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Tetracycline-niosomes versus Tetracycline Hydrochloride-niosomes: How to Modulate Encapsulation and Percutaneous Permeation Properties

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Abstract

Nowdays, the design of the optimalniosomal formulation may ensure the best performance in terms of physico-chemical properties and drug skin permeation. Our study want to propose the loading of Tetracycline or Tetracycline hydrocholoride drugs as strategy to modulate niosomes properties, with particular emphasis on encapsulation and percutaneous permeation behavior. Niosomes were prepared from mixtures of nonionic commercial surfactants with different final value of hydrophile-lipophile balance (HLB). Significant increase in size respect to the corresponding empty vesicles occurred when drugs were encapsulated, while the presence of glyceryl monostearate resulted in a reduction of the diameter. Drugs entrapment efficiencies were found to be directly related to the chemical properties of the system: tetracycline showed better affinity for lipophilic matrices, while tetracycline hydrochloride was better encapsulated in hydrophilic ones. Finally we found drugs permeation across the skin was affected by many variables, such as bilayer composition, niosomes size, nature of the drugs and their entrapment efficiency, where by depending on the intended effect (topical or systemic) the opportune formulation may be selected.

Keywords: Niosomes, Tetracycline, Entrapment efficiency, Permeation.

Introduction

Niosomes are nanosized vesicles composed of one or more lipid layers that enclose aqueous compartments. They differ from lipososomes since niosomes consist of surfactants molecules and not by phospholipids. Because of their biphasic character, niosomes can act as carriers for hydrophilic, lipophilic and amphiphilic drugs. Depending upon their solubility and partitioning characteristics, therapeutics molecules are located differently in the niosomal environment and exhibit different entrapment and release behavior [1]. Often, problems like poor entrapment efficiency, physical and chemical instability have been found to be associated with the encapsulation of drugsin niosomesor other macromolecular systems [2], so that the use of the appropriate drug chemical form can significantly improve the carrier performances.

Drug molecules can be classified into three categories: hydrophilic, lipophilic and those with biphasic solubility (amphiphilic). Hydrophilic drugs (HD) are dissolved in the external aqueous phase during niosomes preparation, and become entrapped in the internal aqueous core within the formed vesicles [3]. Their entrapment efficiency (E%) is difficult to predict since it depends on the preparation method, the bilayer composition, as well as the niosomes size and lamellarity, but it is usually very low [2,4]. In addition, water soluble drugs show relatively fast leakage out of the vesicles, whereby their rapid clearance, suboptimal biodistribution, low intracellular absorption can limit their therapeutic efficacy [5]. High E% is very important for drug delivery because it reduces the cost of formulations [6]. Lipophilic drugs (LD) are entrapped almost completely in the lipid bilayer of the vesicles, often reaching entrapment efficiency of 100% [3]. In addition, sincethey are very poorly soluble in water, problems like loss of entrapped drug on storage are minimal. Drugs with intermediate partition coefficients (amphiphilic drugs, AD) distributed between the lipid and aqueous phases and arevery easily lost from the vesicular systems [7]. Moreover, during the processing of vesicles, the removal of non-encapsulated molecules forms an essentialstep because the entire purpose of niosomaldrug incorporation would be defeated if the unentrapped drug is present in the final product. Procedures such as dialysis and passage through exclusion columns (which are employed for theremoval of non-entrapped material) are often time-consuming, tedious and expensive. In this respect, the use of lipophilic drugs is particularly useful as they are quantitatively incorporated into the niosomes.

Considering these findings, the aim of this work was to make a comparison between the physico-chemical properties of Te and Te-HCl loaded niosomes, with particular emphasis

on encapsulation and percutaneous permeation behaviour. In particular, as reported elsewhwere [8,9], the solubility of tetracicline hydrochloride form in water is different than that of the respective base form ($7x10^{-3}$mol/L and $37x10^{-3}$mol/L respectively), while in organic solvents (i.e. ethanol, acetone) reverse effect occurs. The drug solubility in selected solvents is of key importance for the identification of drug delivery pathways, in order to develop more efficient pharmaceutical formulations. For these reasons, we designed different niosomal formulations starting from mixtures of non ionic commercial surfactants. Glyceryl mono stearate (GMS) is the glycerolester of stearic acid and occurs naturally in the body as a by-product of the breakdown of fats; Tween 60 (T60, polyoxyethylenesorbitanmonostearate) or Tween 80 (T80, polyoxyethylenesorbitanmonooleate), belong to the class of Polysorbates and are traditionally used in pharmaceutical fields because their least toxicity and irritant potential. Tetracycline (Te) or tetracycline hydrochloride (Te-HCl), were loaded in the niosomallipidic film or in the acqueous core, respectively (Figure 1).

