series. The intensity autocorrelation function was measured at a 165-degree angle using a viscosity of 0.8878 Pa · s and a refractive index of 1.3328 for the bulk medium. The samples were appropriately diluted with water before the analysis.

2.4. Nuclear magnetic resonance (NMR) studies

^{1}H NMR studies were performed at 298 K in a Bruker® Advance II High Resolution Spectrometer equipped with a Broad Band Inverse probe (BBI) and a Variable Temperature Unit (VTU) using 5-mm sample tubes. Spectra were obtained by diluting a 0.1 ml volume of the empty, SMR-loaded and INM-loaded PC:CHO 3:1 THF liposomes to 1 ml D_2O. In order to acquire the spectra of the pure lipids, plain liposomes containing only PC or CHO were prepared and diluted to a 1 mM concentration with D_2O. All the studies were carried out at 400.16 MHz and the data were processed with the Bruker® TOPSPIN 2.0 software. The residual solvent signal (4.80 ppm) was used as the internal reference. Induced changes in the ^{1}H NMR chemical shifts (Δδ) for the drugs and the liposomes components originated due to their interaction were calculated according to the following equations:

$$\Delta\delta = \delta_{\text{loaded liposomes}} - \delta_{\text{unloaded liposomes}}$$

and

$$\Delta\delta = \delta_{\text{drug in liposomes}} - \delta_{\text{drug}}$$

2.5. Measurement of liposome integrity

The integrity of liposomes was evaluated by measuring the retention (%) of calcein in the vesicles. For this, the lipid film was hydrated with

the appropriate volume of a solution of calcein, at 40 °C. After complete hydration, during which spontaneous formation of liposomes occurred, separation of liposomes from non-encapsulated calcein was achieved by ultracentrifugation (at 50,000 rpm, 5 cycles for 30 min). Liposomes were stored at 4 °C before use. Samples were incubated at 37 °C for 48 h suspended in PBS and the latency and retention were determined at different time points measuring the fluorescence intensity (EM 470 nm, EX 520 nm) before and after the addition of Triton X-100 at a final concentration of 1% (v/v) (to disrupt the liposomes), using a Shimatzu RF-1501 spectrofluorimeter.

The percentage of calcein latency (% Latency) was calculated using the following equations:

$$\%\ \text{Latency} = 1.1(F_{AT}-F_{BT})/(1.1\ \ F_{AT}) \times 100$$

where by F_{BT} and F_{AT} represent calcein fluorescence values measured before and after the addition of Triton X-100, respectively.

The retention of calcein in the liposomes (% Retention) was calculated from the latency at each time point (Latency $_t$) and the corresponding initial latency value (Latency $_0$).

$$\%\ \text{Retention} = \left(\text{Latency}_{t)/}\text{Latency}_0\right) \times 100$$

2.6. Encapsulation efficiency studies

Drug encapsulation in liposomes is determined as the molar ratio of drug over the total lipid concentration [D/L (mmol/mol)] in the drug encapsulating liposomes. For the calculation of drug encapsulation efficiency, the drug content of each liposome preparation was measured using UV-spectroscopy. A phospholipid colorimetric assay (Stewart, 1980) was used to measure the lipid concentration. Calibration curves were constructed by standard solutions of the drugs and the lipids.

2.7. In-vitro drug release from the liposomes

The in-vitro release of SMR and INM from the liposomes was determined using a MicroettePlus® Vertical diffusion Franz cell apparatus with automatic sampling at 37 ± 2 °C and 300 rpm stirring rate (Hanson Research Corporation®). A cellulose acetate membrane with a 0.45-μm pore size and a 1.77-cm^2 exposed area was used (Sigma Aldrich®, USA) with aqueous formulations (0.3 ml). The pure drugs or the ones incorporated into the liposomes in an oral dose whereas loaded in the donor compartment. A 10 mM PBS of pH 7.4 was used as the diffusion medium in the donor and receptor cells, and samples (2.0 ml) were withdrawn from the receiver compartments at fixed intervals and replaced automatically with an equal volume of previously warmed PBS. Drug concentration was spectrophotometrically measured at 240 nm or 225 nm for SMR or INM, respectively. The same initial concentration of the drug alone or in the liposomes in PBS solution was used. Each experiment was performed at least three times and the results and the results represent the experimental average. The data are presented as the mean ± standard deviation. The significant differences of the induced changes in release, due to the incorporation in the liposomes compared with the pure drug, were assessed by means of a one-way analysis of variance (ANOVA). Results were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Particle size, polidispersity and zeta potential

The particle size, polidispersity and zeta potential of PC and PC:CHO 3:1, 2:1 and 1:1 liposomes obtained by the THF technique, were determined in PBS pH 7.4 at 25 °C (Table 1). The mean size of empty and drug-loaded THF liposomes was between 31.1 and 115 nm and the polidispersity was <0.3, indicating that monodispersed small unilamellar vesicles (SUV) were obtained. The zeta potential of empty liposomes was negative and close to neutrality (−0.38 a −0.03 mV), with similar values for SMR-loaded (−2.9 to −0.07 mV) and INM-loaded liposomes (−0.41 to −0.09 mV). From the results it is obvious that these lipids hold a negative surface charge as demonstrated before [43].

