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Effect of some formulation parameters on flurbiprofen encapsulation and release rates of niosomes prepared from proniosomes

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ABSTRACT

Proniosomal gels or solutions of flurbiprofen were developed based on span 20 (Sp 20), span 40 (Sp 40), span 60 (Sp 60), and span 80 (Sp 80) without and with cholesterol. Nonionic surfactant vesicles (niosomes) formed immediately upon hydrating proniosomal formulae. The entrapment efficiency (EE%) of flurbiprofen (a poorly soluble drug) was either determined by exhaustive dialysis of freshly prepared niosomes or centrifugation of freeze-thawed vesicles. The influence of different processing and formulation variables such as surfactant chain length, cholesterol content, drug concentration, total lipid concentration, negatively or positively charging lipids, and the pH of the dispersion medium on flurbiprofen EE% was demonstrated. Also, the release of the prepared niosomes in phosphate buffer (pH 7.4) was illustrated. Results indicated that the EE% followed the trend Sp 60 (C_{18}) > Sp 40 (C_{16}) > Sp 20 (C_{12}) > Sp 80 (C_{18}). Cholesterol increased or decreased the EE% depending on either the type of the surfactant or its concentration within the formulae. The maximum loading efficiency was 94.61% when the hydrating medium was adjusted to pH 5.5. Increasing total lipid or drug concentration also increased the EE% of flurbiprofen into niosomes. However, incorporation of either dicetyl phosphate (DCP) which induces negative charge or stearyl amine (SA) which induces positive charge decreased the EE% of flurbiprofen into niosomal vesicles. Finally, in vitro release data for niosomes of Sp 40 and Sp 60 showed that the release profiles of flurbiprofen from niosomes of different cholesterol contents is an apparently biphasic release process. As a result, this study suggested the potential of proniosomes as stable precursors for the immediate preparation of niosomal carrier systems.

1. Introduction

Like phospholipids, the nonionic surfactants are able to form vesicular delivery systems "niosomes" when dispersed into water. They are known as analogues of liposomes, and have been used in cosmetic formulations and experimentally as drug carriers (Arunothayanun et al., 1999; Barolli et al., 1999). Niosomal vesicles can encapsulate both lipophilic and hydrophilic drugs and protect them against acidic and enzymatic effects in vivo (Yoshida et al., 1992). There are many reported studies showed that niosomes behave in vivo like liposomes as they are able to prolong the circulation of encapsulated drug altering its organ distribution and metabolic stability (Rogerson et al., 1988), or to increase the contact time of drug with the applied tissue in topical preparations (Hofland et al., 1994). They offer several advantages over liposomes such as higher chemical stability, intrinsic skin penetration enhancing properties and lower costs (Manconi et al., 2002). However, there may be problems of physical instability in niosome dispersions during storage like vesicles aggregation, fusion, leaking or hydrolysis of encapsulated drugs, which affected the shelf life of the dispersion (Hu and Rhodes, 2000).

Several methods were reported for the preparation of niosomes, however, still have many disadvantages. The reverse-phase evaporation method and ether or ethanol injection methods require vigorous conditions such as organic solvents, sonication and high temperatures for long time (Weiner, 1994). Proniosomes; semisolid liquid crystal (gel) products of nonionic surfactants easily prepared by dissolving the surfactant in a minimal amount of an acceptable solvent namely ethanol and the least amount of water could solve the problem (Vora et al., 1998). The proniosomal structure was liquid crystalline-compact niosomes hybrid that was converted into niosomes immediately upon hydration (Fang et al., 2001; Varshosaz et al., 2005; Gupta et al., 2007). Lecithin still acts as a component of the proniosomal structure as they reported. However, the incorporation of lecithin into formulations requires special treatment during preparation and storage, which makes the product

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Table 1
Formulation design of flurbiprofen proniosomes

Cholesterol (mmol)	Nonionic surfactant[a] (mmol)	Flurbiprofen (mg)	Ethanol (mg)	Water (ml)
0	1	50	400	0.16
0.1	0.9	50	400	0.16
0.2	0.8	50	400	0.16
0.3	0.7	50	400	0.16
0.4	0.6	50	400	0.16
0.5	0.5	50	400	0.16
0.6	0.4	50	400	0.16

[a] Sp 20, Sp 40, Sp 60, or Sp 80.

less stable and highly expensive. So, it may be suggested to prepare proniosomes devoid of lecithin component.

Proniosomes hydrated by agitation in hot water for a short period of time, offer a versatile delivery concept with the potential for drug delivery via the transdermal route (Hu and Rhodes, 2000). This would be possible if proniosomes form niosomes following topical application under occlusive conditions, due to hydration by water from the skin itself.

The aim of this study is to investigate the feasibility of using proniosomes as stable precursors for the preparation of niosomes as drug carrier systems for poorly soluble drugs. Proniosomes provide the advantages of easy and immediate preparation of niosomes. Also, the niosomal drug delivery systems have a great advantage for a poor soluble drug by increasing its solubility, controling its release and prolonging its activity over long periods of time. Hence, decreasing the frequency of administration and improving patient compliance. The influence of different processing and formulation variables such as cholesterol content, nonionic surfactant structure (Sp 20, Sp 40, Sp 60, or Sp 80), drug concentration, total lipid concentration, negatively or positively charged lipids, and the pH of the hydration medium on flurbiprofen entrapment efficiency will be demonstrated. Also, flurbiprofen release rates from niosomes in phosphate buffer (pH 7.4) will be illustrated.