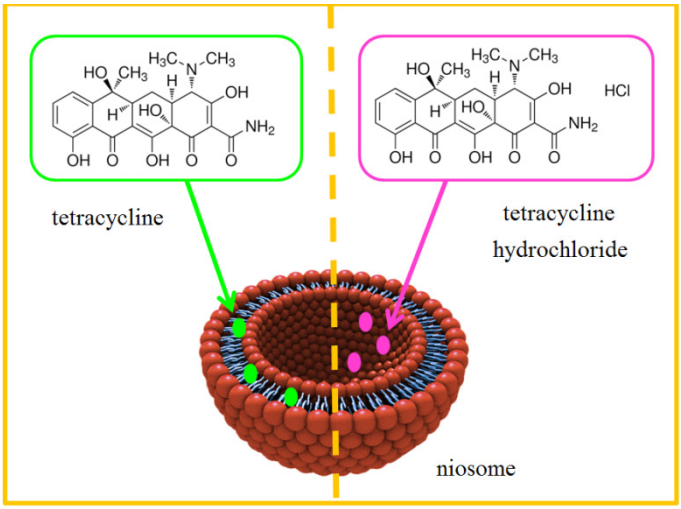

Figure 1: Schematic representation of Te and Te-HCl designed niosomes

All formulations were compared in terms of size, morphology, polydispersity index and drug entrapment efficiency. Moreover, because tetracycline was the first topical antibiotic approved for the treatment of acne and its use has been limited because of the low skin penetration, we further investigated the enhancing effect of niosomeson the *ex vivo* percutaneous permeations of both drugs. These experiments were carried out using a Franz-type diffusion cells and resultswere compared to those obtained by using their drug solutions.

Materials and Methods

Chemicals

Tetracycline (Te) and tetracycline hydrochloride (Te-HCl) were purchased from Fluka (Sigma-Aldrich, Milan, Italy,98% purity). Glycerylmonostearate, Tween 60, Tween 80 were purchased from Sigma-Aldrich (Milan, Italy). Organic solvents were supplied from Sigma-Aldrich (Milan, Italy) and are of high performance liquid chromatography grade. Double-distilled water was used. Absorption spectra were recorded with a UV-Vis JASCO V-530 spectrometer using 1 cm quartz cells.

Preparation of niosomes

Multilamellarniosomal vesicles (MLVs) were prepared by the hydration of lipidic film method, at 10 mM total lipid concentration [10]. Accurately weighed amounts of GMS and Tween 60 or Tween 80 at different molar ratios were dissolved in ethanol in a round-bottom flask as reported in Table 1. After mixing, solvent was evaporated under reduced pressure and constant rotation to form a thin lipid film. This film was then hydrated with 10 mL of distilled water at 60°C for 30 min, to obtain empty large MLV. Tetracycline hydrochloride loaded niosomes were prepared hydrating the lipidic film with 10 mL of Te-HCl aqueous solution containing $9x10^{-6}$ moles of drug, while Tetracycline niosomes were obtained dissolving $9x10^{-6}$ moles of Te in the initial surfactants mixture and then hydrating with 10 mL of distilled water. After preparation, dispersions were left toequilibrate at 25 °C overnight. Small unilamellar vesicles (SUV) were prepared starting from MLV by sonication in an ultrasonicbath for 30 min at 60°C. The purification of Te-HCl and Teniosomes from untrapped materials was carried out by a flow of niosomal suspensions across a Sepharose CL-4B gel. After purification, niosomes were stored at 4°C in the dark, until needed in subsequent.