Furthermore, the particle size, polidispersity and zeta potential of PC:CHO 3:1 DRV liposomes in the presence of βCD, MβCD, HPβCD and MEG were also assessed under the same conditions (Table 2). Even though the polidispersity values were high, suggesting that polidispersed systems were obtained, not being possible to infer onliposomes size, the results were between 139 and 218 nm for unloaded and SMR-loaded DRV liposomes. On the other hand, the INM-loaded DRV liposomes size was between 578.7 and 790.7 nm. The zeta potential values of the unloaded (−8.66 to −0.03 mV), SMR-loaded (−2 to 0.02 mV) and INM-loaded DRV liposomes (−12.9 to −0.2 mV) were negative and no significant difference was observed between different liposomes due to the incorporation of the different ligands, SMR or INM.

Table 1
Particle size, polidispersity index (PDI) and zeta potential of THF liposomes in PBS pH 7.4 at 25 °C.

	Particle size (nm)	PDI	Zeta potential (mV)
	EmptyTHF liposomes		
PC	52 ± 1	0.250	−0.03
PC:CHO 3:1	79.0 ± 0.8	0.220	−0.04
PC:CHO 2:1	71 ± 1	0.184	−0.34
PC:CHO 1:1	54.2 ± 0.5	0.220	−0.38
	SMR-loaded THF liposomes		
PC	59.8 ± 0.3	0.300	−2.0
PC:CHO 3:1	115 ± 1	0.156	−0.07
PC:CHO 2:1	61 ± 3	0.220	−2.9
PC:CHO 1:1	58.9 ± 0.2	0.223	−0.60
	INM-loaded THF liposomes		
PC	31.1 ± 0.6	0.193	−0.15
PC:CHO 3:1	68.7 ± 0.6	0.198	−0.41
PC:CHO 2:1	46.3 ± 0.7	0.206	−0.16
PC:CHO 1:1	36.6 ± 0.1	0.227	−0.09

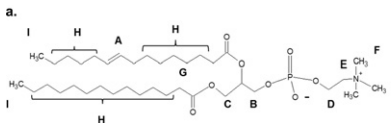
Table 2
Particle size, polidispersity index (PDI) and zeta potential of DRV liposomes in PBS pH 7.4 at 25 °C.

	Size (nm)	PDI	Zeta potential (mV)
	Empty DRV liposomes		
PC:CHO 3:1 (THF)*	214 ± 19	0.194	−0.03
PC:CHO 3:1 + βCD	187.3 ± 31.9	0.400	−8.66
PC:CHO 3:1 + MβCD	166 ± 17	0.701	−1.60
PC:CHO 3:1 + HPβCD	139 ± 10	0.764	−3.40
PC:CHO 3:1 + MEG	173.5 ± 35.8	0.973	−6.30
	SMR-loaded DRV liposomes		
PC:CHO 3:1 (THF)*	97.1 ± 30.3	0.156	−2.0
PC:CHO 3:1 + βCD	135 ± 44	0.502	−0.04
PC:CHO 3:1 + MβCD	132 ± 16	0.586	0.02
PC:CHO 3:1 + HPβCD	218 ± 69	0.999	−1.96
PC:CHO 3:1 + MEG	211.7 ± 83.1	0.973	0.0008
	INM-loaded DRV liposomes		
PC:CHO 3:1 (THF)*	31.1 ± 0.6	0.198	−0.2
PC:CHO 3:1 + βCD	482.6 ± 53.2	0.404	−6.4
PC:CHO 3:1 + MβCD	790.7 ± 101.0	0.979	−12.9
PC:CHO 3:1 + HPβCD	579.2 ± 92.7	0.882	−9.9

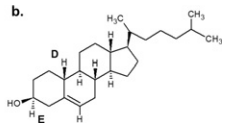
* Liposomes obtained by the thin hydrated film (THF) method are presented for comparison purposes.

Table 3
Chemical shifts (δ) and induced changes in chemical shifts (Δδ) of the signals corresponding to the liposomes components: a) PC and b) CHO, before and after the encapsulation of SMR or INM, determined by ^{1}H NMR.

a.



b.



	δ(ppm)					Δδ		
Signal	PC	CHO	PC:CHO 3:1	PC:CHO 3:1 + SMR	PC:CHO 3:1 + INM	PC:CHO 3:1	PC:CHO 3:1 + SMR	PC:CHO 3:1 + INM
H_A	5.2995	Ø	5.3056	5.2937	5.2565	0.0061	−0.0119	−0.0491
H_B	4.2928	Ø	4.285	4.2601	5.2143	−0.0078	−0.0249	0.9293
H_C	3.6854	Ø	3.691	3.6747	3.8212	0.0056	−0.0163	0.1302
H_D	3.4985	3.500	3.4902	3.4761	3.515	−0.0083	−0.0141	0.0248
H_E	3.355	3.3384	3.3348	3.3155	3.3276	−0.0202	−0.0193	−0.0072
H_F	3.2473	Ø	3.2506	3.2305	3.1905	0.0033	−0.0201	−0.0601
H_G	2.0384	Ø	2.0462	2.0279	1.9929	0.0078	−0.0183	−0.0533
H_H	1.2616	Ø	1.2886	1.2752	1.2524	0.027	−0.0134	−0.0362
H_I	0.8878	Ø	0.8742	0.869	0.8593	−0.0136	−0.0052	−0.0149

Ø: undistinguishable.

