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FORMULATION AND IN VITRO EVALUATION OF FLUFENAMIC ACID LOADED DEFORMABLE LIPOSOMES FOR IMPROVED SKIN DELIVERY

MOHAMED BADRAN[a,b*]
[a]*Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11451, P.O. Box 2457, Saudi Arabia*
[b]*Department of Pharmaceutics, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt*

The aim of this work was to evaluate the deformable liposomes (DLs) containing sodium deoxycholate or cineol to produce two types of liposomes in comparison to conventional liposomes (CL). The effect of these DLs on the *in vitro* transdermal delivery of flufenamic acid (FFA) was investigated. The DLs and CL were characterized in terms of size distribution, zeta potential, polydispersity index and vesicle stability. The influence of ULs on the in vitro skin penetration and skin deposition of FFA was studied by in vitro diffusion experiments in comparison with CL and ethanolic solutions of the drug. Results showed that all of the used vesicular systems were able to have smaller particle size, low polydispersity (PDI<0.3), negative zeta potential and good stability. In vitro skin penetration data showed that DLs were able to give a statistically significant improvement of FFA deposition in the skin in comparison with CLs and drug solutions. Moreover, the DLs, prepared with cineole, were also able to deliver to the skin a higher total amount of FFA thus suggesting that drugs delivery of the drug to the skin was strictly correlated to the vesicle composition.

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1. Introduction

It is reported that enhanced skin delivery following application of the liposomes was observed [1-3]. Liposomal carriers have been successful in improving the topical application of a number of drugs. For instance, tretinoin for the treatment of acne [4], glucocorticoids for the treatment of atopic eczema [5], lignocaine and tetracaine as anesthetics [6]. There have several mechanisms reported in literature for penetration enhancement of liposomes. These include the vesicles that collapse at the skin surface and that vesicle components penetrate into the intercellular lipid matrix, where they mix with the strum corneum (SC) lipids modifying the lipid lamellae [7]. Another mechanism reported that intact vesicles could enter the SC due to the influence of transepidermal osmotic pressure [8]. Cevc et al., reported that conventional liposomes, a systemic biological effect upon transdermal applications was not observed [9]. Only localized or rarely transdermal effects of liposomes was reported.

It is assessed by several researchers that the penetrations enhancing of the vesicles through the skin depend on the membrane flexibility of vesicles. It is noticed that the liposomes with high elasticity membrane could result in enhanced drug transport across the skin as compared to vesicles with rigid membrane [10]. Recently, sequences of new vesicles with elastic membranes were developed in order to improve transdermal delivery of drugs. For example, deformable vesicles containing phospholipids and edge activator such as sodium deoxycholate [11]. An edge activator is often a single chain surfactant that destabilizes lipid bilayers of the vesicles and increases flexibility of the membrane [12]. Deformable vesicles have been shown to penetrate

*Corresponding author: mbadran75@gmail.com

intact skin carrying the drugs efficiently. Also, the elasticity of vesicle membranes could be improved by incorporation of the ethanol in lipids. These vesicles have been reported as an efficient skin penetration enhancer.

However, due to the interdigitation effect of ethanol on lipid bilayers, it was commonly believed that the vesicles could not coexist with high concentrations of ethanol [13]. These described vesicular systems are not plenty sufficient to convey the drugs across the SC. Therefore, many approaches were developed to achieve the successful topical delivery of the substances. Recently, new vesicles were generated after incorporation ethanol and terpenes into the lipids and highly flexible vesicles were obtained. These vesicles firstly optimized by Verma et al. and employed for topical application [14]. In recent years terpenes, which are constituents of essential oils, have widely been used as penetration enhancers. Cineole is a terpene and extensively used as permeation enhancers with both hydrophilic and lipophilic drugs. When skin is treated with cineol, the existing network of hydrogen bonds between ceramides may get loosened and break since cineol enter into the lipid bilayer of SC [15]. Very recently, flexible vesicles prepare using 1% cineole were described as transdermal carrier for systems [16, 17].