2. Material and methods

2.1. Materials

Flurbiprofen was a gift from Egyptian International Pharmaceutical Industries Co. (E.I.P.I.Co), Egypt. Sorbitan monolaurate (span 20), sorbitan monopalmitate (span 40), sorbitan monostearate (span 60), sorbitan mono-oleate (span 80), sodium azide, and cholesterol (Chol; >99%) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Stearylamine (SA) and dicetyl phosphate (DCP) were obtained from Fluka Chemical Co. (Germany). Spectrapore® nitrocellulose membranes (MWCO 2000–15,000) were obtained from Spectrapore Inc., NY, USA. All other chemicals and solvents were of analar grade and obtained from El-Nasr Company for Pharmaceutical Chemicals, Cairo, Egypt.

2.2. Methods

2.2.1. Preparation of proniosomes

Proniosomes were prepared by the method reported by Vora et al. (1998) with some modifications. In glass vials accurately weighed amounts of the surface-active agent were mixed with the appropriate amount of cholesterol to make 1 mmol total lipids (Table 1). The amounts of cholesterol were added as 10% increments varied from 0% to 60% of total lipids. Flurbiprofen was added to the nonionic surfactant/cholesterol mixture. Absolute ethanol (about 400 mg) was added to the mixtures then vials were tightly sealed and warmed in water bath (55–60 °C) for 5 min while shaking until complete dissolution of cholesterol. To each of the formed transparent solutions, about 0.16 ml hot distilled water (55–60 °C) was added while warming in the water bath for 3–5 min till a clear or translucent solution was produced. The mixtures were allowed to cool down at room temperature and observed for the formation of transparent solution, two phases liquid, and translucent, transparent or white creamy proniosomal gel as shown in Table 2. To obtain charged proniosomes, SA or DCP were added to lipid mixtures then dissolved into ethanol as described above. SA or DSP did not affect the final appearance of the developed proniosomes. The obtained formulations were kept in the same closed glass vials in dark for further characterization.

2.2.2. Hydration step and formation of niosomes

Niosomes were prepared by hydration of the gels prepared as described above. About 7 ml of Sorensen's phosphate buffer (pH 7.4) was added into each vial followed by heating for 10 min at a temperature above 60 °C in a water bath. Vortexing of the formulations was done two to three times during the 10 min heating. The final volume was adjusted to 10 ml by the same buffer.

Proniosomal systems of span 20 containing 0% and 10% cholesterol and that of span 80 containing from 0% to 20% cholesterol formed niosomes upon addition of phosphate buffer and shaking for only one minute at room temperature.

2.2.3. Microscopic examination

Small amounts of the formed niosomes were spread on a glass slide and examined for the vesicles structure and the presence of insoluble drug crystals using ordinary light microscope with varied magnification powers (10× and 40×). Photomicrographs were taken for niosomes using Fujifilm Finepix F40fd 8.3 MP digital camera with 3× optical zoom.

2.2.4. Solubility determination of flurbiprofen

An excess amount of flurbiprofen was added to each of distilled water, and phosphate buffer (pH 5.5, 6.5, 6.8, 7.4, and 8). The mixtures were then kept at ambient temperature for 72 h in a shaker water bath to get equilibrium. The equilibrated samples were centrifuged at 3000 rpm for 5 min (Eppendorf Centrifuge 5415C, maximum speed 14,000 min^{-1}, Germany). Aliquot portions of the supernatants were taken and properly diluted with phosphate buffer (pH 7.4) for quantification of flurbiprofen spectrophotometrically at 247 nm (Shimadzu UV-1201, Cat No. 206-62409, Shimadzu Corporation, Japan).

2.2.5. Determination of entrapment efficiency of flurbiprofen in niosomes

2.2.5.1. Dialysis method (Udupa et al., 1993). From the niosomal dispersion formed from proniosomes of Sp 20, Sp 40, Sp 60 and Sp 80, unentrapped free drug was removed by placing 1 ml of the dispersion into a glass tube to which a cellophane membrane was attached to one side and dialyzing exhaustively for 1 h each time against 100 ml of phosphate buffer (pH 7.4). The dialysis of free flurbiprofen was completed after about six changes of buffer solution

Table 2
Composition and appearance of nonionic surfactant/cholesterol/water/absolute ethanol

Cholesterol molar%	Sp 20	Sp 40	Sp 60	Sp 80
0	Transparent liquid (one phase)	White creamy gel	White creamy gel	Two phases liquid
10	Transparent liquid (one phase)	White creamy gel	White creamy gel	Two phases liquid
20	Transparent gel	White creamy gel	White creamy gel	Two phases liquid
30	Transparent gel	White creamy gel	White creamy gel	Translucent gel
40	Translucent gel	White creamy gel	White creamy gel	Translucent gel
50	Translucent gel	White creamy gel	White creamy gel	Translucent gel
60	Translucent gel	White creamy gel	White creamy gel	Translucent gel

where no further flurbiprofen could be detected in the solution. The drug content was determined spectrophotometrically at 247 nm against phosphate buffer (pH 7.4) as a blank. Amount of entrapped drug was obtained by subtracting amount of unentrapped drug from the total drug incorporated (Deepika and Indu, 2005).

