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In vitro retinoic acid release and skin permeation from different liposome formulations

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Abstract

The effect of including charged phospholipids (both negative and positive) and/or cholesterol in liposomal formulations on retinoic acid (RA) in vitro release and permeation through human stratum corneum and epidermis was studied. No significant difference in RA release was observed comparing the different liposomal formulations tested. On the contrary, positively charged liposomes provided significantly higher RA skin permeation compared to negatively charged vesicles which, in turn, showed RA permeation through the skin similar to that obtained from neutral liposomes. Furthermore, the inclusion of cholesterol in charged liposomes did not significantly affect RA skin permeation.

Keywords: Retinoic acid; Skin permeation; Release; In vitro; Liposomes; Charged phospholipids

1. Introduction

Recently, interest has been increasing in the use of liposomes in the pharmaceutical field. Many authors reported that liposomes can be used to deliver active compounds into the skin in greater amounts than conventional topical formulation (such as gels, cream, lotions and ointments), with localization at the desired site of action (Foldvari et al., 1990). To date, the mechanism by which liposomes enhance active compound concentration into the skin is not well known. Recently, Du Plessis et al., 1994 suggested that the phospholipids of liposomes bilayers could mix with the intercellular lipids of the stratum corneum (SC), causing an intracutaneous depot of active compound.

Interest has recently been focused on the use of vitamin A acid (retinoic acid) for the treatment of dermatological diseases such as acne and psoriasis (Suarat, 1985). Unfortunately, some drawbacks such as poor water solubility, photolability and local irritating reactions (Lehman et al., 1988) strongly limit the topical use of vitamin A acid (RA). In order to overcome these disadvantages and to improve the effectiveness of this compound after its topical application, many authors (Masini et al., 1990; Meybeck, 1991; Mezei, 1993) have proposed the use of RA liposomal formulations.

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So, Foong et al., 1990 evaluating RA biodisposition after its topical application in liposomal, cream and gel dosage forms, found that liposome formulations provided greater bioavailability with higher RA concentration in the epidermis and upper dermis of guinea pigs than conventional formulations. Notwithstanding that many papers reported an improved topical effectiveness of RA when formulated in liposomes, to date little work has been carried out to evaluate the influence of liposome composition on retinoic acid skin permeation. The inclusion of charged phospholipds or cholesterol in liposome bilayers has been shown to affect the extent of active compound entrapment within the vesicles (Luxnat and Galla, 1986) and active compound release and skin permeation could be influenced as well. As extensively reported in the literature (Barry, 1983), the topical efficacy of an active compound depends on its ability both to diffuse from the formulation to the skin surface and to penetrate the skin. Both these processes can affect the extent of drug percutaneous absorption and the slowest one will be rate-limiting. So, the purpose of this study was to assess both in vitro release and skin permeation through human stratum corneum and epidermis of retinoic acid in liposome formulations having different phospholipid bilayer composition. Since Burnette and Ongipattanakul, 1987 suggested that the skin could act as a negatively charged membrane, we thought it noteworthy to introduce negatively or positively-charged phospholipids in liposomal bilayers to investigate if this parameter could affect RA skin permeation. Furthermore, since the inclusion of cholesterol in liposomal formulations can alter bilayer permeability, we included cholesterol in negatively or positively-charged liposomes.

2. Materials and methods

2.1. Materials

1, 2 - dipalmitoyl - L - α - phosphatidylcholine (DPPC), 1,2-dipalmitoyl-DL-α-phosphatidylserine (DPPS), cholesterol (CHOL) and stearylamine (SA) were purchased from Sigma-Aldrich (Milan, Italy) and were used as received. The absence of lysophosphatides was checked by two-dimensional thin layer chromatography (TLC). Retinoic acid (RA) was bought from Fluka (Basel, Switzerland). Cellulose acetate membranes (Spectra/Por CE; Mol. Wt cut off 100 000) were supplied by Spectrum (Los Angeles, CA, USA). All other reagents were of analytical grade.