Flufenamic acid (FFA) is a potent non-steroidal anti-inflammatory drug, is known as 2-[[3- (trifluoromethyl) phenyl] amino] benzoic acid. It is a weekly acidic compound (pKa=3. 9), which is also very lipophilic with an octanol/water log partition coefficient of 4.88. FFA is readily soluble in organic solvent such as methanol, ethanol, chloroform, and acetone but practically has a very poor solubility in water, 0.0067 mg/ml at 22 ^{O}C [18-19]. In order to provide new topical application forms suitable for FFA, several techniques should be applied to overcome the barrier properties of stratum corneum (SC) [17]. The purpose of this study was to evaluate and characterize two types of elastic liposomes including deformable liposomes (DLs) carrier systems suitable for FFA and investigate their penetration-enhancing ability.

2. Materials and methods

2.1. Materials

Soybean lecithin Lipoid S75 (Lipoid KG, Germany). 1, 8-cineole was purchased from Sigma Aldrich (Taufkirchen, Germany). Sodium deoxycholate (Serva, Germany). Tween 80 was purchased from Sigma Aldrich (Taufkirchen, Germany). Flufenamic acid was purchased from Sigma (Sigma-Aldrich Inc., St. Louis, Missouri, USA). Methanol (HPLC grade) was purchased from Carl Roth GmbH&Co. (Germany). Methanol and ethanol were purchased from Carl Roth GmbH&Co. (Germany). All other chemicals used in this study were of analytical grade.

2.2. Methods

2.2.1. Preparation of elastic liposomes

The deformable liposomes (DL1) and conventional liposomes (CL) were prepared by a conventional rotary evaporation method [20]. In case of DL1, the lipoids S75 and sodium deoxycholate were used (to get the optimum flexibility of the liposomes, weight ratio of surfactant and lipids should be equal to 0.28) [8, 21]. In case of CL, lipoid S75 was used. Briefly, the lipids, FFA (0.5%) and other components (shown in Table 1) were added in a round bottom flask as solution in organic solvent, chloroform/methanol (1:1). The flask was connected to a rotor evaporator (Rotavapor, Büchi, Germany) and immersed in a water bath preheated at temperature equal or more than the transition temperature of phospholipids that in the case of lipoid S75 is about 45°C. The lipid film was then flashed with nitrogen gas for removal of possible traces of organic solvents. Multilamellar vesicles (MLV) were formed after film hydration with PBS pH 7.4. These MLVs were extruded through polycarbonate membranes with pore size of 100 nm by means of the Avestin-Liposofast device to get liposomes of the preferred size.

The DL2 with the cineole were prepared by ethanol dissolution method [22]. The composition of the liposomes containing cineole is represented in table 1. The liposomes were

prepared by dissolving 1% w/v of cineol in the ethanolic solution of lipoid S75 containing FFA (0.5%). The mixture was vortexed for 5 min and afterwards sonicated for 5 min in order to obtain a clear solution. The PBS pH 7.4 was added to the solution by a syringe under constant vortexing. The vortexing was continued for additional 5 min. The last step was the probe sonication of MLVs as described before. The composition of the liposomes and ethanolic solution containing FFA is represented in table 1.

2.2.2. Particle size measurements

Photon correlation spectroscopy (PCS): Dynamic light scattering was measured at 25 °C with a Zeta plus instrument (Brookhaven Instruments, Brookhaven, USA). The samples were analyzed 24 h after preparation. The liposomes were appropriately diluted with the aqueous phase of the formulations prior to the measurements. The particle size values given are the averages of 4 measurements and are expressed as z-average. The polydispersity index (PDI) measures the size distribution of the liposomes. Zeta-potential (mV) was measured by the instrument from electrophoretic mobility of the particles [23]. The zeta potential of the vesicles was determined by diluting the suspensions in PBS buffer pH 7.4.