$$\text{Entrapment efficiency (\%)} = \frac{\text{amount of drug entrapped}}{\text{total amount of drug}} \times 100$$

2.2.5.2. Freeze thawing/centrifugation method. 1 ml samples of niosomes prepared from Sp 40 and Sp 60 were frozen for 24 h at −20 °C in Eppendorf tubes. The frozen samples were removed from the freezer and let to thaw at room temperature, then centrifuged at 14,000 rpm for 40 min at 4 °C. Niosomal pellets were resuspended in phosphate buffer (pH 7.4) and then centrifuged again. This washing procedure was repeated two times to ensure that the unentrapped drug was no longer present in the void volume between the niosomes. The supernatant was separated each time from niosomal pellets and prepared for the assay of free drug. Each result was the mean of three determinations (±S.D.). The % entrapped flurbiprofen was calculated as mentioned before.

2.2.6. Assessment of flurbiprofen release rates from niosomes

The niosomal pellets prepared from proniosomes of Sp 40 and Sp 60 were separated by centrifugation and washed twice as described above. The pellets were resuspended in 50 ml of phosphate buffer (pH 7.4). The suspension was placed in a 125 ml stoppered glass flask in a shaking water bath at 37 °C. 1 ml samples were withdrawn at the following intervals: 0, 1, 2, 4, 6, 8, 24, 48 and 72 h after incubation. Samples were centrifuged as mentioned before and supernatants were assayed at 247 nm. The percentage of drug release was plotted as a function of time (Sammour and Hassan, 1996).

2.2.7. Statistical analysis

The data were reported as mean ± S.D. ($n = 3$) and statistical analysis of the data was carried out using one way ANOVA followed by LSD test at a level of significant of $P < 0.05$.

3. Results and discussion

3.1. Factors affecting entrapment efficiency of flurbiprofen in niosomal formulations

3.1.1. Effect of surfactant structure

To investigate the influence of surfactant structure on flurbiprofen entrapment efficiency, niosomal formulations of different spans were prepared from proniosomes with the same total lipid concentration (100 μmol/ml). Results listed in Table 3 show that Sp 60 has significant higher entrapment efficiency than other span types ($P < 0.05$). This could be due to the surfactant chemical structure. All span types have the same head group and different alkyl chain. Increasing the alkyl chain length is leading to higher entrapment efficiency (Hao et al., 2002). The entrapment efficiency followed the trend Sp 60 (C_{18}) > Sp 40 (C_{16}) > Sp 20 (C_{12}) > Sp 80 (C_{18}) (Uchegbu and Vyas, 1998). Sp 60 and Sp 80 have the same head groups but Sp 80 has an unsaturated alkyl chain. De Gier et al. (1968) demonstrated that the introduction of double bonds into the paraffin chains causes a marked enhancement of the permeability of liposomes, possibly explaining the lower entrapment efficiency of the Sp 80 formulation. In addition, Sp 80 has the lowest transition temperature ($T_c = -12$ °C) amongst all tested spans (16 °C for Sp 20, 42 °C for Sp 40, and 53 °C for Sp 60) (Kibbe, 2000). The span having the highest phase transition temperature provides the highest entrapment for the drug and vice versa (Yoshioka et al., 1994; Hao et al., 2002).

Table 3
Effect of surfactant type on the entrapment efficiency of flurbiprofen (100 μmol/ml total lipids without cholesterol)

Type of span	Method of free drug separation	Entrapment efficiency (%)
Span 20	Exhaustive dialysis	41.05 ± 0.91
Span 40	Exhaustive dialysis	42.79 ± 0.24
Span 60	Exhaustive dialysis	44.69 ± 1.04
Span 80	Exhaustive dialysis	37.74 ± 0.87
Span 40	Freeze thawing	45.33 ± 2.25
Span 60	Freeze thawing	52.11 ± 1.62

Each result is the mean ± S.D. ($n = 3$).

3.1.2. Effect of method used for the separation of the unentrapped drug

Fig. 1 shows the effect of the method of free drug separation on the entrapment efficiency of flurbiprofen into niosomes. Results clearly revealed that the drug entrapment efficiency determined by the exhaustive dialysis method was lower than that determined by the freeze thawing/centrifugation technique for both Sp 40 and Sp

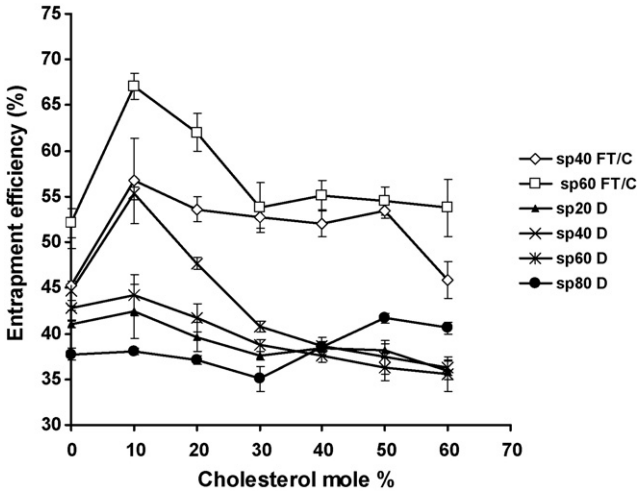

Fig. 1. Effect of cholesterol mol% and the method of free drug separation on the entrapment efficiency of flurbiprofen into niosomes. FT/C: freeze thawing/centrifugation. D: dialysis.