2.2. Preparation of liposomes

Multilamellar vesicles (MLV) were prepared following the 'film' method (Bangham et al., 1974). The composition as molar ratio of the preparations was as follows: DPPC/SA (9:0.25); DPPC/CHOL/SA (4:3:1); DPPC/DPPS (9:1); DPPC/CHOL/DPPS (4:3:1); DPPC/CHOL (9:1). Phospholipids (10 mg) in mixture with RA (400 μg), or without RA (control), were dissolved in chloroform and the solvent was removed at 30°C under a nitrogen stream. The resulting lipid film was kept overnight at 30°C under high vacuum. Liposomes were obtained by adding 200 μl of 0.9% sodium chloride solution. The mixture was heated at a temperature (40°C) above that of its gel-to-liquid crystal phase transition to allow the full hydration of the sample and then vortexed twice for 2 min. The liposomal suspension was centrifiged at 22 000 × *g* at 4°C for 20 min. in a 50 Ti type rotor of a Beckman L8–60 M ultracentrifuge, in order to separate the incorporated RA from the free form. This washing step was repeated twice. The liposomal pellet was finally resuspended in 1 ml of 0.9% sodium chloride solution. All the preparation steps were carried out sheltered from the light to avoid photodegradation.

To determine the incorporation of RA, the supernatant obtained from the two washing steps was diluted to 1 ml with ethanol and RA content was determined as described below. The incorporation of RA was determined by difference from the initial amount of drug added and was expressed as percentage of entrapment (E%), i.e. the fraction of encapsulated drug relative to the initial amount of drug in solution:

$$E(\%) = \frac{[RA]enc}{[RA]tot} \times 100$$

where [RA]enc represents the amount of active compound encapsulated and [RA]tot represents the amount of drug used for liposome preparation.

2.3. Morphology and size analysis

As previously reported (Panico et al., 1993), liposomes were examined under a photomicroscope (Zeiss III RS, Germany) for morphological evaluation. The method employed gave rise to a rather homogeneous population of multilamellar vesicles, no cluster or formation of crystals was observed.

Vesicle size was determined by photon correlation spectroscopy (PCS) light scattering analysis (Douglas et al., 1984). The apparatus consisted of an He-Ne Spectra Physic model 120 Laser (7 mW), a holding sample cell (PC8 Malvern) thermostated at 24°C by a Haake F3-R and equipped with a Microcontrol precise mechanical goniometer and an optical system (Melles-Griot f. 150); Hamamazu R1333 and RCA 8852 photomultipliers were used. All the data from PCS analysis were correlated by a Malvern 4700 C particle analyzer connected to an Olivetti 240 computer. The scattering angles were 20 and 40°. From the scattering behaviour of vesicles, the quality parameter or polydispersity index (PI) (Pusey et al., 1974) was determined. This parameter, which can range from 0 to 9, shows values approaching 0 for a monodisperse system and higher values for a polydisperse system.

2.4. Release studies

In vitro diffusion of RA in different liposomal formulations was measured through cellulose acetate membranes using Franz diffusion cells (Franz, 1975). As reported by Shah et al., 1989 who studied in vitro release of hydrocortisone through cellulose acetate membranes, the use of Franz cells provides an accurate and reliable method for evaluating active compound release from topical formulations. The Franz cells used in this study had a receiver compartment volume of 4.5 ml and an effective diffusion area of 0.75 cm^2. The receptor compartment was filled with a solution consisting of ethanol/water 50:50 which was stirred and thermostated at 37°C throughout the experiments. Before being mounted in Franz cells, cellulose acetate membranes were moistened with the receptor phase; 300 μl of each liposomal suspension entrapping RA or without RA (control) was placed on the membrane surface. A further series of experiments was carried out using RA hydroalcoholic solution (RA 50 μg/ml; ethanol/water 50:50; amount applied on the membrane surface 900 μl) as control. After having applied the formulation on the membrane surface, the diffusion cells were covered completely with aluminum foil to prevent light exposure since other authors reported that RA is photolabile (Lehman et al., 1988). Samples of the receiving solution were withdrawn at intervals and replaced with an equal volume of ethanol/water 50:50. RA content in the receiving solution samples was determined as described below. At the end of the experiments, samples of the donor phase were analyzed for determining RA content (both free and encapsulated) and liposome integrity. RA in the free form was found to be negligible and liposomes did not show any appreciable alteration in size and morphology. RA recovery from donor and receptor compartment accounted for more than 95% of the applied dose.

Studies were performed in triplicate and the mean values were used for the analysis of the data.