2.2.3. Determination of FFA-entrapment efficiency

The free FFA was separated from entrapped by using ultracentrifugation technique (Optima^{TM} Max-E, Ultra Centrifuge, Beckman Coulter, Pasadena, CA) at 50,000 rpm at 4 $^{\circ}$C, for 30 min [24]. Liposome samples were removed when the PBS was clear. Purified sediment was then diluted to the initial volume using PBS (pH= 7.4) in order to keep a final lipid concentration of 5% (w/v), and used directly for in vitro penetration study.

The content of FFA was measured by HPLC. Entrapment efficiency of FFA was calculated indirectly from the amount of free drug, according to the following equation:

Entrapment efficiency (%) = (FFA_t- FFA_f/ FFA_t) x 100

Where FFA_f was the amount of free FFA and FFA_t was the total amount of FFA.

2.2.4. Storage stability studies

In order to determine the physical stability of vesicles, their, the vesicles were stored at 4 °C for up to 2 months under light protection. In predetermined time intervals, the particle sizes of the vesicles and PDI were measured [25].

2.2.5. In-vitro skin penetration studies

The subcutaneous fatty tissue was carefully removed from the abdominal rat skin using a scalpel and surgical scissors. The surface of the skin was cleaned with the PBS solution and the skin was allowed to dry (exposed to normal air for 20 min). Afterwards the skin was wrapped into aluminum foil and stored at -20 °C until use. Prior to the experiments, the skin samples were taken from the freezer and let thaw at room temperature for about 30 min. Skin samples were mounted onto Franz diffusion cells (FDC) with a nominal area for diffusion of 1.76 cm^2 and a receptor volume of about 12 ml. The epidermal side of the skin was exposed to ambient conditions while the dermal side was bathed with BPS buffer pH 7.4 containing tween 80 to keep sink condition. The receptor fluid was kept at 37 ± 1°C throughout the experiments to reach the physiological skin temperature (i.e.32 ± 1°C). The constant stirring was maintained by magnetic stirring at 500 rpm. Care was taken to remove all air bubbles between the underside of the skin (dermis) and the receptor solution throughout the experiment. After equilibration for 30 min, 10 µl/cm^2 of liposomal dispersions or ethanolic solution were applied to the skin surface under non-occlusion and light protection.

Samples were taken from the receptor fluid (1ml) every hour and the withdrawn volume was replaced by the same volume of fresh PBS pH 7.4 to maintain a constant volume. After 6 and 12 h, the formulations were wiped off by the cotton wool pads wetted with PBS buffer 3-4 times.

For determinations of the drug deposition in the different skin layers, the skin was fixed onto cork plates and stretched using small pins. The SC was then subsequently removed by tape stripping. Transpore tape (3M TransporeTM tape, St. Paul, MN, USA) with a surface area of approximately 4 cm^2 was applied on the SC surface of the skin. The tape was firmly pressed on skin surface and pulled off immediately with one smooth stroke. Each skin sample was stripped with 10 pieces of adhesive tape to confirm the removal of the SC [26]. The amount of FFA in the stripped skin was determined by cutting into small pieces. The tapes and stripped skin were placed each in PBS pH 7.4: methanol (1:2) overnight following by 5 min vortexing and 5 min sonication for complete extraction of FFA following by filtration. The tapes, stripped skin and receptor fluid were assayed for the content of FFA by HPLC. All experiments were done in triplicate. The extraction method was validated by spiking with a known amount of the FFA. The recoveries were about 90% from tapes and stripped skin.

2.2.6. HPLC analysis

The quantification of FFA in this study was performed using Water HPLC system, which was equipped with a Dual Absorbance detector, a Binary HPLC pump and a reversed-phase C18 column (4.6 x 150 mm, Hypersil). The HPLC system was monitored by "Empower (Water)" software. The mobile phase was a mixture of McIlvaine buffer pH 2.2: Methanol (20:80), filtered through 0.45 μm membrane filter and eluted at a flow rate of 1.2 ml/min, injection volume, 20 μl and retention time (3.2 ± 0.2) min. Effluents were monitored at 290 nm [27].