Size and distribution analysis

The niosomes diameter and size distribution were determined by dynamic light scattering (DLS), using a 90 Plus Particle Size Analyzer (Brookhaven Instruments Corporation, New York, USA) at 25.0 ± 0.1°C. We measured the autocorrelation function at 90°. The laser beam was operating at 658 nm. The polydispersity Index (P.I.) was used as a measure of the size distribution. It was directly obtained from the instrumental data fitting procedures by the inverse "Laplace transformation" and by Contin methods [11]. P.I. values ≤0.3 indicate homogenous and mono-disperse populations in the case of colloidal systems. The samples were analyzed 24 h after preparation and before each

Table 1: Composition, hydrodynamic diameter and polydispersity index of empty vesicular systems at 25°C. Values represent mean ± SD (*n*=3)

Formulation	(X) mg		GMS (Y) mg	Moles ratio X:Y	Diameter (nm)	P.I.
	Tween 60	Tween 80				
A	131	-	-	1:0	441±11	0.267
B	87	-	12	2:1	411±10	0.283
C	65	-	18	1:1	213±14	0.233
D	-	60	-	1:0	462±12	0.260
E	-	40	12	2:1	416±11	0.237
F	-	30	18	1:1	338±13	0.278

following characterization step. They were diluted with distilled water before measurements were run. 50 mL of each vesicle dispersion were diluted to 10 mL with distilled water. Each sample was measured three times and the results are expressed as mean ± standard deviation. The Z-potential of the formulations was measured with the laser Doppler electrophoretic mobility measurements using the Zetasizer ZS (Malvern Instruments Ltd., Malvern, U.K.), at 25.0 ± 0.1°C. All analyses were done in triplicate. Z-potential values and standard deviations were elaborated directly from the instrument.

Drug entrapment efficiency

Te and Te-HCl entrapment efficiencies (E%) into niosomes were expressed as the percentage of the drug entrapped into purified niosomes, referred to the total amount of drug present in the non-purified samples. It was determined by diluting 1 mL of purified and 1 mL of non-purified niosomes in 25 mL of methanol, followed by the measurement of maximum absorbance of these solutions at 268 nm and 272 nm for Te and Te-HCl, respectively. Methanol allows the breaking of niosomal membranes and the release of encapsulated drug. Absorption spectra were recorded with a UV±vis JASCO V-530 spectrometer using 1 cm quartz cells. Each experiment was carried out in triplicate and the results are expressed as mean ± standard deviation.

Ex vivo permeation studies

Ex vivo permeation experiments were carried out in the vertical Franz diffusion cells for 24 h at 37°C, through rabbit ear skin obtained from a local slaughterhouse, following procedure reported elsewhere [12]. The skin, previously frozen at -18°C, was pre-equilibrated in physiological solution at room temperature for 2 h before the experiments. A circular piece of this skin was sandwiched securely between the receptor and donor compartments with the dermal side in contact with the receiver medium and the epidermis side in contact with the donor chamber (contact area= 0.416 cm^2). The donor compartment was charged with an appropriate volume of sample to keep constant the drug moles ($1.9x10^{-7}$moles) while the receptor compartment was filled with 5.5 mL of distilled water. As reported in literature, in fact, Te possess a certain water solubility and the total moles of drugloaded in the donor compartment are lower of the maximum amount permitted. At regular intervals up to 24 h, the medium in the receiver compartment was removed and replaced with an equal volume of pre-thermostated (37 ± 0.5°C) fresh one. The complete substitution of the medium was needed to ensure sink conditions and quantitative determination of the small amounts of permeated drug. The content of drug in the samples was analyzed by UV–vis spectrometry. Each experiment was carried out in triplicate and the results were in agreement within ± standard deviation.

Results and Discussion

Novel niosomal formulations loading Te or Te-HCl were prepared starting from mixtures of GMS/Tween 60 and GMS/Tween 80 surfactants, with the objective to widen knowledge on the influence that the drug hydrophilic/hydrophobic nature may exercise of the niosomesphysico-chemical properties. GMS, Tween 60 and Tween 80 are nonionic surfactant with well-know hydrophile-lipofile balance (HLB). GMS possess HLB of 3.8, suggesting lipid soluble behaviour, while in the case of Tween 60 and Tween 80 this parameter is 14.9 and 15.0, respectively, predicting water affinity. As vesicles formation depends on both surfactant structure and balance between its hydrophilic and hydrophobic portions, we eliged mixtures of Polysorbates and GMS giving final HLB ranged from 15 to 9, as reported in Table 1. All matrix compositions were able to produce vesicles without the presence of any membrane additive. GMS alone was not able to form vesicles, because its lower HLB and its not adequate critical packing parameter, whereby molar ratio of 0:1 have not been reported.