60 systems. Statistical analysis showed that freezing the prepared niosomes for 24 h at −20 °C followed by centrifugation resulted in a significant increase in flurbiprofen entrapment ($P < 0.05$) compared to that of the non-frozen exhaustively dialyzed niosomes. The mechanism, which explains the events happen during the freeze thawing cycle, was reported by Ohsawa et al. (1984). In freezing, drug and vesicles are concentrated; particles are closely packed in contact with each other resulting in fusion of niosomal vesicles. Therefore, large aggregates which include the drug are formed which after shaking, niosomal vesicles are formed entrapping efficiently the drug. On the other hand, the non-frozen niosomes of Sp 40 and Sp 60 were devoid of such large aggregates and therefore lower drug contents were entrapped. Furthermore, because of long dialysis periods, flurbiprofen may be released from the niosomal vesicles to the external buffer solution and led to lower drug encapsulation. This could be considered as a disadvantage of exhaustive dialysis technique.

3.1.3. Effect of cholesterol

Various techniques may be used to optimize the drug loading and this is very important in industrial settings. One method used to maximize drug loading is to increase cholesterol content into niosomal systems. Incorporation of cholesterol was known to influence vesicle stability and permeability (Rogerson et al., 1987; Gregoriadis, 1993). It is also one of the common and essential additives in niosome formulation in the present study. The influence of added cholesterol within the lipid composition on flurbiprofen (poorly water soluble drug, logP 4.1) EE% was determined by varying cholesterol molar% from 0% to 60% (Fig. 1). The effect of cholesterol on flurbiprofen entrapment was varied according to the nonionic surfactant used. Cholesterol was found to have little effect on the flurbiprofen entrapment into Sp 20 and Sp 80 niosomes. However, a significant increase in entrapment efficiency of flurbiprofen was obtained when 10% of cholesterol was incorporated into niosomes prepared from Sp 40 and Sp 60 ($P < 0.05$) followed by a decrease in encapsulation efficiency of the drug upon further increase in cholesterol content.

For Sp 20, entrapment of flurbiprofen is non-significantly increased ($P = 0.272$) from 41.05% to only 42.4% by increasing cholesterol content from 0% to 10%, respectively. Further increase in cholesterol content resulted in a significant decrease in entrapment efficiency ($P < 0.05$). Cholesterol at 60% concentration decreased flurbiprofen incorporation to 35.97%.

On the other hand, a significant increase in flurbiprofen entrapment efficiency from 37.74% to 41.4% ($P = 0.000$) upon increasing cholesterol concentration from 0% to 50%, respectively, was observed in Sp 80 niosomes. This could be explained on the basis that Sp 80 has the lowest transition temperature due to unsaturation double bond present in the oleate side chain. Above the phase transition temperature cholesterol made the membrane more ordered and abolish the gel to liquid phase transition of niosome system, hence, it was able to effectively prevent leakage of drug from niosomes (Cable, 1989; Hao et al., 2002). Also, niosomes prepared from Sp 80 was found to be unstable (separation of surfactant large globules after 48 h of preparation) when cholesterol content was below 40%. More than 40% cholesterol was essential to stabilize the preparations formed. Based on the above explanation, entrapment efficiency of Sp 80 at 50% and 60% cholesterol content are greater than that of other spans at the same cholesterol content. The difference in entrapment efficiency of Sp 80 at 50% and 60% cholesterol was non-significant ($P = 0.109$). The lower entrapment efficiency of Sp 20 at 50%, and 60% cholesterol content compared to Sp 80 in spite of its low transition temperature and cholesterol effect should be the same as in Sp 80 might be due to smaller side chain length (C_{12}) of span 20.