3.2. Drug-component interaction studies

In order to determine the possible location of the drugs in the liposome, the chemical shifts (δ) and the induced chances (Δδ) for the liposome components (PC and CHO), and for SMR or INM after the incorporation in the vesicles, were determined by ^{1}H NMR experiments for unloaded and drug-loaded liposome. The results are presented in Tables 3 and 4. These studies revealed the interaction between PC and CHO with the drugs, mainly evidenced by upfield displacements of the protons corresponding to the side chain of PC and, in the presence of INM, downfield displacements of the protons adjacent to the phosphate moiety of PC; this could be due to their proximity to the carbonyl moiety of INM, which presents electronic density interacting with the amine group of choline by electrostatic attractions (Table 4). Meanwhile, most of the drug protons presented downfield displacements, probably due to their proximity to the ester and phosphate groups of PC. These observation may indicate that Van Der Waals or hydrophobic interactions occurred, suggesting that SMR and INM were located in the lipidic bilayer of the liposomes.

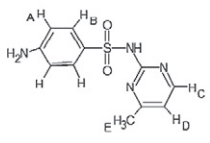
3.3. Measurement of liposome integrity

The retention of vesicle encapsulated calcein in PC and PC:CHO 3:1, 2:1 and 1:1 liposomes containing SMR or INM was determined after incubation in PBS at 37 °C for up to 48 h. The plain liposomes suspended in PBS showed high stability, since the initially encapsulated calcein, which remains in vesicles after 48 h of incubation, was higher than 80% (Fig. 1 a). The integrity of PC:CHO 3:1 liposomes containing SMR or INM was also evaluated, and it was observed that the retention percentages were over 70 and 95%, respectively, indicating that the vesicles remained stable even after the encapsulation of the drugs (Fig. 1 b).

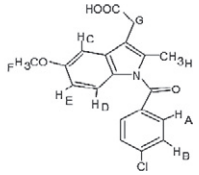
On the other hand, the integrity of DRV liposomes containing βCD, MβCD, HPβCD or MEG was also tested (Fig. 1 c), and it was observed that the retention percentages were over 80%. Exceptionally, an exponential diminution on the retention was observed in the presence of MβCD, probably because this CD acts as a surfactant, forming micels with the lipids, thus causing the loss of liposomes stability, as it was previously reported by Hatzi et al. [20] It has been also been proposed by Piel et al. that MβCD can interact with the lipids from the inner side of

Table 4
Chemical shifts (δ) and induced changes in chemical shifts (Δδ) of the signals corresponding to: a) SMR and b) INM, after the encapsulation in liposomes, determined by ^{1}H NMR.

a.



b.



	δ(ppm)				Δδ	
Signal						
A	pure SMR	pure INM	SMR in LIP3:1	INM in LIP3:1	SMR in LIP3:1	INM in LIP3:1
B	8.1764	7.7464	8.0113	7.6653	−0.1651	−0.0811
C	7.7369	7.6335	7.6256	7.5328	−0.1113	−0.1007
D	6.867	7.1147	6.7893	7.02545	−0.0777	−0.08925
E	6.8091	7.1006	6.6318	6.6643	−0.1773	−0.4363
F	2.3921	6.7834	2.267	5.2565	−0.1251	−1.5269
G	/	3.902	/	Ø	/	x
H	/	3.61	/	Ø	/	x
I	/	2.2563	/	2.2306	/	−0.0257

Ø: undistinguishable; /: no signal assignment in the molecule; x: correspond to undistinguishable proton.