All formulations appeared in translucent white dispersions without sedimentation, indicating that the niosomes were physically stable due to small and uniform sizes obtained after sonication process. All niosomal preparations were stable when kept at room temperature (25°C) over a period of 8 months. Table 1 also shows diameter, P.I. and Z-potential of all developed niosomes. Polydispersity index (P.I.) of less than 0.28 indicates a narrow size distribution of the niosomes and consequently homogeneous formulations. Empty vesicles exhibited negative values of the Z-potential which might be attributed to the negative charge onto the surface of niosomes: these values ranged from -16.4 mV (absence of GMS) to -24.5 mV (higher amount of GMS). No relevant difference between T60 and T80 based vesicles were achieved.

Tween 60 and Tween 80-based niosomes were characterized by mean sizes of 441 and 462 nm, respectively. Addition of increasing amount of GMS produced a remarkable reduction of the size.

Generally surfactants with high HLB value were not able to form vesicles because the high hydrophilicity of the molecules [13]: in the case of Tween 60 and Tween 80 this not occurred, but larger vescicles were obtained. The hydrophobicity increase of the systems produced by GMS may contribute toenhance the stability of the bilayer and its cohesive energy, restricting the Polysorbates chains motion, as reported elsewhere [14]. Slightly higher diameters were obtained with Tween 80 respect to the corresponding formulations based on Tween 60: both surfactants possess 18 C atoms in their structure, but Tween 80 presents a double bond, capable tomodify bilayer fluidity andthe chains packing, resulting in higher vesicles diameter.

Following the experimental procedures previously described, Te and Te-HCl loaded niosomes were prepared and size, polydispersity index and entrapment efficiency of all formulations are reported in Table 2 and Table 3. Loaded vesicles were stored in transparent vials covered with aluminum cap in the dark and their physical characteristics (colour, sedimentation and particle size) were monitored for several months. All formulations appeared as translucent pale yellow suspensions without sedimentation, creaming or flocculation up to 8 months. In addition, diameters values generally keep constant. P.I. values are lower than 0.3, as reported for empty vesicles, confirming homogeneous distributions. Both Te and Te-HCl loaded niosome sgave Zeta-potential values similar to those obtained by empty vesicles showing that this parameter was not influenced either by the encapsulation of the drug.

Tetracycline is a broad spectrum medicinal drug active against a

Formulation	Drug	Diameter (nm)	P.I.	E%
A-Te	tetracycline	690±18	0.272	17±0.99
B-Te	tetracycline	456±14	0.288	31±1.21
C-Te	tetracycline	222±11	0.293	45±1.64
A-Te-HCl	tetracycline-HCl	501±12	0.280	44±1.56
B-Te-HCl	tetracycline-HCl	411±11	0.299	26±1.09
C-Te-HCl	tetracycline-HCl	270±10	0.282	23±1.44

Table 2: Composition, hydrodynamic diameter, polydispersity index and entrapment efficiency of Tween 60-based niosomesat 25°C. Values represent mean ± SD (*n*=3)

Formulation	Drug	Diameter (nm)	P.I.	E%
D-Te	tetracycline	476±16	0.250	33±2.03
E-Te	tetracycline	391±13	0.207	40±2.40
F-Te	tetracycline	357±15	0.238	41±1.98
D-Te-HCl	tetracycline-HCl	606±12	0.296	80±2.76
E-Te-HCl	tetracycline-HCl	577±11	0.288	69±2.33
F-Te-HCl	tetracycline-HCl	367±15	0.289	55±2.08

Table 3: Composition, hydrodynamic diameter, polydispersity index and entrapment efficiency of Tween 80-based niosomesat 25°C. Values represent mean ± SD (*n*=3)

number of gram-positive and gram-negative bacteria, successfully used worldwide in human and veterinary medicine. It decrease the production and activity of inflammatory cytokines and inhibit matrix metalloproteinases production and activation, as requested in the treatment of rosacea, acne, diabetes and various types of neoplasms [15-17]. Tetracycline is composed of several interlinked aromatic rings and multiple substituents containing heteroatoms (N and O) liable to a variety of specific interactions with other formulation constituents, biological membranes and fluids. It is an amphoteric compound with characteristic pH values and it forms crystalline hydrates and salts with acids and bases [18].