Incorporation of cholesterol into Sp 40 and Sp 60 showed an initial increase in entrapment efficiency of flurbiprofen as cholesterol content was increased from 0 to 10 mol% followed by a decrease upon further increase in cholesterol content. Flurbiprofen entrapment in Sp 40 preparations has been increased non-significantly ($P = 0.250$) from 42.79% to 44.18% then significantly decreased to 35.55% ($P = 0.000$) when cholesterol content was 0%, 10% and 60%, respectively; (as determined by exhaustive dialysis) and increased significantly from 45.33% to 56.74% ($P = 0.000$) then decreased to 45.82% at the same cholesterol contents when determined by centrifugation of freeze-thawed niosomes. The same trend was exhibited by Sp 60 as the entrapment efficiency of flurbiprofen showed maximum percent at 10% cholesterol (55.37% by exhaustive dialysis, and 67.04% by centrifugation of freeze-thawed niosomes) and minimum at 60% cholesterol (36.3% by exhaustive dialysis and 53.75% by centrifugation of freeze-thawed niosomes). A similar result was reported by Mohammed et al. (2004) and Mohammed and Perrie (2005) who studied the effect of cholesterol incorporation into liposomes on the entrapment efficiency of the poorly soluble drug ibuprofen. It was suggested that, the improvements in drug loading by cholesterol increase (20 mol%) and the major reductions in drug incorporation when cholesterol content was further increased may be due to two conflicting factors: (1) with increasing cholesterol, the bilayer hydrophobicity and stability increased (Bernsdorff et al., 1997; Gregoriadis and Davis, 1979) and permeability decreased (Kirby et al., 1980) which leads to efficiently trapping the hydrophobic drug into bilayers as vesicles formed. (2) In contrast, higher amounts of cholesterol may compete with the drug for packing space within the bilayer, hence excluding the drug as the amphiphiles assemble into vesicles. Another study suggested that decreasing the entrapment efficiency with increasing cholesterol ratio above a certain limit may be due to the fact that increasing cholesterol beyond a certain concentration can disrupt the regular linear structure of vesicular membranes (EL-Samaligy et al., 2006). Moreover, both Sp 40 and Sp 60 have higher phase transition temperatures being solids at room temperature. Below transition temperature cholesterol made the membrane less ordered and increasing cholesterol has been found to increase membrane fluidity to the extent where the phase transition is abolished (Ladbrooke et al., 1968; Yoshioka et al., 1994; Arunothayanun et al., 2000). This explains why Sp 80 at 50% and 60% cholesterol showed higher entrapment efficiencies than those showed by Sp 40 and Sp 60 at high cholesterol contents.

3.1.4. Effect of pH of the hydration medium

The EE% of flurbiprofen in niosomes prepared by hydration of proniosomal gels of Sp 60/cholesterol (9:1) was found to be greatly affected by the pH of the hydrating medium. Acidic and alkaline pH conditions were established using phosphate buffers of pH 5.5, 6.5, 6.8, 7.4, and 8. Fig. 2a shows flurbiprofen entrapment efficiency at its highest level at acidic pH conditions. The maximum loading efficiency was 94.61% when the hydrating medium was adjusted to pH 5.5. The fraction of flurbiprofen encapsulated was increased to about 1.5 times as the pH decreased from pH 8 to 5.5. Statistical analysis showed no significant difference in EE% of flurbiprofen as the pH was changed from pH 5.5 to 6.5 ($P = 0.094$). However the decrease in EE% was significant as the pH was shifted to pH 6.8 and more ($P = 0.001$). Moreover, flurbiprofen showed the lowest entrapment at pH 7.4 and 8 with no significant difference between them ($P = 0.191$). The increase in the EE% of flurbiprofen by decreasing the pH could be attributed to the presence of the ionizable carboxylic group in its chemical structure. Decreasing the pH could increase the proportions of the unionized species of flurbiprofen, which have higher partitioning to the bilayer lipid phase compared to the ionized species (Abu-Zaid et al., 2003a). The solubility of flur-

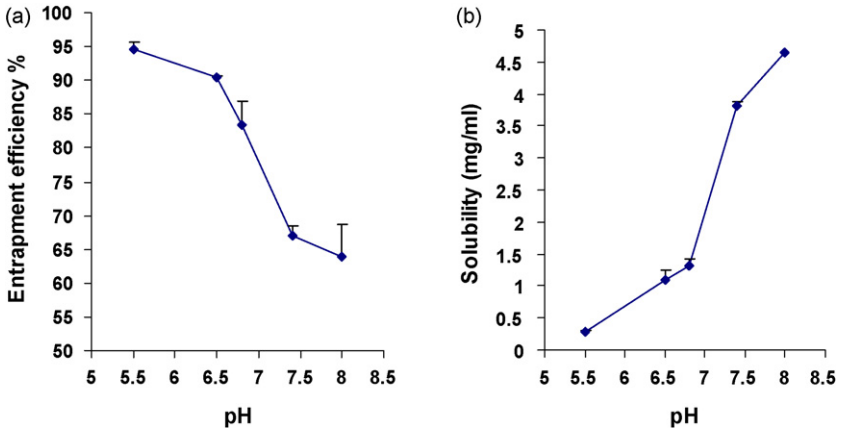

Fig. 2. Effect of pH of the hydrating medium on both EE% of flurbiprofen into niosomes (a) and flurbiprofen solubility (b) ($n = 3$).

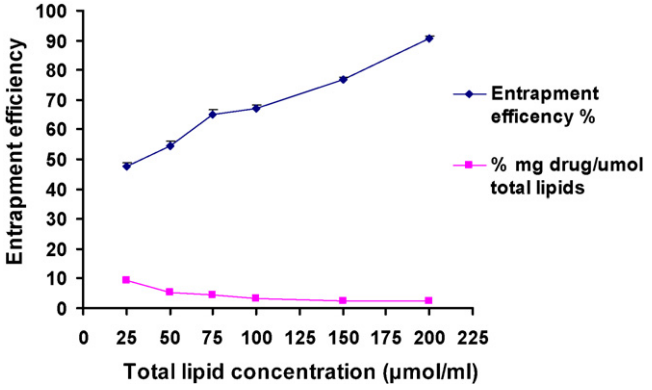

Fig. 3. Effect of total lipid concentration on entrapment efficiency of flurbiprofen in niosomes of Sp 60 ($n = 3$).

biprofen at different pH conditions confirmed the results as it goes in contrast with the entrapment efficiency of this drug (Fig. 2b).