2. 3. Statistical data analysis

Data analysis was carried out with the software package Microsoft Excel, Version 2003 and origin software, version 6. Results are expressed as mean ± standard error (n = 3 independent samples).

3. Results and discussion

3.1. Particle size investigations

In the present study, the additions of such surfactants sodium deoxycholate in vesicle membranes lead to flexibility of the vesicles membrane, which was called deformable liposomes [28]. Also, cineole is widely used to prepare deformable liposomes [16]. Recently, flexible liposomes prepared using 1% cineole as penetration enhancers with both hydrophilic and lipophilic drugs were developed [16, 29]. The efficiency of these deformable vesicles on delivering FFA was investigated and compared with an ethanolic solution and CL. The compositions of these different vesicular systems, their particle size distribution, PDI and zeta potential are presented in Table 1.

The size distribution of the vesicles was determined by dynamic light scattering. The particle size of deformable liposomes DL1 and DL2 were 122 ± 1.01 and 172 ± 1.32, respectively. The particle size of conventional liposomes (CL) was 147 ± 1.25 nm. As expected a slight increase in the vesicle size was detected in the case the deformable liposomes containing cineol (DL2), was due to the incorporation of cineol, this result was in agreement with literature [17]. All the formulations used in this study showed an optimum polydispersity index (PDI) below 0.3, which indicate the good homogeneous of the prepared liposomes [16, 17, 28]. The PDI of the investigated formulations was in the range from 0.159±0.019 (DL2) to 0.212 ± 0.037 (CL). Regarding the zeta potential, the formulated liposomes possessed a negative surface charge, indicating that the formulations are more stable and more homogeneous (Table 1). DL2 produced high negative charged vesicles due to presence of ethanol and cineol [16-17]. FFA loaded liposomes of small particles obtained give a good opportunity to achieve an enhanced skin penetration effect, as the vesicular size has been reported to affect the penetration parameters [3].

Table I: Composition of the different types of liposomes and ethanolic solution

Code	Lipids/components		Particle size	PDI	zeta potential
				(nm)	
CL	Lipoid-S75	5%	147 ± 1.25	0.212 ± 0.037	- 14.6±0.2
Cholesterol	1%				
DL1	Lipoid-S75	5%	122 ± 1.01	0.173 ±0.014	- 26.4±1.1
	Sodium cholate	1.4%			
DL2	Lipoid-S75	5%	172 ± 1.33	0.159±0.019	- 37.2±1.1
Cineole	1%				
	Ethanol	1.65%			
ET	PBS (pH 7.4) Ethanol 50%				

CL: conventional liposomes; DL1: deformable liposomes containing sodium deoxycholate; DL2: deformable liposomes containing cineole and ethanol and ET: the controls present the ethanolic solution.

3.2. Determination of FFA-entrapment efficiency

The entrapment efficiency of FFA in DL1 and DL2 reported, 85.4, and 79.2% respectively, compared with 55.3 % reported for CL (Fig. 1). It is obvious that deformable liposomes represented the largest EE% which is accompanied with increasing in a zeta potential of the vesicles. A number of studies found that presence of ethanol, sodium deoxycholate and cineole increased the zeta potential of liposomes [16, 28, 29, 30]. The reason for that was attributed to the high value of zeta potential which frequently lead to increase the repulsion forces of the bilayer structure of the vesicles which consequently increasing the loading of the FFA in the liposomes.