As reported in literature, the loading of a drug greatly influence vesicles size: its chemical nature and then its localization into the niosomal structure, in fact, affect in a relevant manner both niosomes physico-chemical properties and skin permeation [19].

In our case, significant increase in vesicles size respect to the corresponding empty formulations occurred, independently from the chemical form of the drug, both for Tween 60 and Tween 80 based samples. Most important, any variation of vesicles morphology was detected. Interesting, as found for empty niosomes, the presence of GMS resulted in a reduction of the average diameter since it helps in forming self-closed bilayers, leading to appropriate molecular geometry and hydrophobicity for vesicles formation.

From obtained data, it seems that the drug chemical nature may not be used to predict in absolute the increment of vesicles size. For Tween 80-based niosomes, the use of Te-HCl always gave an higher increase, while in the case of Tween 60, the behavior is unpredictable. So, this increment is not in relation with the compartment in which the drug was located: bilayer for Te and aqueous core for Te-HCl.

Because its chemical structure, tetracycline has pk_a values of about 3, 7 and 9 in water, attributed to different structural grouping. At our experimental condition, (about pH= 6.8), mainly the first dissociation assigned to the tricarbonyl system in position 1, 2 and 3 occurred. Therefore drug molecules presented a negativecharge, liable to cause electrical repulsion among them and then an increase of niosomal size [20]. This trend could be described on the basis of different mechanisms in forming niosomal vesicles, which spontaneously occurred during hydration process: when hydrating with a certain medium pH, some charges may develop on the drug molecules, causing the orientation of nonionic surfactant into bilayers membrane. Subsequently this bilayer would curve and split up to form closed vesicles so as to reduce its free energy. Eventually, the increment of size allow to keep the charges distant.

The encapsulation efficiencies of Te and Te-HCl into niosomal formulations are given in Table 2. Usually, the chemical structure of drugs and the presence of charged moieties strongly affect the drug entrapment efficiency [21]. As expected, we observed that Te E% into Tween 60 based vesicles may be directly related to the hydrophobicity of the system: higher GMS content corresponded to higher entrapment efficiency values, that ranged from 17% (A-Te) up to 45% (C-Te), respectively. Conversely, Te-HCl, due its hydrophlic nature, showed higher E% (44%)in the formulations not containing GMS, while lowest E% was obtained by C-Te-HCl sample (22%). Similar trends were obtained in the case of Tween 80 based vesicles. In particular Te-HCl entrapment efficiencies were found to be 80%, 69% and 55% for D-Te-HCl, E-Te-HCl and F-Te-HCl samples, respectively, while the corresponding E% of Te were found to be 33%, 40% and 41%. Anyhow, the use of Te-HCl generally ensured higher entrapment efficiencies. These results were unexpected because, generally, lipophilic compounds give higher E% than the corresponding hydrophilic forms [3].

Also particle size may be directly related to the entrapment efficiency values [22]. We observed inverse relationship in the case of Te-loaded niosomes: a decrease in the size vesicles corresponded to an increase of E%. Since Te was located into the bilayer, higher amount of drug may cause an increase of matrix cohesion and then a reduction of the size. Conversely, direct relationship occurred for Te-HCl loaded vesicles. In this case, Te-HCl was located into the aqueous compartment and, due to the electrical repulsion and free motion in the internal core, the higher amount of drug inside, the larger the vesicles.

Percutaneous permeation studies

The optimization of drug delivery through skin is important in modern therapy. The topical route offers an attractive alternative to the conventional administration ones (i.e. oral, parenteral), and it represents one of the most innovative research area in pharmaceutical field [23]. The main advantages of using niosomes as topical drug delivery systems arise from their peculiar features, such as their small size, composition and architecture. In addition these vesicles have been claimed to serve as a local depot for sustained drug release, permeation enhancers

of dermally active compounds or as rate-limiting barrier for the modulation of systemic absorption of drugs [24].