At lower pH conditions niosomal formulations were examined for the presence of drug precipitates by optical microscopy at 40× magnification, both before and after centrifugation and washing. No drug precipitates was observed before and after centrifugation and washing (drug concentration before centrifugation and washing was 5 mg/ml). This observation lead to the assumption that at pH 5.5 the poorly soluble flurbiprofen drug was completely incorporated within niosomes and that the concentration of the free drug was lower or equal its solubility in phosphate buffer of pH 5.5 (Yu-Kyoung et al., 1995).

3.1.5. Effect of total lipid concentration

Fig. 3 shows the effect of total lipid concentration on the amount of flurbiprofen entrapped in Sp 60/cholesterol (9:1) niosomes prepared from proniosomal gel. The EE% of flurbiprofen was increased from 47.64% to 90.45% as the lipid concentration was increased from 25 to 200 μmol/ml, respectively. The increase in EE% of flurbiprofen as a function of total lipid concentration was linear ($R = 0.991$). Similar results were obtained by Yoshioka et al. (1994) who reported a linear increase in the entrapment efficiency of 5(6)-carboxyfluorescein with increasing total lipid concentration. On the other hand, the amount of flurbioprofen entrapped was decreased from 9.53% to 2.26% mg drug/μmol total lipids on increasing the lipid concentration from 25 to 200 μmol/ml, respectively. This leads to the fact that the fraction of lipid taking part in encapsulation decreases as the concentration of lipid increases (Abu-Zaid et al., 2003b).

Table 4
Effect of flurbiprofen concentration on niosomal encapsulation efficiency

Flurbiprofen concentration mg/mmol lipids	EE%	% (mg drug/μmol of total lipids)
25	55.99 ± 2.28	1.40 ± 0.06
50	67.04 ± 1.41	3.35 ± 0.14
75	72.25 ± 2.3	5.41 ± 0.17

Each result is the mean value ± S.D. ($n = 3$).

3.1.6. Effect of flurbiprofen concentration

Increasing flurbiprofen concentration from 25 to 75 mg/mmol lipids in the proniosomes prepared from Sp 60/cholesterol (9:1) showed an increase in both EE% and the amount of drug encapsulated per μmol total lipids (Table 4) upon hydration and formation of niosomes. The entrapment efficiency of flurbiprofen was increased from 55.99% to 72.25% and the amount of drug entrapped increased from 1.4% to 5.42% mg/μmol total lipid, as the drug concentration was increased from 25 to 75 mg/mmol lipids, respectively. Statistical analysis clearly revealed that the differences

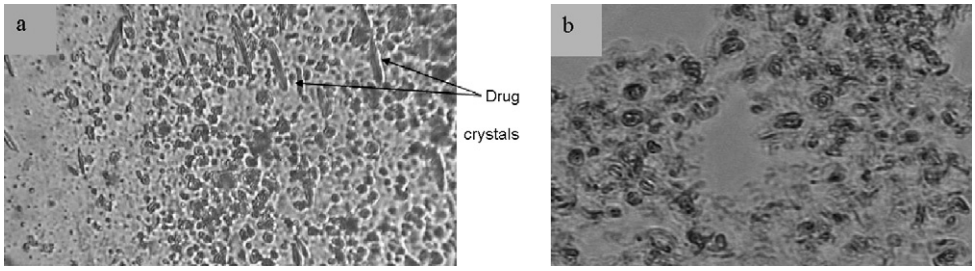

Fig. 4. Niosomal vesicles photomicrographs of Sp 60/cholesterol (9:1) containing flurbiprofen as seen under microscope (40×). (a) Flurbiprofen precipitated crystals and (b) vesicles after washing the crystals by phosphate buffer pH 7.4.

in EE% upon increasing drug concentration from 25 to 75 mg/mmol lipids were significant ($P < 0.05$). The increased amount of encapsulated flurbiprofen with increasing its added amount during the formulation could be due to the saturation of the media with flurbiprofen that forces the drug to be encapsulated into niosomes (EL-Samaligy et al., 2006).

Increasing drug concentration to 100 mg/mmol lipids showed drug crystals dispersed in-between the niosomal pellets after centrifugation and washing when seen under optical microscope (Fig. 4a). This leads to the assumption that niosomal formulations could enhance the solubility of certain poorly soluble drugs but to a maximum limit after which any increase in the drug concentration leads to drug precipitation. Further washing has removed drug crystals (Fig. 4b). The maximum amount of flurbiprofen to be entrapped in 1 ml of the niosomal preparation which contains 100 μmol of lipids was determined to be 6.1 ± 0.17 mg. Interestingly, all the drug was dissolved into the proniosomal gels even when its concentration was 150 mg/mmol lipids. However, upon hydration with buffer solution (pH 7.4) the excess non-entrapped drug was greater in concentration than the flurbiprofen solubility limit into the buffer solution and was precipitated.