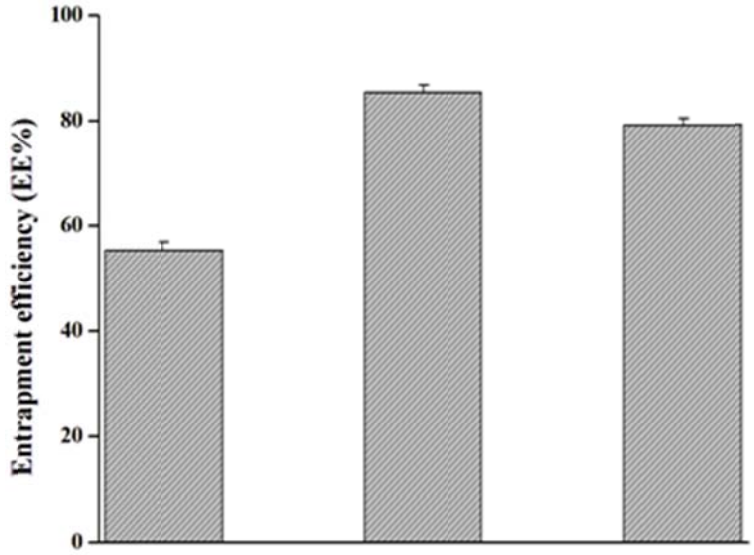

Fig. 1. Entrapment efficiency of FFA loading liposomes.

3.3. Storage stability

In the different liposomal dispersions, the storage temperature had low effect on the physical stability of the vesicles, i.e. the particle size and PDI values showed a smaller change

during storage at 4°. The formulated liposomes showed a small change during the storage at 4° and they could be considered physically stable when stored at 4° for two months (Fig. 2 A and B).

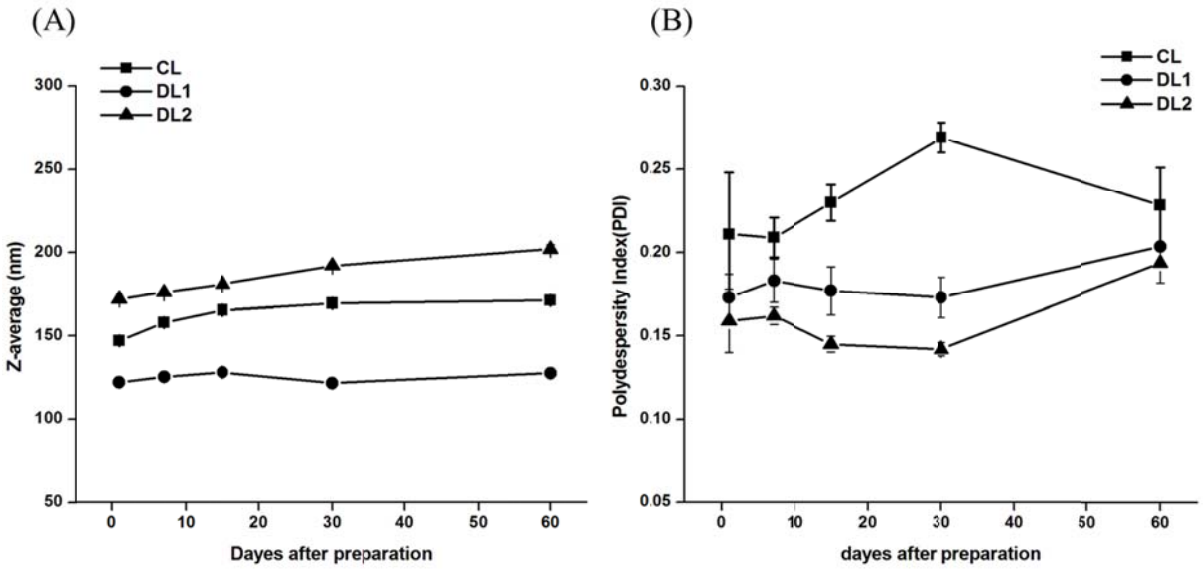

Fig. 2. Stability study of FFA loaded liposomal dispersions stored at 4 °C over 2 months duration. (A) Change of particle size (z-average), (B) change of the polydispersity index (PDI).

3.4. *In vitro* penetration studies

In order to investigate the ability of deformable liposomes to deliver FFA into the skin using a standardized skin stripping technique with a Franz diffusion cell.