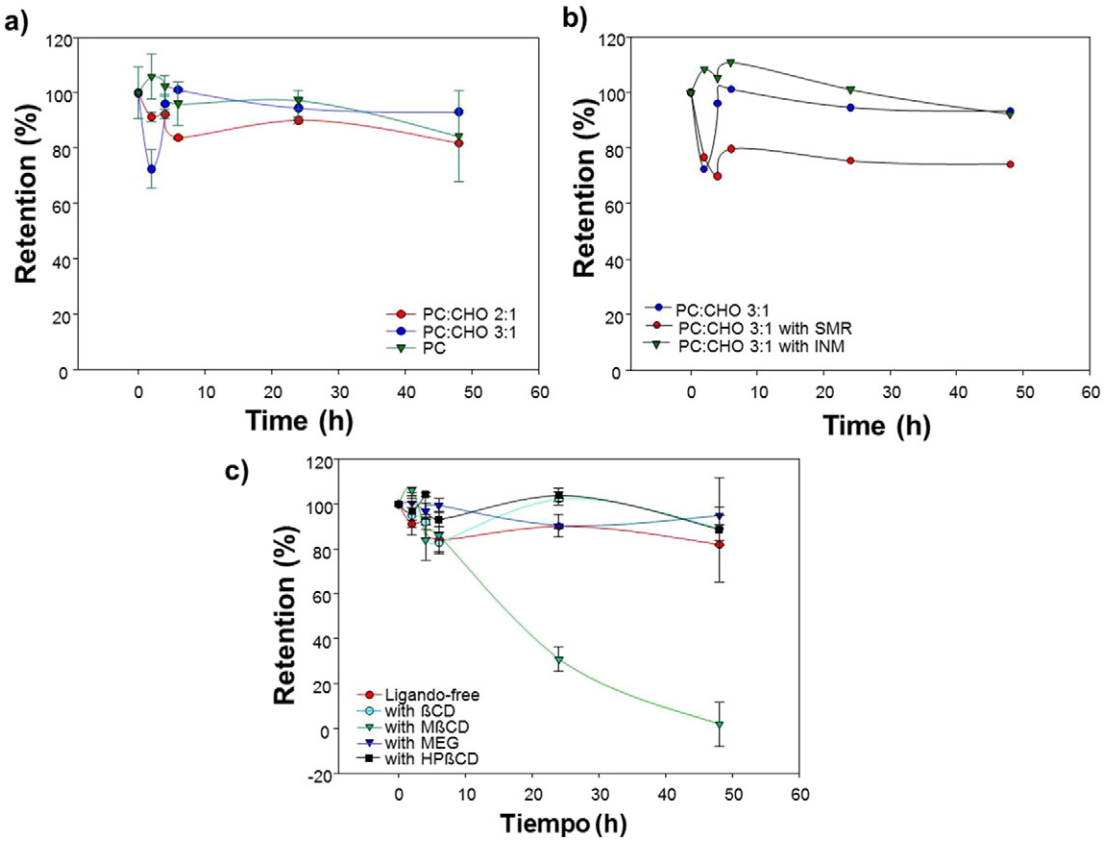


Fig. 1. Integrity of: a) empty, b) SMR- and INM-loaded liposomes obtained by the thin film layer method and: c) SMR-loaded and d) INM-loaded DRV liposomes during incubation in PBS at 37 °C for 48 h.

the bilayer due to the higher lipophilicity of this CD, which allows it to be internalized by the liposomes membrane [11].

3.4. Encapsulation efficiency studies

The entrapment of SMR and INM in all the liposome formulations was determined as the molar ratio of drug over the total lipid concentration [D/L (mmol/mol)] and as the drug concentration (mg/ml) in the drug encapsulating liposomes. The THF liposomes (Fig. 2 A and B) showed higher encapsulation amounts, as the PC:CHO ratio increased, achieving the highest entrapment with pure PC liposomes, with drug per lipid concentration values (D/L) of 308.98 and 10.14 mmol/mol for SMR and INM, respectively. This may be not only due to PC lipophilicity, but also due to the fact that this lipid presents the amine group positively charged at pH 7.4, being able to interact with both active ingredients that are mainly ionized and present negative charge at this media [44, 45].

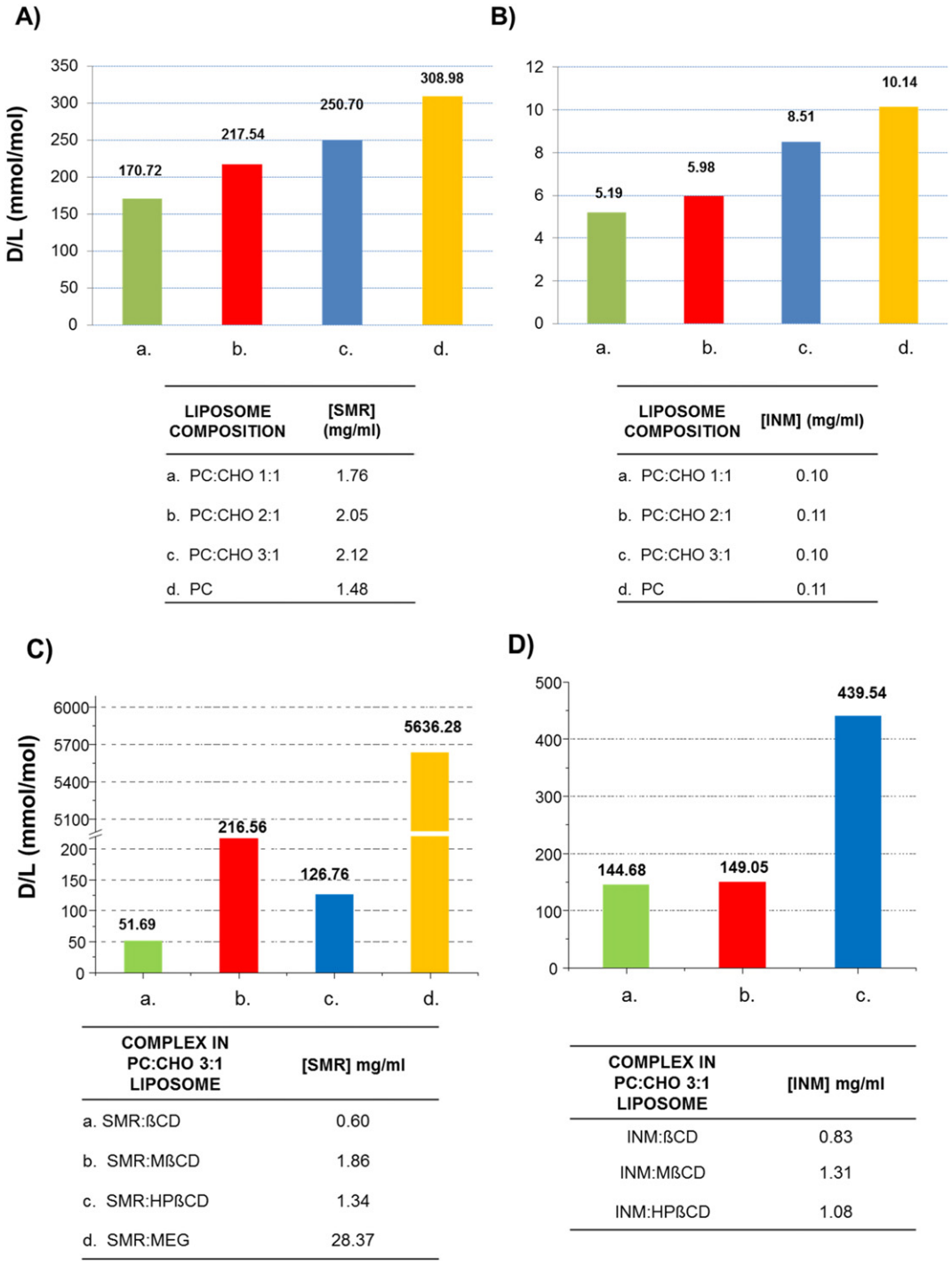
On the other hand, the vesicles prepared by the DRV method (Fig. 2 C and D) showed the highest entrapment values for SMR and INM when MEG or HPβCD were used, respectively (5636.28 and 439.54 mmol/mol), being able to achieve an incorporation of SMR and INM 18 and 43 times higher, in comparison with the plain THF liposomes, owing to the solubilization of the poor soluble drugs inside the inner aqueous phase of the vesicles due to complexation with the CD or MEG. The reason for which CD did not seem to improve the uptake of the drug for SMR may be due to the fact that this hydrophobic drug present high affinity for the lipid bilayer of the liposomes and the presence of the CD in the aqueous phase favors the complex formation equilibrium, producing a displacement of the drug outside the bilayer.