The cumulative amounts of Te and Te-HCl permeated from different formulations were investigated for a period of 24h: each sample was analyzed in triplicate. Figure 2a and Figure 2b show the *ex vivo* percutaneous permeation profiles of Te and Te-HCl from T60-based niosomal formulations, respectively. Te and Te-HCl free solutions were used as controls. As illustrated, for all formulations, the cumulative amounts of drugs permeated through skin increased with times. In the case of Te-HCl, these values are always higher than those obtained in the case of free drug solutions, while in the case of Te, drug vehiculated into niosomes permeated more slowly than the free tetracycline.

Niosomes characterized by great hydrophilia (A-Te and A-Te-HCl) exhibited the highest permeation percentages (about 100%) comparing to the formulations containing GMS. In particular, when Te-HCl was loaded, samples prepared with 2:1 molar ratio between Tween 60 and GMS (B-Te-HCl), reached intermediate values, while increasing this molar ratio up to 1:1 (C-Te-HCl), drug penetrated more slowly. Conversely, no significant difference between B-Te and C-Te percutaneous permeation was observed up to 24 h. Additionally, A-Te and B-Tesamples showed similar biphasic release patterns: a significant initial burst releasewas observed up to 6 h, followed by a plateau. In the others cases, permeation curves show classic profiles: drug permeation rates were almost constant. This trend may be due to the bilayer composition. Several studies reported that the presence of a surfactant with low HLB value caused a significant increment of the systems hydrophobicity: this results in a higher drug retention capacity and in a more delayed permeation [25]. In our case tetracycline, because its affinity for hydrophobic environment, possess greater difficulties to leave the bilayer, where by this behaviour was more pronounced. Unexpectedly, this trend also applied for the Te-HCl. Despite the more hydrophilic character, in fact, Te-HCl vesicles showed permeation percentages lower than the corresponding Te-loaded ones, due to higher retention into more hydrophobic niosomal matrix (B-Te-HCl and C-Te-HCl).

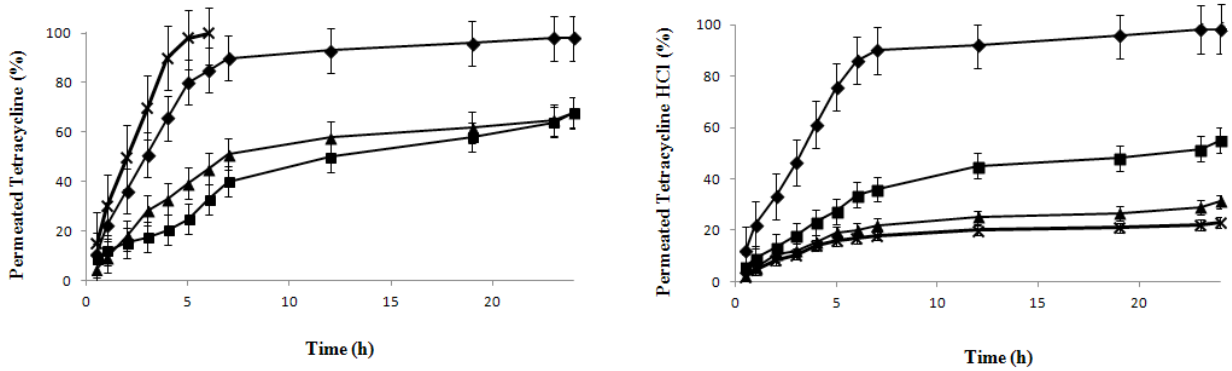

Figure 2: *Ex vivo* Te and Te-HCl permeation from the different formulations through rabbit skin at 37°C: a) (♦) A-Te, (■) B-Te, (▲) C-Te, (×) Te solution; b)(♦) A-Te-HCl, (■) B-Te-HCl, (▲) C-Te-HCl, (×) Te-HCl solution

Regarding Tween 80-based formulations, niosomes with the highest hydrophilia (D-Te and D-Te-HCl) exhibited the highest permeation percentages, but this trend was more pronounced when Te-HCl was loaded into the vesicles, as illustrated in Figure 3a and Figure 3b.