3.1.7. *The influence of charged lipids*

The effects of charge inducing agents on the EE% of flurbiprofen in niosomal vesicles of Sp 60/10% cholesterol were examined. Both DCP and stearyl amine (SA) were used to impart negative and positive charges on niosomal vesicles, respectively. The amounts of the charge inducing agent were varied from 5 to 30 μmol/ml while the amounts of Sp 60 was varied from 85 to 60 μmol/ml, respectively and the concentration of cholesterol was kept constant at 10 μmol/ml. SA at a concentration of 30 μmol/ml resulted in an unstable formulation in which a large floating clot was formed after hydration of the proniosomal formulation. This may be as a result of the destruction of niosomal vesicles upon increasing SA concentration (Kulkarni et al., 1997).

Fig. 5 shows that the incorporation of either DCP or SA resulted in a decrease in the EE% of flurbiprofen in niosomal vesicles. In case of DCP the decrease in EE% of flurbiprofen was about 1.7%, 7.87%, 5.24%, and 6.47 % by using 5, 10, 20, and 30 μmol/ml DCP, respectively, at constant drug concentration. Statistical analysis showed no significant decrease in EE% at 5 μmol/ml DCP ($P = 0.411$). The highest effect could be seen when DCP concentration was increased from 5 to 10 μmol/ml. however, further increase in DCP amounts from 10 to 30 μmol/ml significantly decreased the EE% of flurbiprofen into niosomal vesicles ($P < 0.05$). Moreover, no significant differences in EE% of flurbiprofen ($P > 0.05$) was observed on changing DCP concentration from 10 to 30 μmol/ml.

Incorporation of 5 μmol/ml of SA showed the highest decrease in EE% of flurbiprofen, however, further increase in SA concentration showed lower potential in decreasing EE% of flurbiprofen. The EE% decreased by about 17%, 8.91%, and 4.75% as the SA concentration was increased from 5, 10 to 20 μmol/ml, respectively. Statistical analysis revealed a significant decrease in EE% of flurbiprofen upon addition of varied amounts of SA ($P < 0.05$). However, both SA and DCP containing formulations produced a main reduction in the EE% of flurbiprofen, formulation containing SA at 5 μmol/ml showed highly significant decrease in EE% compared to DCP containing niosomes at the same molar% ($P = 0.000$). Moreover, no significant difference between niosomal formulations containing 10 and 20 μmol/ml of SA and those containing 10, 20, and 30 μmol/ml of DCP.

The reduction in EE% upon the addition of DCP could be due to the repulsive interactions between the negatively charged lipid and the carboxyl group present in flurbiprofen molecules which influence drug incorporation within lipid bilayers (Mohammed et al., 2004). Although SA carries an opposite charge to that of carboxyl group in flurbiprofen, its presence in place of DCP also decreased EE% of the drug. This could be explained by electrostatic induced chain tilt and the subsequent changes in the lateral packing of the bilayers by the effect of charge inducing agents (Jahnig et al., 1979). It should be observed that addition of SA to niosomal formulations gave significantly higher EE% at 10 and 20 μmol/ml compared to formulation containing 5 μmol/ml ($P < 0.05$). This could be due to the electrostatic attraction between the positively charged head group in stearylamine and the carboxyl group in dissociated flurbiprofen molecules (Yamamoto et al., 2002).

3.2. *Flurbiprofen release rates from niosomes*

Results of an in vitro study of the release of flurbiprofen from Sp 40 and Sp 60 niosomes are shown in Figs. 6 and 7. The release profiles of flurbiprofen from niosomes of different cholesterol contents were apparently biphasic release processes. Rapid drug leakage was observed during the initial phase where about 25–55% of the entrapped drug was released from various formulations in the first hour of niosomal incubation in 50 ml of phosphate buffer (pH 7.4). However, during the following 71 h a slow release occurred in which only further 5–10% of flurbiprofen was lost from different niosomal preparations. This could be because the drug is mainly incorporated between the fatty acid chains in the lipid bilayers of niosomal vesicles which leads to rapid ionization and release upon dispersing niosomes in large buffer (pH 7.4) volumes until reaching equilib-

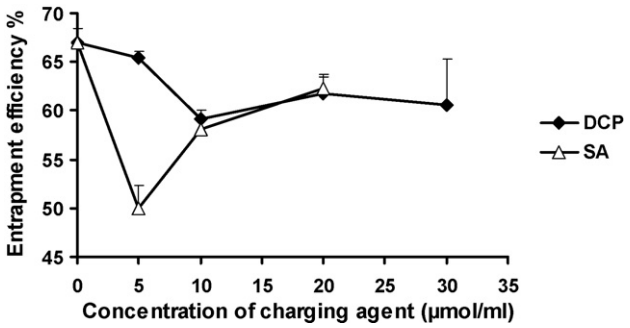

Fig. 5. Effect of charging lipids on the EE% of flurbiprofen into niosomal formulations of Sp 60/10% cholesterol ($n = 3$).