2.5. In vitro skin permeation experiments

Samples of human adult skin (mean age 38 $\pm$ 9 years) were obtained from breast reduction operations. Stratum corneum and epidermis (SCE) were removed from the dermis in accordance with the procedure described by Kligman and Christophers, 1963. SCE membranes were dried, stored and assessed for barrier integrity as previously reported (Bonina and Montenegro, 1992). To obtain reproducible results, SCE samples showing similar tritiated water permeability coefficient ($1.5 \pm 0.1 \times 10^{-3}$ cm/h) were used. In vitro RA skin permeation from different liposomal formulations and from hydroalcoholic solutions was assessed using the same Franz diffusion cells described

Table 1
Charge, mean size, polydispersity index (P.I.) and RA entrapment of liposome formulations

Composition[a]	Charge	Mean[b] size (μm)	P.I.	Entrapment (%) ± S.D.
DPPC/CHOL 9:1	Neutral	0.5	0.9	98.78 ± 0.41
DPPC/SA 9:0.25	Positive	0.2	0.4	98.83 ± 0.70
DPPC/CHOL/SA 4:3:1	Positive	0.4	1.7	98.52 ± 0.80
DPPC/DPPS 9:1	Negative	0.6	1.2	97.44 ± 1.59
DPPC/CHOL/DPPS 4:3:1	Negative	0.9	1.5	98.60 ± 0.71

[a]Molar ratios.
[b]By light scattering.

above. The receptor phase consisted of water/ethanol 50:50 for ensuring sink conditions. Other authors (Mueller, 1988; Touitou and Fabin, 1988) studying in vitro percutaneous absorption of hydrophobic compounds used water/ethanol solution as receptor phase to ensure their solubility. The receptor phase was stirred and thermostated at 37°C during the experiments. A 300-μl sample of liposomal formulation (entrapping RA or without active compound) or 900 μl of RA hydroalcoholic solution (RA 50 μg/ml; ethanol/water 50:50) was placed on the skin surface and the same procedure described for in vitro diffusion studies was followed.

2.6. Retinoic acid quantitative determination

Retinoic acid was determined spectrophotometrically at 360 nm (Varian 640 Spectrophotometer). A standard working curve was constructed daily from known concentration of RA in the suitable solvent. Ethanol/water 50:50 solution was used as reference for analyzing samples from release and percutaneous absorption studies. Empty liposomes were used as reference standards in order to correct for the turbidity effects when RA content in liposomal suspensions was determined.

3. Results and discussion

Using the lipid 'film' method, a quite homogeneous population of multilamellar vesicles (MLVs) was obtained. The sizes of the prepared MLVs detected by light-scattering procedures are reported in Table 1. Liposome mean size ranged between 0.2 and 0.9 μm and a narrow dimensional distribution (PI range 0.4–1.7), indicating almost monodisperse systems, was observed.

As reported in Table 1, the entrapment efficiency of RA was close to 98% and no significant difference in RA encapsulation could be detected comparing the different liposome formulations prepared. The high percentage of RA entrapment observed in our study agree well with that reported by Nastruzzi et al., 1990 who found an entrapment efficiency over than 95%.

All the liposomal formulations used in this study showed to be stable over 2 days since no significant leakage of RA from the bilayers was observed during this period of time. This last finding is in agreement with the data reported by Ganesan et al., 1984 who reported that the in vitro leakage of very hydrophobic compounds, such as progesterone, from liposome bilayers was imperceptible.

The amount of RA released after 24 h through cellulose acetate membranes, RA flux and the percentage of applied dose permeated from different liposomal formulations are reported in Table 2. The same parameters obtained from a RA hydroalcoholic solution used as control are also reported in Table 2. Plotting the amount of RA released from each liposomal formulation as a function of time (see Fig. 1) a linear relationship ($r > 0.99$) was obtained, thus indicating that RA release followed a pseudo-first order kinetic. As may be noted in Table 2, no significant difference in RA release was observed comparing the different liposomal formulations tested. These results suggested that RA release from liposomal formulation was not affected by the presence of charged

Table 2
RA release from different liposome formulations and hydroalcoholic solutions

Formulation	Amount permeated[a] (μg ± S.D.)	% Dose ± S.D.	Flux ± S.D. μg cm^{-2} h^{-1}
RA HA[b]	26.220 ± 1.593	58.26 ± 3.54	1.421 ± 0.089
DPPC/CHOL	16.579 ± 1.241	13.95 ± 1.11	1.011 ± 0.077
DPPC/SA	16.141 ± 0.088	13.67 ± 0.10	0.985 ± 0.008
DPPC/DPPS	17.852 ± 1.656	15.10 ± 1.39	1.076 ± 0.102
DPPC/CHOL/SA	15.795 ± 1.133	13.89 ± 1.20	0.957 ± 0.081
DPPC/CHOL/DPPS	18.666 ± 1.866	15.76 ± 1.44	1.115 ± 0.121