Tables 2 showed that DLs revealed a significantly enhanced deposition of FFA in the SC after 6 h compared to CL and ET. Namely, the amount of FFA delivered by DL2 was 1.7-fold higher than the amount delivered by DL1 and 5.2-fold higher than the amount delivered by CL in the SC (Fig. 3, Table 2). In addition, DL2 also showed a 3.6-fold higher deposition of FFA in the SC compared to ethanolic solution. These data revealed the synergistic effect of DL2 containing cineol and ethanol and its higher penetration enhancing ability compared to the formulated liposomes. Also table 2 showed the skin depth of topically applied FFA liposomes after 6 h. These results revealed almost the same as the SC strip, i.e. DL2 showed the highest skin deposition. The amount of FFA in the deeper skin was 1.8, 3.8 and 2.2-fold higher for DL2 as compared to DL1, CL and ethanolic solution, respectively. Also, the amount of FFA recovered in the receptor compartment was 3.4, 6.6 and 5.6-fold higher for DL2. Furthermore, the quantity of skin deposited FFA after 12 h (the last time point studied) of non-occlusive application was significantly higher for all formulations than after 6h. DL2 showed highest accumulation of FFA in the SC, deeper skin layers, and also in the receptor compartment of the Franz diffusion cell, as compared to other formulations. It is attractive to note that the DL2 resulted in the highest amounts of FFA in the skin layers as compared to other formulations (Figs. 3, table 2). The amounts of FFA deposited in skin for all formulations increased within 12 h compared to 6 h which indicate that the skin deposition of FFA loaded in all formulations and control did not reach the steady-state during 6 h. These results mean that the investigated formulations need more time to reach the steady state of skin deposition.

The possible explanation of these results for DL2 depends in the addition of ethanol and cineole, which makes the phospholipid bilayer greatly deformable vesicles. Furthermore, ethanol and cineol may also act as penetration enhancer by disturbing the SC lipid structure [33-34] and the penetration enhancing effect of deformable liposomes containing ethanol and cineol has been

explained by fluidizing the SC lipid structures thus disturbing the organization of the SC lipids [17, 20]. Due to the deformability of the vesicle bilayer, penetration into the SC may be facilitated particularly under non-occlusive conditions although the mechanism of the penetration of deformable vesicles is not yet completely understood [28, 29]. Concerning DL1, it is clear that the deposition of FFA is high in the SC and deeper layers after application of deformable liposomes comparing with conventional liposomes. The proposed mechanism for penetration enhancement that these vesicles can act as penetration promoters, in which vesicle bilayers interact with the stratum corneum causing modification in the intercellular lipid lamellae. This effect will facilitate the partitioning the drug molecule into and penetration through the stratum corneum [12-29]. These results were in agreement with other studies using ultradeformable vesicles containing sodium cholate for transdermal carrier systems [22, 35].

Table 2: Amounts of FFA (expressed as amount delivered, n = 3) in the different layers of rat skin after 6 and 12 hours of non-occlusive incubation.

Code	SC	Stripped Skin	Receptor
	6 h		
CL	27.067 ± 4.773	6.110 ± 1.345	4.433 ± 0.127
DL1	15.586 ± 1.042	3.406 ± 0.339	1.307 ± 0.199
DL2	5.192 ± 0.129	1.624 ± 0.037	0.675 ± 0.059
ET	7.602 ± 1.272	2.719 ± 0.118	0.796 ± 0.125
	12 h		
CL	45.537 ± 3.435	10.280 ± 2.145	7.458 ± 0.645
DL1	26.233 ± 2.616	5.731 ± 0.797	2.198 ± 0.512
DL2	8.769 ± 1.115	2.532 ± 0.088	1.216 ± 0.408
ET	12.803 ± 2.261	4.587 ± 0.319	1.239 ± 0.193

Drug deposition in the different skin layers was dependent on the compositions of elastic liposomes. Skin incubation with liposomes containing cineole and ethanol led to the highest drug deposition in the SC compared to liposomes containing cholate.