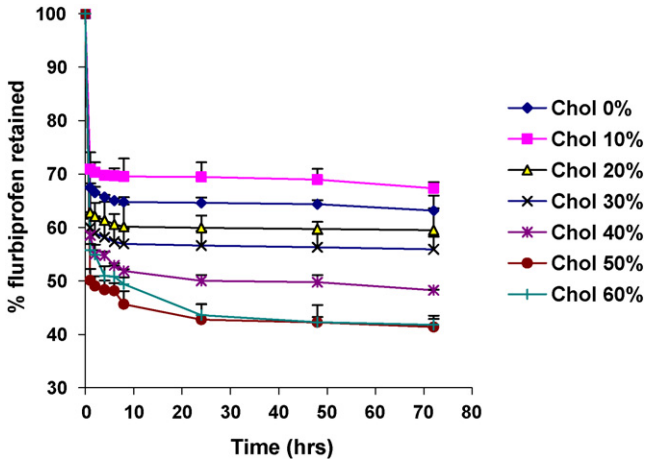

Fig. 6. In vitro release of span 40 niosomes containing flurbiprofen expressed as percentage drug retained in niosomes after incubation in phosphate buffer "pH 7.4" ($n = 3$).

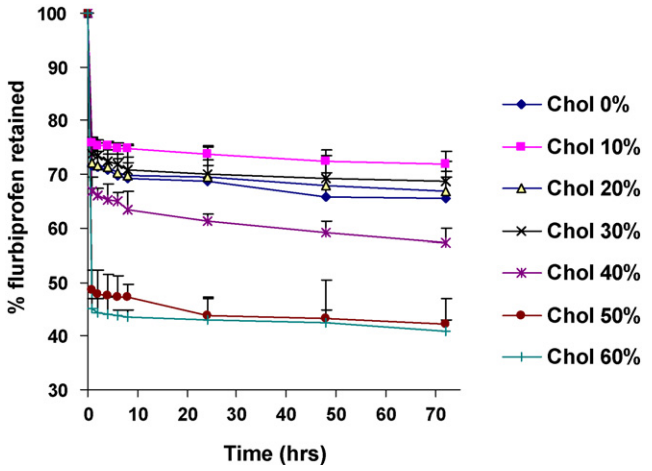

Fig. 7. In vitro release of span 60 niosomes containing flurbiprofen expressed as percentage drug retained in niosomes after incubation in phosphate buffer "pH 7.4" ($n = 3$).

rium. Also, it has been reported that, a highly ordered lipid particles cannot accommodate large amounts of drug and is the reason for drug expulsion (Wissing et al., 2004). Figs. 6 and 7 show that niosomal formulations of both Sp 40 and Sp 60 containing 10% cholesterol displayed the lowest extent of drug release after 72 h. The percent flurbiprofen retained after 72 h was 71.96% and 68.28% for Sp 60 and Sp 40 niosomes, respectively. Cholesterol free niosomes of either Sp 40 or Sp 60 showed higher release rates compared to those containing 10% cholesterol. The decrease in release rates of flurbiprofen from Sp 60/10% cholesterol formulation was statistically significant ($P = 0.048$) compared to that of cholesterol free formulation. However, non-significant difference was observed between Sp 40/10% cholesterol and cholesterol free preparations ($P = 0.19$). Moreover, no significant difference was observed between both cholesterol free preparations of Sp 40 and Sp 60 niosomes ($P = 0.441$) and also between niosomes containing 10% cholesterol of the same surfactants ($P = 0.144$). A non-significant increase in the percent flurbiprofen released could be noted from Sp 60 niosomal formulations when cholesterol content was increased from 10% to 30% ($P > 0.05$), however, further increase in cholesterol content up to 60% significantly increased the drug release ($P < 0.05$). In case of Sp 40 a significant increase in flurbiprofen release was observed as cholesterol content was increased from 10% to 60% ($P < 0.05$).

Addition of cholesterol to a niosomal formulation composed of span/cholesterol (9:1 molar ratio) decreased flurbiprofen release in comparison with cholesterol free formulations. This result could be due to the decreased leakage and permeability of niosomal formulations in presence of cholesterol at this molar ratio 9:1. Cocera et al. (2003) reported that cholesterol produced an optimum hydrophobicity which decreased the formation of the transient hydrophilic holes, by decreasing membrane fluidity, responsible for drug release through liposomal layers. On the other hand, further increase in cholesterol amounts into niosomal formulations could increase the release of flurbiprofen. The result is in accordance with EL-Samaligy et al. (2006) and New (1990) who reported that increasing cholesterol beyond a certain concentration can disrupt the regular linear structure of the vesicular membrane and increase the drug release.

4. Conclusion

Concluding the above mentioned results, the EE% of flurbiprofen (as a model for poor soluble drugs) into niosomes prepared by the proniosomal method was a function of the formulation processing variables such as the method of drug separation, cholesterol content, total lipid concentration, pH of the hydration medium and drug concentration. The electrostatically induced change in bilayers packing and the electrostatic interactions between flurbiprofen and the charged head groups of SA and DCP could influence the incorporation of flurbiprofen into niosomal vesicles. Niosomal formulations could enhance the solubility of certain poorly soluble drugs but to a maximum limit after which any increase in the drug concentration may lead to drug precipitation. It is clear that niosomal formulations containing 10% cholesterol are the most stable among other tested formulations. However, further increase in cholesterol amounts into niosomal formulations could increase the release of flurbiprofen and display much lower stability.

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