[a]RA cumulative amount permeated after 24 h (n = 3).
[b]RA hydroalcoholic solution (ethanol/water 50:50; RA concentration: 50 μg/ml).

phospholipids (negative or positive) and cholesterol in liposomal bilayers. RA release from hydroalcholic solutions was significantly higher than that determined from all the liposomal formulations tested comparing both the cumulative amount permeated and the flux ($P < 0.05$ for all the comparisons). Similar findings have been reported by Thibault and Poelman, 1992 who, studying in vitro RA release from hydrophilic polymeric coated liposomes through Silastic, attributed the slower RA release from liposomes

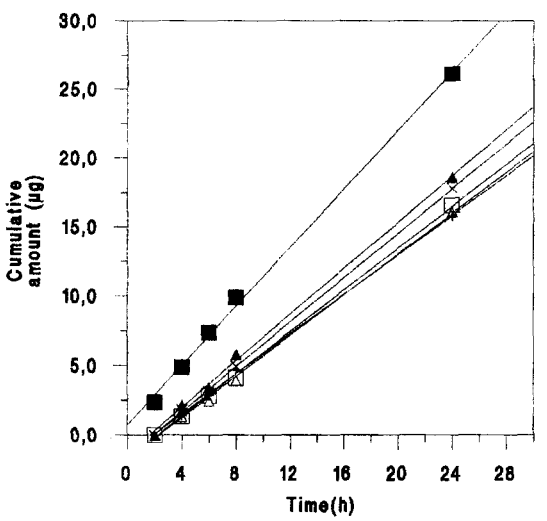

Fig. 1. In vitro retinoic acid release from hydroalcoholic solution and from different liposome formulations. (■) hydroalcoholic solution; (□) DPPC/CHOL; (+) DPPC/CHOL/SA; (▲) DPPC/CHOL/DPPS; (△) DPPC/SA; (x) DPPC/DPPS. Each point represents the mean value of the different determinations. Standard deviation for each point was about 10% of the mean value.

formulations to the fact that RA did not diffuse free but entrapped in the vesicles. This hypothesis could explain only partially the results obtained in our in vitro release study since the mean pore size of the membrane we used (cut-off 100 000 Daltons) was about 10 nm and only very small liposomes could diffuse intact across this membrane. Our results could be more satisfactory explained by the presence of ethanol in the receiving compartment. When a hydroalcoholic solution is used as receptor phase in in vitro release experiments, there is always a back diffusion of alcohol through the artificial membrane which can alter the formulation in the donor compartment (Shah and Skelly, 1993). Shah et al., 1992 evaluating betamethasone valerate release from cream formulations using a synthetic membrane and 60% ethanol/water as receiving phase found a small amount of alcohol in the donor compartment at the end of the experiment. In our experiments, ethanol back diffusion into the donor compartment could have induced a partial vesicle fracture with RA leakage into the outside medium. This hypothesis could reasonably explain the greater lag time values and the lower RA penetration rate through cellulose acetate membranes observed for RA liposome formulations. To assess the effect of receiving solution composition on RA penetration through cellulose acetate membrane, we carried out similar experiments using normal saline as receiving phase. The results of these experiments showed that RA penetration from hydroalcoholic solution was significantly lower than that observed using water/ethanol as receiving solution and RA permeation from liposome vehicles was

Table 3
In vitro RA skin permeation from different liposome formulations and hydroalcoholic solutions

Formulation	Amount permeated[a] (μg ± S.D.)	% Dose ± S.D.
RAHA[b]	2.712 ± 0.318	6.03 ± 0.71
DPPC/CHOL	1.093 ± 0.148	0.92 ± 0.09
DPPC/SA	1.641 ± 0.190	1.37 ± 0.10
DPPC/DPPS	0.871 ± 0.099	0.74 ± 0.08
DPPC/CHOL/SA	1.854 ± 0.260	1.56 ± 0.30
DPPC/CHOL/DPPS	1.026 ± 0.138	0.86 ± 0.11

[a]RA cumulative amount permeated after 24 h ($n = 3$).
[b] RA hydroalcoholic solution (ethanol/water 50:50; RA concentration: 50 μg/ml).

negligible, therefore no permeation rate could be calculated (data not shown). The lower penetration rate of RA observed using normal saline as receiving solution could be due to RA poor water solubility, thus indicating that the presence of ethanol in the receiving phase may play an important role in determining RA penetration through cellulose acetate membrane.