3.5. In-vitro release of SMR and INM from liposomes

The ability of the liposomes to transport and release the drugs was tested by evaluating the diffusion of the drugs incorporated in PC:CHO liposomes, 1:0, 1:1, 2:1, and 3:1, suspended in PBS pH 7.4 across ~~and~~ cellulose acetate membrane at 37 °C. The release profile of both pharmaceutical active ingredients, encapsulated in the different liposomes, were similar among them, with a strong retention effect compared with the control formulation as being observed, indicating that all the systems allow a controlled release of both drugs, regardless of the proportion of PC and CHO present (Fig. 3 a and b). In addition, the in-vitro release and transport of SMR and INM from PC:CHO 3:1 liposomes containing 1.8% βCD, 12% MβCD, 2.5% HPβCD or 5% MEG, in the aqueous phase, was evaluated. Although a strong retention effect was observed from 0 to 6 h (Fig. 3 c), the systems containing SMR in the presence of the less hydrophilic CD (βCD and MβCD) presented a similar behavior with respect to the ligand free formulation. Moreover, the liposomes containing INM, in the presence of the three CD, exhibited a stronger retention effect compared with the ligand free system (Fig. 3 d). On the other hand, the release profiles of SMR from the liposomes containing HPβCD or MEG, presented higher percentages of drug released, regarding the other formulations and similar to the free drug, attaining values close to 40% at 6 h (Fig. 3 c). These results indicated a strong influence of the ligands over the release of the drugs from the studied systems, demonstrating the importance of the incorporation of the CD or MEG to the liposomes formulations for the delivery of poor soluble drugs.

4. Conclusion

These studies allowed the development of liposomes for the delivery of the poor water-soluble drugs, SMR and INM. The vesicles obtained by



LIPOSOME COMPOSITION	[SMR] (mg/ml)
a. PC:CHO 1:1	1.76
b. PC:CHO 2:1	2.05
c. PC:CHO 3:1	2.12
d. PC	1.48

LIPOSOME COMPOSITION	[INM] (mg/ml)
a. PC:CHO 1:1	0.10
b. PC:CHO 2:1	0.11
c. PC:CHO 3:1	0.10
d. PC	0.11

COMPLEX IN PC:CHO 3:1 LIPOSOME	[SMR] mg/ml
a. SMR:ßCD	0.60
b. SMR:MßCD	1.86
c. SMR:HPßCD	1.34
d. SMR:MEG	28.37

COMPLEX IN PC:CHO 3:1 LIPOSOME	[INM] mg/ml
INM:ßCD	0.83
INM:MßCD	1.31
INM:HPßCD	1.08

Fig. 2. Encapsulation efficiency of liposomes obtained by the thin film method, with different compositions: **a.** PC:CHO 1:1; **b.** PC:CHO 2:1; **c.** PC:CHO 3:1 and **d.** pure PC; containing: A) SMR; B) INM; or liposomes obtained by the Dehydration-Rehydration method containing complexes of: C) SMR and D) INM with: **a.** βCD; **b.** MβCD; **c.** HPβCD or **d.** MEG (only for SMR). D: drug concentration; L: total lipid concentration;

the THF method presented a small size (SUV) and this parameter remained hardly modified after the incorporation of the active ingredients. The unloaded and SMR-loaded DRV liposomes presented a small size (SUV) while the INM-loaded DRV liposomes were higher (MLV or LUV). The ^{1}H NMR studies revealed that both drugs presented interaction with lipids of the liposomes obtained by the THF method thus suggesting the location of the drugs in the lipid bilayer. The liposomes presented high stability in PBS at 37 °C after 48 h, before and after the encapsulation of the drugs and when βCD, HPβCD or MEG were incorporated by the DRV method. The drug encapsulation efficiency was high, mainly after the addition of βCD and MEG and allowing a great solubilization enhancement of the drugs, thus resulting in promising formulations to increase the bioavailability of poorly soluble drugs.