More hydrophobic formulations gave lower permeation rates, as found for Tween 60-based samples, whereby the same hypothesis formulated for the previous niosomalset may be still valid.

No significant differences were found between their permeation curves.

However, another consideration need to be made: the amount of drugs permeated from Tween 80-based formulations were lower respect to the corresponding Tween 60 ones, indicating thepresence of unsaturated chainsmay influence in a relevant manner the transdermal release of entrapped molecules [26]. Our hypothesis was confirmed by the permeation percentages

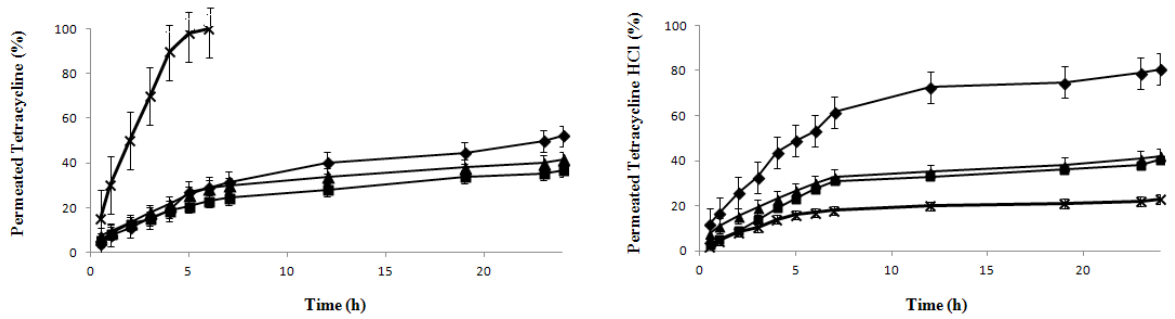

Figure 3: *Ex vivo* Te and Te-HCl permeation from the different formulations through rabbit skin at 37°C: a) (♦) D-Te, (■) E-Te, (▲) F-Te, (×) Te solution; b) (♦) D-Te-HCl, (■) E-Te-HCl, (▲) F-Te-HCl, (×) Te-HCl solution

achieved by Te-niosomes: in these cases, the cumulative amounts of drug permeated up to 24 h were similar, independently from the presence of GMS. This means that double bond in Tween 80 may beresponsible both for the drug retention effect into the bilayer (confirmed by the highest E%) and the subsequently delayed permeation.

Anyway, many variables contributed to the drugs permeation across the skin. Depending on the effect to be obtained (local or systemic), the appropriate formulation may be selected. To achieve systemic effect, Polysorbates-based formulations may be preferred, while niosomes containing high amount of GMS could be used to obtain a delayed permeation of drug. Considering the chemical nature of the drug, we can apologize that only slightly difference occurred: for T60 based formulations, best results were obtained by using Te, while for Tween 80 based vesicles, best modulation may be obtained encapsulationg Te-HCl.

Conclusions

The modulation of niosomes physico-chemical properties and *ex vivo* drug percutaneous permeations were obtained by loading Te or Te-HCl into vesicles. The effects of different bilayer compositions (based on mixtures of nonionic commercial surfactants such as GMS, Tween 60 and Tween 80) have been also evaluated. Vesicles size were significantly affected by niosomal matrices HLB values: increasing amount of glyceryl monostearate resulted in a reduction of the diameter. This trend was also confirmed in presence of drugs. Moreover, despite drug molecules, loaded vesicles were found to be larger than the empty ones and entrapment efficiencies were found to be directly related to the chemical properties of the system. Tetracycline showed better affinity for lipophilic matrices, while tetracycline hydrochloride was better encapsulated in hydrophilic ones, reaching the value of 80% in the case of mixture Tween 80/ GMS in the ratio 1:0. Increased percutaneous permeation across the rabbit skin, respectto control drugs solutions, suggests that niosomal formulations havepotential for transdermal delivery and enhanced drugs release fromthe carriers. In particular we found drugs permeation was affected by bilayer composition, niosomes size, nature of drugs and their entrapment efficiency, whereby depending on the intended effect (topical or systemic) the opportune formulation may be selected.

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