Additional explanations for the difference observed between RA flux from hydroalcoholic solutions and from liposomal suspensions could be the different thermodynamic activity of RA in these formulations and the different amount of formulation applied on the skin.

The results of in vitro RA skin permeation from different liposomal formulations and from hydroalcoholic solutions used as control are reported in Table 3. Results are expressed in terms of cumulative amount permeated after 24 h because the sensitivity of the analytical method (detection limit 0.05 μg/ml) did not allow us to detect RA in the receiving solution before 8–9 h and no flux could be calculated in these conditions. The results of in vitro RA skin permeation from different liposomal formulations and from hydroalcoholic solutions used as control are reported in Table 3. Skin permeation experiments were carried out using a membrane consisting only of stratum corneum and epidermis since the barrier function of the skin resides mainly in the stratum corneum and the dermis in vitro can act as an additional artificial barrier to the absorption of hydrophobic compounds (Bronaugh and Stewart, 1984).

Comparing release study results to skin permeation data, it may be noted that RA amount permeated through the skin both from all the liposomal formulations tested and from alcoholic solutions was significantly lower ($P < 0.05$) than that released from the same formulations. These results indicate that in this study the rate-limiting step in RA percutaneous absorption process was skin permeation rather than release from the formulation.

As shown in Table 3, in vitro RA amount penetrated through the skin from DPPC/CHOL liposomes was lower than that obtained from hydroalcoholic solutions. These results agree well with the data reported by other authors (Ganesan et al., 1984) who observed that skin permeation of lipophilic active compounds was lower when they were applied in the liposomal form than when applied in solutions.

DPPC/SA/CHOL liposomes provided significantly higher ($P < 0.05$) RA amount in the receptor phase compared to DPPC/DPPS/CHOL liposomes which, in turn, showed a RA skin permeation close to that obtained from DPPC/CHOL liposomes ($P > 0.05$). A similar trend was observed comparing the percentage of applied RA dose penetrated after 24 h. Since after topical application, liposomal bilayers can mix with the SC lipids forming a lipid depot in this skin layer (Du Plessis et al., 1994), the greater RA skin permeation observed using positively-charged liposomes could be attributed to a greater accumulation of this type of liposomes within the SC, probably due to the negative charge of the skin surface. Our results are different from that reported by Ganesan et al., 1984 who found that the inclusion of positively-charged phospholipids in liposomes containing progesterone did not affect in vitro skin permeation of this drug compared to neutral liposomes. This discrepancy could be due to the different experimental condi-

tions used in these studies since Ganesan et al., 1984 performed their in vitro experiments using full-thickness hairless mouse skin. The use of whole skin, due to the presence of the dermis which in vitro can act as an additional barrier to the permeation of lipophilic compound (Bronaugh and Stewart, 1984), could have levelled off the effect of charged phospholipids on progesterone skin permeation from liposomal suspension.

In order to evaluate the influence of cholesterol inclusion in charged liposome bilayers on RA skin permeation, we determined the amount of RA permeated from DPPC liposomes containing only charged phospholipids (DPPC/SA, DPPC/DPPS). As shown in Table 3, no significant difference was observed comparing the amount of RA permeated from liposomes containing cholesterol to that determined from cholesterol free liposomes ($P > 0.05$ for all the comparisons). These findings suggest that the inclusion of positively-charged phospholipids in liposome bilayers may play a role more important than the inclusion of cholesterol in determining RA skin permeation.

Recently, Masini et al., 1993 studying in vitro permeation through hairless mouse skin of radiolabeled RA from DPPC liposomal suspension and gel formulation, reported that RA absorption was higher from the gel but the percentages of drug found in the epidermis and dermis were higher from liposomal suspensions which, therefore, affected RA skin distribution.

Similar findings have been reported by Foong et al., 1990 who found greater RA concentration in the epidermis and the dermis after topical application of RA liposomal formulation compared to conventional cream. In our study, the use of positively or negatively-charged liposomes could have differently influenced RA distribution within the skin layers. So, further in vivo and in vitro studies, using radiolabeled RA, are planned to determine RA content in the different skin layers after topical application of charged liposomes.

In conclusion, the results of our study show that RA release was not significantly affected by the inclusion of charged (positively or negatively) phospholipids and cholesterol in DPPC liposomes entrapping RA. On the contrary, positively-charged liposomes provided greater RA skin permeation compared to neutral or negatively-charged liposomes. A better understanding of the mechanism by which charged liposomes could affect RA skin distribution and permeation could be helpful in designing more effective RA topical formulations.

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