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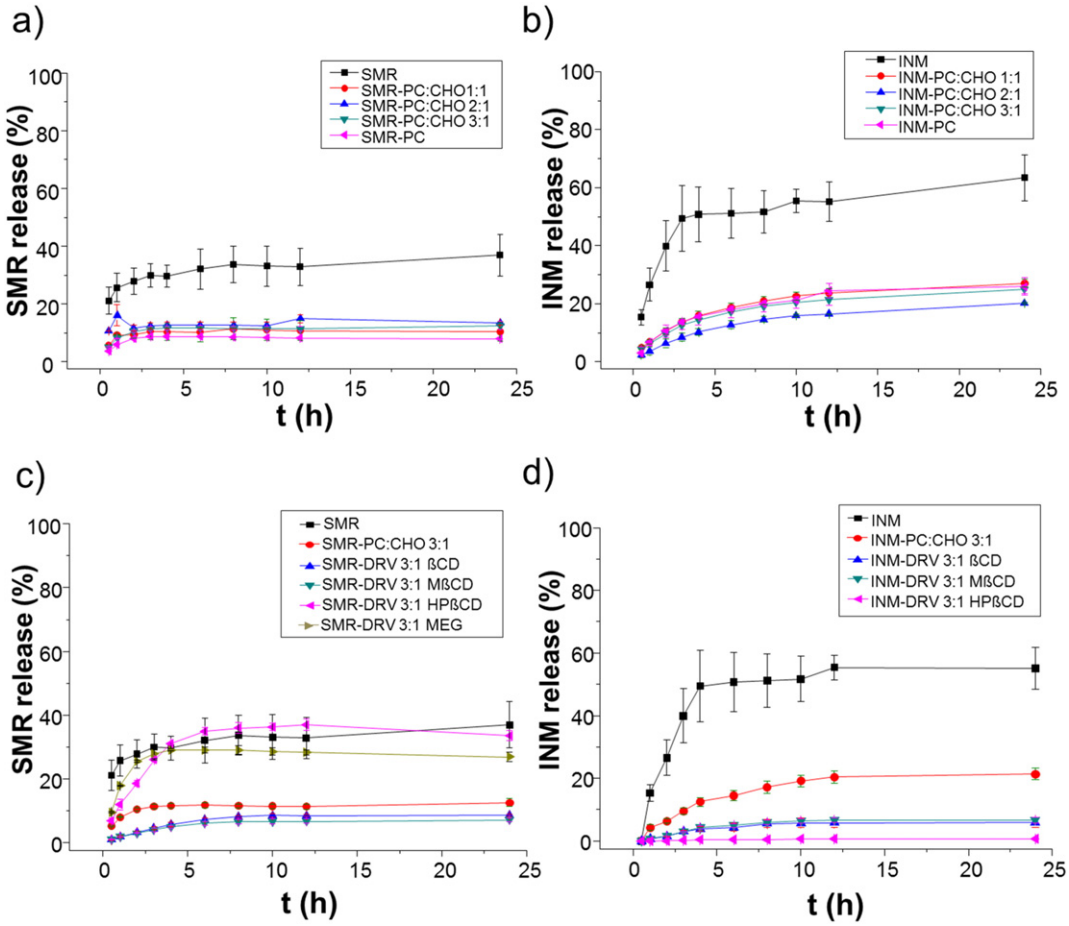

Fig. 3. Release profiles of: a) SMR and b) INM, from liposomes obtained by the thin film method or liposomes obtained by the Dehydration-Rehydration method containing complexes of: c) SMR and d) INM with: **a.** βCD; **b.** MβCD; **c.** HPβCD or **d.** MEG (only for SMR) (each value represents the mean ± S.D. of $n \geq 3$).