All data presented revealed that lipophilic character of FFA generally present high partition in the SC especially incorporated in lipid matrix. However, the penetration of FFA in viable epidermis is low, presumably due to highly accumulation in the SC, which might result in high amount of the drug in the SC.

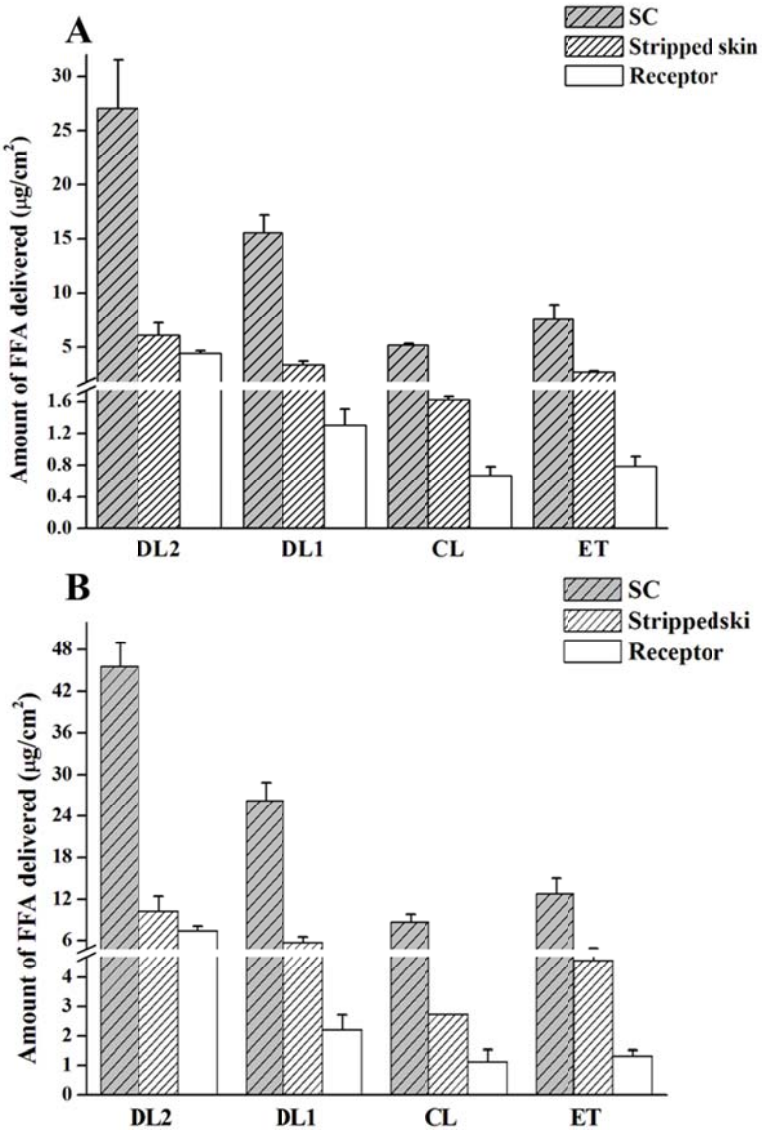

Fig. 3. Amount of FFA delivered into the SC, Stripped skin and the receptor fluid after 6 and 12 h of incubation with rat skin, (A) 6 h and (B) 12 h.

4. Conclusion

In the present study, two types' deformable vesicular systems containing FFA in comparison to conventional liposomes were developed and characterized. The ability of the deformable liposomes to deliver FFA was investigated. The disruption of the SC barrier could be confirmed by the presence of deformable vesicles. Consequently, the deposition of FFA was enhanced after skin incubated with deformable liposomes particularly when containing cineole and ethanol. The results of the penetration study revealed that deformable liposomes were most effective in delivering the FFA into the SC and skin layers. The deformable vesicles incorporating cineole and ethanol when applied non-occlusively onto rat skin, significantly increased distribution of FFA in the SC, stripped skin compared with conventional liposomes.

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