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References

[1] C.-C. Chen, T.-H. Tsai, Z.-R. Huang, J.-Y. Fang, Effects of Lipophilic Emulsifiers on the Oral Administration of Lovastatin from Nanostructured Lipid Carriers: Physicochemical Characterization and Pharmacokinetics, Eur. J. Pharm. Biopharm., 74(3), Elsevier B.V., 2010 Mar 474–482.
[2] C. Mugabe, A.O. Azghani, A. Omri, Preparation and characterization of dehydration-rehydration vesicles loaded with aminoglycoside and macrolide antibiotics, Int. J. Pharm. 307 (2) (2006 Jan 13) 244–250.
[3] T. Tsukamoto, K. Hironaka, T. Fujisawa, D. Yamaguchi, K. Tahara, Y. Tozuka, et al., Preparation of bromfenac-loaded liposomes modified with chitosan for ophthalmic drug delivery and evaluation of physicochemical properties and drug release profile. Asian, J. Pharm. Sci. 8 (2) (2013 Apr) 104–109.
[4] B.J. Crielaard, S. van der Wal, H.T. Le, A.T.L. Bode, T. Lammers, W.E. Hennink, et al., Liposomes as carriers for colchicine-derived prodrugs: vascular disrupting nanomedicines with tailorable drug release kinetics, Eur. J. Pharm. Sci. 45 (4) (2012 Mar 12) 429–435.
[5] E. Markoutsa, G. Pampalakis, A. Niarakis, I.a. Romero, B. Weksler, P.O. Couraud, et al., Uptake and Permeability Studies of BBB-Targeting Immunoliposomes Using the hCMEC/D3 Cell Line, Eur. J. Pharm. Biopharm., 77(2), Elsevier B.V., 2011 Feb 265–274.
[6] A. Skouras, S. Mourtas, E. Markoutsa, M.C. De Goltstein, C. Wallon, S. Catoen, et al., Magnetoliposomes with High USPIO Entrapping Efficiency, Stability and Magnetic Properties, Nanomed Nanotech Biol Med., 7(5), Elsevier Inc., 2011 Oct 572–579.
[7] I. MacLachlan, Liposomal Formulations for Nucleic Acid Delivery, in: Taylor & Francis Group (Ed.), Antisense Drug Technology: Principles, Strategies, and Applications 2007, p. 237.
[8] Y. Chen, Y. Lu, J. Chen, J. Lai, J. Sun, F. Hu, et al., Enhanced Bioavailability of the poorly Water-Soluble Drug Fenofibrate by Using Liposomes Containing a Bile Salt, Int. J. Pharm., 376(1-2), Elsevier B.V., 2009 Jul 6 153–160.
[9] P. Paixão, L.F. Gouveia, M. JAG, Prediction of the Human Oral Bioavailability by Using in Vitro and in Silico Drug Related Parameters in a physiologically Based Absorption Model, Int. J. Pharm., 429(1–2), Elsevier B.V., 2012 Jun 15 84–98.
[10] S.C. Mourão, P.I. Costa, H.R.N.H.R.N. Salgado, M.P.D. Gremião, Improvement of antischistosomal activity of praziquantel by incorporation into phosphatidylcholine-containing liposomes, Int. J. Pharm. 295 (1–2) (2005 May 13) 157–162.
[11] G. Piel, M. Piette, V. Barillaro, D. Castagne, B. Evrard, L. Delattre, Betamethasone-in-cyclodextrin-in-liposome: the effect of cyclodextrins on encapsulation efficiency and release kinetics, Int. J. Pharm. 312 (1–2) (2006 Apr 7) 75–82.
[12] Y. Koda, M.T. Liang, J.T. Blanchfield, I. Toth, In vitro stability and permeability studies of liposomal delivery systems for a novel lipophilic endomorphin 1 analogue, Int. J. Pharm. 356 (2008 May 22) 37–43.
[13] P. Langguth, A. Kubis, G. Krumbiegel, W. Lang, H.P. Merkle, W. Wächter, et al., Intestinal absorption of the quaternary trospium chloride: permeability-lowering factors and bioavailabilities for oral dosage forms, Eur. J. Pharm. Biopharm. 43 (3) (1997 Jun) 265–272.
[14] D. Chen, D. Xia, X. Li, Q. Zhu, H. Yu, C. Zhu, et al., Comparative study of Pluronic(®) F127-modified liposomes and chitosan-modified liposomes for mucus penetration and oral absorption of cyclosporine A in rats, Int. J. Pharm. 449 (2013 Jun 5) 1–9.
[15] M. Niu, Y. Tan, P. Guan, L. Hovgaard, Y. Lu, J. Qi, et al., Enhanced oral absorption of insulin-loaded liposomes containing bile salts: a mechanistic study, Int. J. Pharm. 460 (2014 Jan 2) 119–130.
[16] J. Kowapradit, A. Apirakaramwong, T. Ngawhirunpat, T. Rojanarata, W. Sajomsang, P. Opanasopit, Methylated N (4-*N*,*N*-dimethylaminobenzyl) chitosan coated liposomes for oral protein drug delivery, Eur. J. Pharm. Sci. 47 (2) (2012 Sep 29) 359–366.
[17] S. Hu, M. Niu, F. Hu, Y. Lu, J. Qi, Z. Yin, et al., Integrity and stability of oral liposomes containing bile salts studied in simulated and ex vivo gastrointestinal media, Int. J. Pharm. 441 (2013 Jan 30) 693–700.
[18] D. Fatouros, O. Gortzi, P. Klepetsanis, S.G. Antimisiaris, M.C. Stuart, A. Brisson, et al., Preparation and properties of arsonolipid containing liposomes, Chem. Phys. Lipids 109 (2001) 75–89.
[19] P. Kallinteri, S.G. Antimisiaris, D. Karnabatidis, C. Kalogeropoulou, I. Tsota, D. Siablis, Dexamethasone incorporating liposomes: an in vitro study of their applicability as a slow releasing delivery system of dexamethasone from covered metallic stents, Biomaterials 23 (24) (2002) 4819–4826.
[20] P. Hatzi, S. Mourtas, P.G. Klepetsanis, S.G. Antimisiaris, Integrity of liposomes in presence of cyclodextrins: effect of liposome type and lipid composition, Int. J. Pharm. 333 (2007 Mar 21) 167–176.
[21] M.L. Manca, G. Loy, M. Zaru, A.M. Fadda, S.G. Antimisiaris, Release of rifampicin from chitosan, PLGA and chitosan-coated PLGA microparticles, Colloids Surf. B: Biointerfaces 67 (2) (2008 Dec 1) 166–170.

[22] D.G. Fatouros, K. Hatzidimitriou, S.G. Antimisiaris, Liposomes encapsulating prednisolone and prednisolone-cyclodextrin complexes: comparison of membrane integrity and drug release, Eur. J. Pharm. Sci. 13 (3) (2001) 287–296.
[23] M. Zaru, M.L. Manca, A.M. Fadda, S.G. Antimisiaris, Chitosan-coated liposomes for delivery to lungs by nebulisation, Colloids Surf. B: Biointerfaces 1 (71) (2009 Jun) 88–95.
[24] P. Zagana, M. Haikou, P. Klepetsanis, E. Giannopoulou, P.V. Ioannou, S.G. Antimisiaris, In vivo distribution of arsonoliposomes: effect of vesicle lipid composition, Int. J. Pharm. 347 (2008 Jan 22) 86–92.
[25] K. Morand, a.C. Bartoletti, A. Bochot, G. Barratt, M.L. Brandely, F. Chast, Liposomal amphotericin B eye drops to treat fungal keratitis: Physico-chemical and formulation stability, Int. J. Pharm. 344 (2007 Nov 1) 150–153.
[26] C. Chen, T.D. Johnston, H. Jeon, R. Gedaly, P.P. McHugh, T.G. Burke, et al., An in vitro study of liposomal curcumin: stability, toxicity and biological activity in human lymphocytes and Epstein-Barr virus-transformed human B-cells, Int. J. Pharm. 366 (2009 Jan 21) 133–139.
[27] B. Stark, F. Andreae, W. Mosgoeller, M. Edetsberger, E. Gaubitzer, G. Koehler, et al., Liposomal vasoactive intestinal peptide for lung application: protection from proteolytic degradation, Eur. J. Pharm. Biopharm. 70 (2008 Sep) 153–164.
[28] S.G. Antimisiaris, P. Klepetsanis, V. Zachariou, E. Giannopoulou, P.V. Ioannou, In vivo distribution of arsenic after i.p. injection of arsonoliposomes in balb-c mice, Int. J. Pharm. 289 (1–2) (2005 Jan 31) 151–158.
[29] P. Zagana, P. Klepetsanis, P.V. Ioannou, P.M. Loiseau, S.G. Antimisiaris, Trypanocidal activity of arsonoliposomes: effect of vesicle lipid composition, Biomed. Pharmacother. 61 (8) (2007 Sep) 499–504.
[30] M. Taylor, S. Moore, S. Mourtas, A. Niarakis, F. Re, C. Zona, et al., Effect of Curcumin-Associated and Lipid Ligand-Functionalized Nanoliposomes on Aggregation of the Alzheimer's α- Peptide, Nanomed. Nanotechnol. Biol. Med., 7(5), Elsevier Inc., 2011 Oct 541–550.
[31] T. Nii, F. Ishii, Encapsulation efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method, Int. J. Pharm. 298 (2005 Jul 14) 198–205.
[32] X. Ge, Z. Huang, S. Tian, Y. Huang, C. Zeng, Complexation of carbendazim with hydroxypropyl-β-cyclodextrin to improve solubility and fungicidal activity, Carbohydr. Polym. 89 (1) (2012 Jun 5) 208–212.
[33] M.V. Araújo, E.K. Barbosa, G. Lázaro, L. Conegero, L.E. Almeida, L. Barreto, et al., Sulfadiazine/hydroxypropyl-β-cyclodextrin host-guest system: characterization, phase-solubility and molecular modeling, Bioorg. Med. Chem. 16 (2008) 5788–5794.
[34] L. Ribeiro, D. Ferreira, F. Veiga, Physicochemical investigation of the effects of water-soluble polymers on vinpocetine complexation with β-cyclodextrin and its sulfobutyl ether derivative in solution and solid state, Eur. J. Pharm. Sci. 20 (3) (2003) 253–266.
[35] T. Taupitz, J.B. Dressman, C.M. Buchanan, S. Klein, Cyclodextrin-water soluble polymer ternary complexes enhance the solubility and dissolution behaviour of poorly soluble drugs. Case example: Itraconazole, Eur. J. Pharm. Biopharm. 83 (2013) 378–387.
[36] B. McCormack, G. Gregoriadis, Drugs-in-cyclodextrins-in-liposomes: an approach to controlling the fate of water insoluble drugs in vivo, Int. J. Pharm. 162 (1–2) (1998 Mar) 59–69.
[37] P. Gupta, A.K. Bansal, Ternary amorphous composites of celecoxib, poly(vinyl pyrrolidone) and meglumine with enhanced solubility, Pharmazie 60 (11) (2005) 830–836.
[38] C. Aloisio, A. Gomes de Oliveira, M. Longhi, Characterization, inclusion mode, phase-solubility and in vitro release studies of inclusion binary complexes with cyclodextrins and meglumine using sulfamerazine as model drug, Drug Dev. Ind. Pharm. 40 (7) (2014 Jul) 919–928.
[39] F. Frézard, P.S. Martins, A.P.C.O. Bahia, L. Le Moyec, A.L. de Melo, A.M.C. Pimenta, et al., Enhanced oral delivery of antimony from meglumine antimoniate/β-cyclodextrin nanoassemblies, Int. J. Pharm. 347 (2008 Jan 22) 102–108.
[40] P. Gupta, A.K. Bansal, Modeling of drug release from celecoxib-PVP-meglumine amorphous system, PDA J. Pharm. Sci. Technol. 59 (6) (2005) 346–354.
[41] P. Gupta, A. Bansal, Molecular interactions in celecoxib-PVP- meglumine amorphous system, J. Pharm. Pharmacol. 57 (2005) 303–310.
[42] C. Kirby, G. Gregoriadis, Dehydration- rehydration vesicles: a simple method for high yield drug entrapment in liposomes, Biotechnology 2 (1984) 979–984.
[43] D.G. Fatouros, P. Klepetsanis, P.V. Ioannou, S.G. Antimisiaris, The effect of pH on the electrophoretic behaviour of a new class of liposomes: arsonoliposomes, Int. J. Pharm. 288 (1) (2005 Jan 6) 151–156.
[44] Z. Qiang, C. Adams, Potentiometric determination of acid dissociation constants (pKa) for human and veterinary antibiotics, Water Res. 38 (2004) 2874–2890.
[45] Nokhodchi A. The effect of type and concentration of vehicles on the dissolution rate of a poorly soluble drug (indomethacin) from liquisolid compacts. J. Pharm. Pharm. Sci. 8(1):18–25.